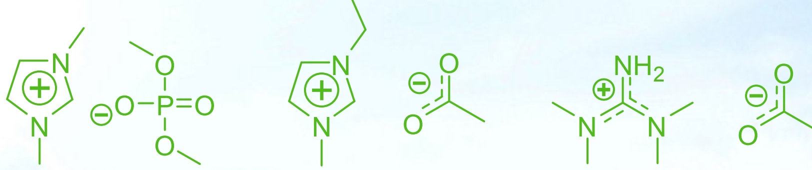

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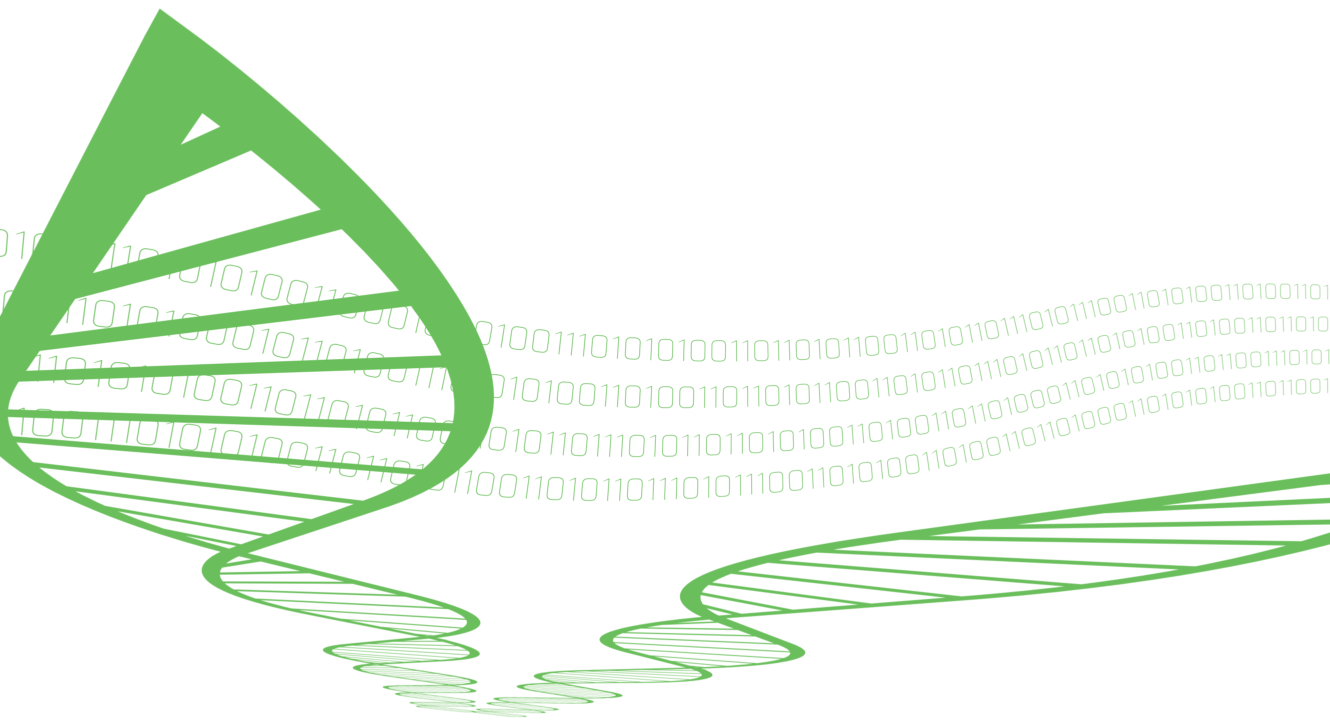
Enzymatic hydrolysis of cellulose in aqueous ionic liquids

Ronny Wahlström



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Enzymatic hydrolysis of cellulose in aqueous ionic liquids

Ronny Wahlström

VTT Technical Research Centre of Finland

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VTT
PL 1000 (Tekniikantie 4 A, Espoo)
02044 VTT
Puh. 020 722 111, faksi 020 722 7001

VTT
PB 1000 (Teknikvägen 4 A, Esbo)
FI-02044 VTT
Tfn. +358 20 722 111, telefax +358 20 722 7001

VTT Technical Research Centre of Finland
P.O. Box 1000 (Tekniikantie 4 A, Espoo)
FI-02044 VTT, Finland
Tel. +358 20 722 111, fax +358 20 722 7001

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Enzymatic hydrolysis of cellulose in aqueous ionic liquids

Enzymatisk cellulosahydrolys i vattenhaltiga jonvätskor.
Selluloosan entsymaattinen hydrolyysi vesipitoisissa ioninesteissä. **Ronny Wahlström.**
Espoo 2014. VTT Science 52. 102 p. + app. 57 p.

Abstract

Total enzymatic hydrolysis of the polysaccharides in lignocellulosic biomass to monosaccharides is currently a focus research area. The monosaccharides obtained from lignocellulose hydrolysis can be used for the production of platform chemicals and biofuels, most notably ethanol. One major challenge in the commercialization of lignocellulosic ethanol production is the recalcitrance of lignocellulosics towards enzymatic hydrolysis, necessitating efficient pretreatment of the lignocellulosic feedstock. Certain ionic liquids (ILs, salts with melting points below 100 °C) dissolve cellulose and even lignocellulosic biomass and are as such interesting candidates for pretreatment technology. However, cellulose-dissolving ILs have been found to severely inactivate the hydrolytic enzymes (cellulases) employed in cellulose hydrolysis. This work focuses on elucidating how certain ILs affect the action of cellulases in cellulose hydrolysis. The main emphasis was on the action of purified monocomponent *Trichoderma reesei* cellulases, but some commercial cellulase preparations were also studied in IL matrices.

Hydrolysis experiments were made in solutions containing up to 90% of the two cellulose-dissolving ILs 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO) and 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP). The presence of increasing amounts of IL led to decreasing yields of solubilised saccharides in enzymatic hydrolysis. Depending on the IL and cellulase, no soluble saccharides were released in hydrolysis matrices containing over 40–50% IL. There were clear differences in the severity of the effects of different cellulose-dissolving ILs on cellulase action. [EMIM]AcO was generally more harmful for cellulase action than [DMIM]DMP. Pure [EMIM]AcO completely inactivated *T. reesei* endoglucanase in 4 h in residual activity measurements, whereas pure [DMIM]DMP supported considerable cellulase activity for at least three days. These results were confirmed by time curves of microcrystalline cellulose (MCC) hydrolysis in matrices containing the two ILs. Cellulose-dissolving ILs based on carboxylate salts of the organic superbases 1,1,3,3-tetramethylguanidine (TMG) and 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) have recently become available. These compounds are distillable under relatively mild conditions and are thus recyclable. However, these ILs were found to be at least as harmful for cellulase action as the studied imidazolium-based ILs and did thus not offer any benefits in terms of enzyme compatibility. *T. reesei* endoglucanases were unable to reduce the molecular weight of MCC in buffer or in any aqueous matrix containing IL, except in 90% (v/v) [DMIM]DMP in which the MCC was partially dissolved.

Cellulose-dissolving ILs were found to be basic in aqueous solution. According to the results in this work, the pH increase caused by IL basicity was not the main reason for the observed cellulase inactivation. Cellulases with confirmed activity at high pH did not perform better than acidic or neutral cellulases in IL solutions. Some indications were however obtained that cellulase thermostability may be associated with better activity in cellulose-dissolving ILs.

The studied ILs were found to have very detrimental effects on saccharide analytics. A capillary electrophoresis (CE) method was developed for the analysis of mono- and oligosaccharides in matrices containing ILs. With this CE method, the yields and product distribution of cello-oligomers produced in the hydrolysis experiments could be determined. It was found that the presence of ILs shifted the product distribution to larger cello-oligomers for some cellulases. The CE method was also used to monitor the hydrolysis of cello-oligomers with *Aspergillus niger* β-glucosidase in IL matrices. This β-glucosidase was found to be very IL sensitive.

ILs were found to affect the cellulose binding of *T. reesei* cellulases. The cellulase binding to MCC in solutions with [DMIM]DMP and [EMIM]AcO was studied with radiolabeled *T. reesei* Cel5A (endoglucanase II) and Cel7A (cellobiohydrolase I) and their respective core domains. Cel7A was able to bind to MCC with its core domain, whereas it was shown that Cel5A was very dependent on its CBM for efficient substrate binding. High cellulose binding affinity was not necessary for all the cellulases in order for them to be hydrolytically active. [EMIM]AcO interfered more with cellulase substrate binding than [DMIM]DMP. The binding ability of the *T. reesei* carbohydrate-binding modules (CBMs) was very IL sensitive.

Keywords Ionic liquid, cellulase, hydrolysis, carbohydrate, cellulose, inactivation, carbohydrate-binding module, cellulase binding, glycoside hydrolase

Enzymatisk cellulosahydrolys i vattenhaltiga jonvätskor

Enzymatic hydrolysis of cellulose in aqueous ionic liquids.
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Sammanfattning

Enzymatisk totalhydrolys av lignocellulosans polysackarider till monosackarider är för tillfället ett mycket aktivt forskningsområde. De sålunda producerade monosackariderna kan användas som råvara vid tillverkningen av plattformkemikalier och biobränslen, av vilka särskilt kan nämnas etanol. En av de största utmaningarna i kommersialiseringen av etanoltillverkning från lignocellulosa är lignocellulosans motståndskraft mot enzymatisk hydrolys. Därför behövs effektiva förbehandlingsmetoder då lignocellulosan används som råvara. Vissa jonvätskor (definierade som salt med smältpunkt under 100 °C) löser cellulosa och till och med fullständig lignocellulosa. Jonvätskorna utgör sålunda ett intressant alternativ som förbehandlingsteknologi för lignocellulosa. Jonvätskorna har emellertid i hög grad konstaterats inaktivera de hydrolytiska enzymer, cellulaser, som används i cellulosahydrolys. Detta arbete har haft som målsättning att klargöra hur cellulosalösande jonvätskor påverkar cellulasernas funktion i cellulosahydrolys. I första hand undersöktes hur funktionen hos cellulaser renade till enkomponentpreparat från *Trichoderma reesei*, men också hos kommersiella cellulaspreparat, påverkades i vissa jonvätskelösningar.

Hydrolysexperimenten utfördes i lösningar med upp till 90 % jonvätska (1-etyl-3-metylimidazolium acetat ([EMIM]AcO) eller 1,3-dimetylimidazolium dimetylfosfat ([DMIM]DMP)). En ökande mängd jonvätska ledde till avtagande hydrolysutbyten i form av lösliga sackarider i enzymatisk hydrolys. Beroende på kombinationen av jonvätska och cellulas observerades ingen tillkomst av lösliga sackarider när jonvätskekoncentrationen steg över 40–50 %. De olika jonvätskorna var i olika utsträckning skadliga för cellulasernas funktion. [EMIM]AcO var i allmänhet mer skadlig än [DMIM]DMP för cellulasernas funktion. Ren [EMIM]AcO inaktiverade *T. reesei* endoglukanas fullständigt på mindre än 4 h, medan betydande restaktiviteter mättes efter inkubation i [DMIM]DMP under åtminstone tre dygn. Detta resultat understöddes av hydrolyskurvorna när mikrokristallin cellulosa (microcrystalline cellulose, MCC) hydrolyserades i lösningar med dessa två jonvätskor. Cellulosalösande jonvätskor som består av karboxylater av de organiska superbaserna 1,1,3,3-tetrametylguanidin (TMG) och 1,5-diazabicyklo[4.3.0]non-5-en (DBN) har nyligen blivit tillgängliga. Dessa jonvätskor är speciellt intressanta eftersom de är destillerbara under relativt milda förhållanden och sålunda är återvinningsbara. I hydrolysexperimenten konstaterades dessa jonvätskor dock vara åtminstone lika skadliga för cellulasernas funktion som de imidazoliumbaserade jonvätskorna, så dessa jonvätskor medförde ingen nytta i form av ökad enzymkompatibilitet. *T. reesei*s

endoglukanaser kunde inte reducera MCC:s molmassa i buffertlösning eller i någon jonvätskelösning, förutom i 90 % (v/v) [DMIM]DMP, vari MCC partiellt löste sig.

Jonvätskor som löser cellulosa befanns vara basiska i vattenlösning. Enligt resultaten i detta arbete skulle det stigande pH-värdet, som förosakades av de cellulosalösande jonvätskornas basiskhet, inte vara en av huvudorsakerna för den observerade inaktiveringen hos cellulaserna. Cellulaser med aktivitet i höga pH-värden presterade inte bättre i jonvätskelösningar än sura eller neutrala cellulaser. Däremot observerades det att cellulaser med ökande termostabilitet verkade bevara sin förmåga att katalysera cellulosahydrolys i jonvätskelösningar bättre, än cellulaser som är temperaturkänsliga.

De studerade jonvätskorna konstaterades vara mycket skadliga för många av de vanliga metoderna som används i kolhydratanalytik. En kapillärelektroforesmetod utvecklades för att analysera mono- och oligosackarider i jonvätskelösningar. Med den här analysmetoden kunde både hydrolysutbytena och produktdistributionen av lösliga cello-oligomerer bestämmas i jonvätskelösningar. För endel cellulaser ledde närvaron av jonvätska under hydrolysen till att produktdistributionen skiftades mot längre oligomerer, jämfört med situationen i optimumförhållanden. Kapillärelektroforesmetoden användes också för att följa med hur *Aspergillus niger*s β-glukosidas hydrolyserade cello-oligomerer i jonvätskelösningar. Detta β-glukosidas konstaterades vara mycket känsligt för närvaron av jonvätska.

Jonvätskor konstaterades påverka cellulosabindandet hos *T. reesei*s cellulaser. *T. reesei* Cel5A (endoglukanas II), Cel7A (cellobiohydrolas I) och deras respektive katalytiska domäner märktes med radioaktivt tritium och dessa cellulasers förmåga att binda till MCC studerades i lösningar innehållande [DMIM]DMP och [EMIM]AcO. Cel7A kunde binda sig till MCC direkt via sin katalytiska domän, medan det kunde påvisas att Cel5A var ytterst beroende av sin kolhydratbindande modul för att binda till cellulosa. En hög grad av bindning till cellulosa var inte nödvändig för Cel5A för att hydrolys skulle äga rum. [EMIM]AcO konstaterades påverka cellulasernas bindingsgrad till MCC mer än [DMIM]DMP. Bindningsförmågan hos *T. reesei*s kolhydratbindande moduler konstaterades vara synnerligen känslig för de studerade jonvätskorna.

Nyckelord Ionic liquid, cellulase, hydrolysis, carbohydrate, cellulose, inactivation, carbohydrate-binding module, cellulase binding, glycoside hydrolase

Selluloosan entsymaattinen hydrolyysi vesipitoisissa ioninesteissä

Enzymatic hydrolysis of cellulose in aqueous ionic liquids.
Enzymatisk cellulosahydrolys i vattenhaltiga jonvätskor. **Ronny Wahlström.**
Espoo 2014. VTT Science 52. 102 s. + liitt. 57 s.

Tiivistelmä

Lignoselluloosan entsymaattista totaalihydrolyysiä tutkitaan nykyisin hyvin aktiivisesti. Lignoselluloosassa olevien polysakkaridien hydrolyysistä syntyviä monosakkarideja voidaan käyttää raaka-aineina kemikaalien, polymeerien ja biopolttoaineiden, erityisesti etanolin, tuotannossa. Iso haaste lignoselluloosapohjaisen etanolituotannon kaupallistamisessa on lignoselluloosan monimutkainen rakenne, joka vaikeuttaa entsymaattista hydrolyysiä. Tehokkaiden, lignoselluloosaa avaavien esikäsittelymenetelmien kehittäminen on siis tärkeää. Tietyt ioninesteet, jotka määritellään suoloiksi, joiden sulamispiste on alle 100 °C, liuottavat selluloosaa ja jopa lignoselluloosaa. Ne ovatkin hyvin mielenkiintoisia käytettäviksi lignoselluloosan esikäsittelyssä. Selluloosaa liuottavien ioninesteiden on kuitenkin todettu inaktivoivan hydrolyyttisiä entsyymejä, sellulaaseja, joita käytetään selluloosan totaalihydrolyysissä. Tässä työssä selvitettiin, miten tietyt ioninesteet vaikuttavat sellulaasien toimintaan selluloosan hydrolyysissä. Työssä tutkittiin pääasiassa *Trichoderma reesei* -homeen tuottamien ja puhdistettujen sellulaasien sekä myös joidenkin kaupallisesti saatavien sellulaasituotteiden toimintaa vesipitoisissa ioninesteliuoksissa.

Hydrolyysikokeita tehtiin selluloosalla vesiliuoksissa, joiden ioninestepitoisuus vaihteli; suurimmillaan se oli 90 % (joko 1-etyyli-3-metyylimidatsoliumi asetaatti ([EMIM]AcO) tai 1,3-dimetyylimidatsoliumi dimetyylifosfaatti ([DMIM]DMP)). Kasvavat ioninestepitoisuudet aiheuttivat hydrolyysisaannon pienenemisen selluloosan entsyymaattisessa hydrolyysissä. Riippuen sellulaasin ja ioninesteen yhdistelmästä liukenevia mono- ja oligosakkarideja ei syntynyt lainkaan hydrolyyseissä, joissa oli enemmän kuin 40–50 % ioninestettä. Selluloosaa liuottavien ioninesteiden vaikutuksessa sellulaasien toimintaan oli selviä eroja. [EMIM]AcO haittasi enemmän sellulaasien toimintaa kuin [DMIM]DMP. Puhtaassa [EMIM]AcO:ssa *T. reesei*n endoglukanaasi inaktivoitui täysin neljän tunnin käsittelyssä jäännösaktiivisuusmittauksen perusteella, kun taas aktiivisuus aleni hyvin vähän ja hitaasti [DMIM]DMP:ssa kolmen vuorokauden aikana. Nämä tulokset vastasivat hyvin samojen ioninesteiden vesiliuoksissa tehtyjen mikrokiteisen selluloosan (microcrystalline cellulose, MCC) entsymaattisten hydrolyysien tuloksia. Äskettäin on kehitetty selluloosaa liuottavia ioninesteitä, jotka perustuvat orgaanisten superemästen 1,1,3,3-tetrametyyliguanidiinin (TMG) ja 1,5-diatsabisyklo[4.3.0]non-5-eenin (DBN) karboksylaattisuoloihin. Nämä ioninesteet ovat tislattavia suhteellisen miedoissa olosuhteissa ja näin ollen kierrätettäviä. Hydrolyysikokeiden perusteella nämä uudet selluloosaa liuottavat ioninesteet eivät kuitenkaan olleet paremmin yhteensopivia sellulaasien kanssa kuin perinteiset imidatsoliumi-pohjaiset ioninesteet. *T. reesei*n endoglukanaasit eivät pystyneet vähentämään MCC:n molekyylipainoa puskurissa eivätkä missään muussa io-

ninestettä sisältävässä liuoksessa, paitsi 90-prosenttisessa (v/v) [DMIM]DMP:ssa, johon MCC oli osittain liuennut.

Selluloosaa liuottavien ioninesteiden todettiin olevan emäksisiä vesiliuoksessa. Tämän työn tulosten perusteella ioninesteiden aiheuttama pH-arvon nousu ei kuitenkaan ollut sellulaasien inaktivoitumisen pääsyy. Sellulaasin kyky toimia korkeissa pH-arvoissa ei tehnyt sellulaasista tehokkaampaa selluloosan hydrolyysissä ioninestematriiseissa. Sen sijaan sellulaasien termostabiilisuus vaikutti johtavan kasvavaan ioninestetoleranssiin.

Tutkittujen ioninesteiden havaittiin olevan hyvin haitallisia hiilihydraatti-analytiikkamenetelmille. Työssä kehitettiin kapillaarielektroforeesimenetelmä mono- ja oligosakkaridien analyysiin ioninestepitoisissa matriiseissa. Hydrolyysien saannot ja liuenneiden oligosakkaridien tuotejakaumat määriteltiin tällä menetelmällä ioninestepitoisista hydrolysaateista. Analyysien perusteella havaittiin, että ioninesteiden läsnäolo entsymaattisessa selluloosan hydrolyysissä sai tuotejakauman siirtymään pitempiin oligosakkarideihin joillakin sellulaaseilla. Elektroforeesimenetelmää käytettiin myös sello-oligomeerien hydrolyysin seuraamiseen *Aspergillus niger*in β-glukosidaasilla ioninestematriiseissa. Tämä β-glukosidaasi havaittiin hyvin ioninesteherkäksi.

Ioninesteiden havaittiin vaikuttavan *T. reesei*n sellulaasien selluloosaan sitoutumiseen. Sellulaasien sitoutumista MCC:aan tutkittiin radioleimatuilla *T. reesei* Cel5A:lla (endoglukanaasi II), Cel7A:lla (cellobiohydrolaasi I) ja niiden katalyyttisillä domeeneillä puskuriliuoksissa [DMIM]DMP:n ja [EMIM]AcO:n läsnä ollessa. Cel7A pystyi sitoutumaan MCC:aan pelkällä katalyyttisellä domeenillaan, kun taas Cel5A oli hyvin riippuvainen hiilihydraatteja sitovasta modulistaan sitoutuakseen tehokkaasti selluloosaan. Korkea sitoutumisaste ei kuitenkaan ollut tarpeellinen Cel5A:lle, jotta se olisi toiminut selluloosan hydrolyysissä. [EMIM]AcO vaikutti [DMIM]DMP:a voimakkaammin sellulaasien selluloosaan sitoutumiseen. Yleisesti tutkittujen ioninesteiden todettiin vaikuttavan herkästi *T. reesei*n hiilihydraatteja sitovien moduulien toimintaan.

Avainsanat Ionic liquid, cellulase, hydrolysis, carbohydrate, cellulose, inactivation, carbohydrate-binding module, cellulase binding, glycoside hydrolase

Preface

The work in this thesis was carried out at VTT Technical Research Centre of Finland during the years 2010–2013. As VTT has a very long experience in cellulase research, it has been a true privelege to work together with so many top scientists in this field. This work was funded by the Finnish Bioeconomy Cluster's (FiBiC) Future Biorefinery (FuBio) programme and by VTT Graduate School. In addition, the Academy of Finland Graduate School for Biorefining (BIOREGS) has provided funding for participating in conferences and financed many interesting courses. COST action FP0901 "Analytical tools for biorefineries" provided the funding to carry out a short term scientific mission to the University of Natural Resources and Life Sciences (BOKU) in Vienna in 2010. I am deeply grateful to all my funders for providing the possibility to have such a rich time in learning, networking, conferencing, studying new topics and developing as a scientist during my PhD studies.

At VTT I wish to thank Vice President, Dr. Anu Kaukovirta-Norja, my current and former Technology Managers Dr. Raija Lantto and Dr. Niklas von Weymarn, for providing me with good working facilities. I am forever thankful to my thesis supervisor at VTT, Dr. Anna Suurnäkki, for all the time she has given me even though her time schedules have been extremely tight during the last years. I greatly appreciate our regular meetings during which Anna has not only given me guidance for my scientific work but also helped me understand how our research environment and the scientific world works: in brief, being not only a supervisor, but also a mentor. In addition, I am grateful for the scientific guidance given to me by my thesis advisory board with Prof. Reija Jokela, Research Prof. Kristiina Kruus, Dr. Jarmo Ropponen and Hannu Mikkonen, who sadly passed away during the time of my thesis work. I am greatly thankful to Prof. Reija Jokela for being my supervising professor at Aalto University; I am really happy for the smooth cooperation we have had in this work. Also the Planning Officer for doctoral affairs at Aalto University, Sirje Liukko is thanked for guiding me through the university bureaucracy and Prof. Matti Leisola is thanked for supervising my minor studies. Dr. Kristiina Poppius-Levlin, the coordinator of VTT Graduate School, is thanked for her support and positive attitude in guiding us graduate school students towards the ultimate goal.

I wish to thank the pre-examiners of my thesis, Prof. Jack Saddler at University of British Columbia, Canada, and Deputy Director, Dr. Blake Simmons from Sandia

National Laboratories, US, for their extremely precious comments on the thesis manuscript. Based on their comments we had many new great ideas on how to study this topic further. I deeply believe my thesis was much improved based on the feedback I had during the preliminary examination process. Päivi Vahala and VTT publication services are thanked for helping with technical editing and Michael Bailey for carrying out linguistic corrections.

I am grateful to my team leader Dr. Terhi Hakala and my whole team at VTT for their supportive and positive attitudes towards my work. Matti Siika-aho has been a never-ending source of good advice for the practical laboratory experiments, and I hope my office mate Stina Grönqvist has enjoyed our fruitful and usually also diverting discussions as much as I have. Of the technical staff I want to thank especially Mariitta Svanberg, Riitta Alander, Jenni Lehtonen, Pirkko Saarelainen, Ulla Vornamo, Eila Turunen and Nina Vihersola for technical assistance in carrying out many of the experiments. I also want to acknowledge Dr. Tarja Tamminen for challenging a quite, at that time, reluctant young man to start conducting PhD studies in the first place. The so called “lunch group” with PhD students and young scientists has been very important for me as a forum for exchanging ideas, opinions and experiences. Jenni Rahikainen, Katariina Kemppainen, Katariina Rommi, Heini Virtanen, Piritta Niemi, Outi Santala and Kirsi Kiiveri can be counted among the most active members in this group.

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Finally, I want to thank my family, my relatives and all my friends for always being supportive to me in my pursuit of the doctoral degree.

Espoo, December 2013

Ronny Wahlström

Academic dissertation

Supervisor, custos	Professor Reija Jokela Department of Chemistry, Aalto University Espoo, Finland
Thesis advisers	Principal Scientist, Dr. Anna Suurnäkki VTT Technical Research Centre of Finland Espoo, Finland
	Research Professor Kristiina Kruus VTT Technical Research Centre of Finland Espoo, Finland
Preliminary examiners	Deputy Director, Dr. Blake Simmons Biofuels and Biomaterials Science and Technology, Sandia National Laboratories Livermore, CA, USA
	Professor John Saddler Department of Wood Science, University of British Columbia, Vancouver, British Columbia, Canada
Opponent	Professor Antje Spiess Enzyme Process Technology, RWTH-Aachen University Aachen, Germany

List of publications

This thesis is based on the following original publications which are referred to in the text as I–V. The publications are reproduced with kind permission from the publishers. Additional unpublished data is also presented.

I Wahlström R, Rovio S, Suurnäkki A. 2012. Partial enzymatic hydrolysis of microcrystalline cellulose in ionic liquids by *Trichoderma reesei* endoglucanases. RSC Adv 2:4472–4480.

II Wahlström R, Rovio S, Suurnäkki A. 2013. Analysis of mono- and oligosaccharides in ionic liquid containing matrices. Carbohydr Res 373:42–51.

III Wahlström R, King A, Parviainen A, Kruus K, Suurnäkki A. 2013. Cellulose hydrolysis with thermo- and alkali-tolerant cellulases in cellulose-dissolving superbase ionic liquids. RSC Adv 3:20001–20009.

IV Wahlström R, Rahikainen J, Kruus K, Suurnäkki A. 2013. Cellulose hydrolysis and binding with *Trichoderma reesei* Cel5A and Cel7A and their core domains in ionic liquid solutions. Biotech Bioeng, in Press.

V Ebner G, Vejdovszky P, Wahlström R, Suurnäkki A, Schrems M, Kosma P, Rosenau T, Potthast A. 2014. The effect of 1-ethyl-3-methylimidazolium acetate on the enzymatic degradation of cellulose. J Mol Cat B 99:121–129.

Author's contributions

I The author participated in planning the work and carried out all the laboratory work including both hydrolysis experiments and analyses. The author interpreted the results and wrote the article together with the co-authors.

II The author carried out the method development together with Dr. Stella Rovio. The laboratory work for the application examples was carried out by the author. The author interpreted the results and wrote the article together with the co-authors.

III The author planned the experiments together with the co-authors and carried out the laboratory work including both hydrolysis experiments and analyses. The author interpreted the results and wrote the article together with the co-authors.

IV The author planned the experiments together with the co-authors. The author guided the execution of the hydrolysis experiments, carried out the Tr-labeling of cellulases and the binding experiments. The author interpreted the results and wrote the article together with the co-authors.

V The author participated in the planning and execution of the cellulase inactivation experiments (endoglucanase activity measurements on soluble substrates) and the hydrolysis of beech dissolving pulp in buffer, including the analysis of the hydrolysate. The author interpreted the results from these experiments and wrote the corresponding parts of the article together with the co-authors. Results from this article have also been used in the PhD thesis of Dr. Gerald Ebner at the University of Natural Resources and Life Sciences (BOKU), Vienna, Austria.

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Appendices

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List of abbreviations

ABEE	4-Aminobenzoic acid ethyl ester
ABN	4-Aminobenzonitrile
AFEX	Ammonia freeze (or fibre) explosion
ANTS	8-Aminonaphthalene-1,3,6-trisulphonic acid
APTS	8-Aminopyrene-1,3,6-trisulphonic acid
ARP	Ammonia recycle percolation
AGU	Anhydroglucose unit
BGE	Background electrolyte
BASIL	Biphasic Acid Scavenging utilizing Ionic Liquids
BSA	Bovine serum albumine
CE	Capillary electrophoresis
CBM	Carbohydrate-binding domain
CMC	Carboxymethylcellullose
CBH	Cellobiohydrolase
CD	Core domain
DP	Degree of polymerization
DBN	1,5-Diazabicyclo[4.3.0]non-5-ene
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DMAc	Dimethylacetamide
DMF	Dimethylformamide
DMI	1,3-Dimethyl-2-imidazolidinone
DMSO	Dimethylsulphoxide
DNS	3,5-Dinitrosalicylic acid
EG	Endoglucanase
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GPC	Gel permeation chromatography
HPAEC-PAD	High-performance anion exchange chromatography with pulsed amperometric detection

HPLC	High performance liquid chromatography
PAHBAH	*para*-Hydroxybenzoic acid hydrazide
HEC	Hydroxyethylcellulose
HMF	5-Hydroxymethylfurfural
IL	Ionic liquid
LOI	Lateral order index
LCC	Lignin-carbohydrate complex
LOQ	Limit of quantification
LSC	Liquid scintillation counting
LPMO	Lytic polysaccharide mono-oxygenase
NMMO	*N*-Methylmorpholine-*N*-oxide
4-MUC	4-Methylumbelliferyl-β-D-cellobioside
4-MUL	4-Methylumbelliferyl-β-D-lactoside
MCC	Microcrystalline cellulose
PEG	Polyethylene glycol
PHK DP	Pre-hydrolysis kraft dissolving pulp
RC	Regenerated cellulose
REACH	Registration, Evaluation, Authorisation and restriction of CHemicals
rpm	Revolutions per minute
RT	Room temperature
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDP	Sulphite dissolving pulp
TMG	1,1,3,3-Tetramethylguanidine
TCI	Total crystallinity index
VOC	Volatile organic compounds

Abbreviations of ionic liquids

[AMIM]Cl	1-Allyl-3-methylimidazolium chloride
[BzPy]Cl	Benzylpyridinium chloride
[BMIM]Ace	1-Butyl-3-methylimidazolium acesulphamate
[BMIM]DBP	1-Butyl-3-methylimidazolium dibutylphosphate
[BMIM]Cl	1-Butyl-3-methylimidazolium chloride
$[BMIM]PF_6$	1-Butyl-3-methylimidazolium hexafluorophosphate
$[BMIM]BF_4$	1-Butyl-3-methylimidazolium tetrafluoroborate
[BMPy]Cl	1-Butyl-3-methylpyridinium chloride
[DBNH]AcO	1,8-Diazabicyclo[5.4.0]undec-7-enium acetate
[DBNH]EtCOO	1,8-Diazabicyclo[5.4.0]undec-7-enium propionate
[DMIM]DEP	1,3-Dimethylimidazolium diethylphosphate
[DMIM]DMP	1,3-Dimethylimidazolium dimethylphosphate
[EMBy]DEP	1-Ethyl-3-methylbutylpyridinium diethylphosphate
[EMIM]AcO	1-Ethyl-3-methylimidazolium acetate
[EMIM]Br	1-Ethyl-3-methylimidazolium bromide
[EMIM]DBP	1-Ethyl-3-methylimidazolium dibutylphosphate
[EMIM]DEP	1-Ethyl-3-methylimidazolium diethylphosphate
[EMIM]DMP	1-Ethyl-3-methylimidazolium dimethylphosphate
$[EMIM]MeSO_4$	1-Ethyl-3-methylimidazolium methanesulphonate
[HPy]Cl	1-Hexylpyridinium chloride
[E(OH)MIM]AcO	1-Hydroxyethyl-3-methylimidazolium acetate
[DBNMe]DMP	1-Methyl-1,8-diazabicyclo[5.4.0]undec-7-enium dimethylphosphate
[P4444]OH	Tetrabutylphosphonium hydroxide
[TMGH]AcO	1,1,3,3-Tetramethylguanidinium acetate
[TMGH]*n*-PrCOO	1,1,3,3-Tetramethylguanidinium butyrate
[TMGH]COO	1,1,3,3-Tetramethylguanidinium formiate
[TMGH]EtCOO	1,1,3,3-Tetramethylguanidinium propionate
[P8881]AcO	Trioctylmethylphosphonium acetate
HEMA	Tris-(2-hydroxyethyl)methylammonium methylsulphate

1. Introduction

Cellulose is the most abundant biopolymer in the world. Different estimations of annual global cellulose production range between 9×10^{10} ton/a (Pinkert, et al. 2009) and 1.5×10^{12} ton/a (Ha, et al. 2011). Together with lignin and hemicelluloses, cellulose builds up lignocellulosic biomass into a highly complex matrix. Due to greenhouse gas emissions leading to global warming, limited supply, price stability and energy and chemical feedstock security issues, there is a need to replace fossil petroleum as raw material for fuels, chemicals and materials. Lignocellulosic biomass has been recognized as the raw material of the future, as it is renewable, carbon neutral and widely available in different forms around the world. The polysaccharides in lignocellulosic biomass can be hydrolysed to monosaccharides which can be used as raw materials for the production of a variety of commodities, such as ethanol, butanol, fatty acid ethyl esters, lactic acid and hydrogen gas (Barr, et al. 2012; Bokinsky, et al. 2011; Hofvendahl and Hahn–Hägerdal 2000; Maeda, et al. 2007).

Bioethanol production is currently the most studied process for lignocellulosic raw material conversion to value-added chemicals. Bioethanol has been used as a liquid transport fuel *e.g.* in Brazil for decades and its use is constantly growing as a liquid fuel or liquid fuel component in North America, Europe and China. However, first generation bioethanol has to a great extent relied on the use of starch as raw material source in addition to sugar cane (mainly in Brazil), and have thus directly been competing with agricultural food production. The use of food raw material for fuel production is not ethically acceptable in the long run and therefore the next generation biofuels must be produced from non-food sources, such as cellulose and hemicellulose present in lignocellulosic biomass (second generation bioethanol).

Renewable non-food sources of lignocellulosic biomass include forestry sidestreams such as logging and wood processing mill residues, removed biomass from forest management and land clearing operations, and agricultural sources such as crop residues (corn stovers, straw), perennial grasses and energy and woody crops (Perlack, et al. 2005). It has been estimated that 5–8% of the annually produced biomass would be sufficient to completely replace the consumption of fossil petroleum, other fossil resources not included (Stark 2011).

Biotechnical ethanol production from cellulosic sources consists of three distinct steps: pretreatment, total enzymatic hydrolysis and fermentation (Lozano, et al. 2012). This approach is known as separate hydrolysis and saccharification (SHF). In addition, simultaneous saccharification and fermentation (SSF), in which the hydrolysis and fermentation steps are carried out in the same vessel simultaneously, is also an increasingly studied option (Wilson 2009). The pretreatment step is needed to increase the substrate digestibility, as lignocellulosic biomass is very recalcitrant towards enzymatic hydrolysis due to various structural factors (Chandra, et al. 2007). The total hydrolysis of polysaccharides in lignocellulosic biomass is carried out by a mixture of polysaccharide-hydrolysing enzymes, cellulases and hemicellulases. Of the commercial enzymes, cellulases are the third most important group due to their diverse applications in *e.g.* cotton processing, additives in animal feed, paper recycling and detergents (Wilson 2009). When large-scale hydrolysis of lignocellulosics becomes established commercially, cellulases are expected to become the largest commercial enzyme group. Cellulases constitute the second largest operational cost factor in bioethanol production after the feedstock (Klein-Marcuschamer, et al. 2010), even though a 20-fold decrease in cellulase costs in bioethanol production has been reported for the last decade (Aden and Foust 2009). Even though some technical and economical challenges remain to be solved for economical production of lignocellulosic ethanol, several demonstration plants are currently already in operation, including in Europe *e.g.* Inbicon in Denmark and Abengoa in Spain, both of which use agricultural waste as feedstock (Larsen, et al. 2012) and many plants in North America and East Asia. Several commercial scale ethanol production facilities are under construction in different countries (Menon and Rao 2012).

1.1 Structure and enzymatic hydrolysis of cellulose

Lignocellulosic biomass consists of three major components: cellulose, hemicellulose and lignin, the ratio of which varies depending on the biomass origin. The lignocellulosic components make up a complex structural matrix which is highly recalcitrant towards hydrolysis (Figure 1). The structure and properties of cellulose, as well as cellulose hydrolysis, are described in the following sections. Hemicelluloses are heteropolymers which consist of a variety of different saccharides (Sjöström 1993). Common hemicelluloses are xylans, mannans and galactans, named after the main sugars of their backbone structure. Hemicelluloses often have branched structures. Lignin is an irregular and complex, branched polymer built up from three different phenylpropanoid monomers: 4-hydroxycinnamyl alcohol, coniferyl alcohol, and sinapyl alcohol, which differ from each other in the degree of methoxylation of the aromatic ring (Campbell and Sederoff 1996). Consisting of aromatic monomers, lignin has a considerable hydrophobic character, in contrast to polysaccharides. Lignin and polysaccharides have also been shown to form covalent bonds with each other, resulting in lignin-carbohydrate complexes (LCCs) (Björkman 1957).

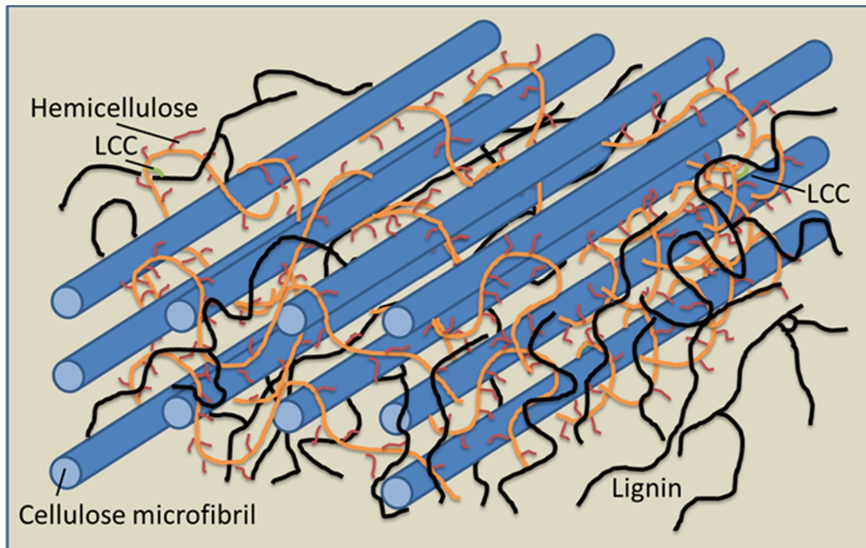

Figure 1. Schematic view of lignocellulosic biomass. The three main components, cellulose, hemicelluloses and lignin form a highly complex and entangled matrix. LCC = lignin-carbohydrate complex.

Biomass polysaccharides, cellulose and hemicelluloses, may be hydrolysed to their constituent monosaccharides either by enzymatic or mineral acid hydrolysis. Enzymatic hydrolysis offers several benefits over acid hydrolysis: 1) no need for corrosion-resistant processing equipment, 2) less acid waste and 3) less formation of undesirable by-products such as 5-hydroxymethylfurfural (HMF), which may be detrimental in downstream processing (Binder and Raines 2010; Dadi, et al. 2006). Further benefits associated with enzymatic hydrolysis includes the potential for almost complete polysaccharide conversion (Wyman, et al. 2005).

1.1.1 Structure of cellulose

Cellulose is a linear, unbranched homopolymer consisting of β-D-glucopyranose units linked by (1→4)-β-glycosidic linkages. The degree of polymerization (DP, the number of polymerized glucose units) of cellulose can vary from 20 (laboratory synthesized cellulose) up to 10 000 anhydroglucose units (AGUs) for some bacterial celluloses (Pinkert, et al. 2009). The AGUs are rotated 180° to each other and thus the (anhydro)cellobiose unit is the smallest structural element of cellulose (Figure 2).

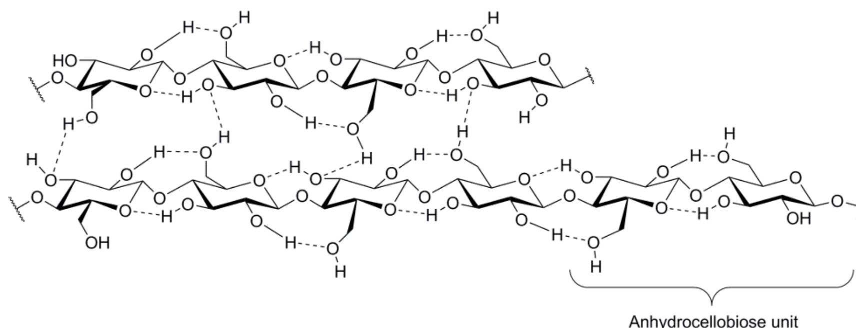

Figure 2. Schematic view of cellulose I. The anhydrocellobiose unit is the smallest repeating structure of the cellulose chain. Each anhydroglucose unit (AGU) forms two intramolecular hydrogen bonds and one intermolecular hydrogen bond.

The cellulose chains form both intra- and intermolecular hydrogen bonds, which makes the cellulose crystallites extremely rigid (Figure 2). Cellulose chains aggregate with each other to form elementary fibrils with a width of 10–20 nm, which contain both highly ordered, crystalline regions and less ordered, *i.e.* amorphous regions (Sjöström 1993). These elementary fibrils build up larger fibrils with which hemicelluloses and lignin finally form lignocellulosic fibers. Cellulose in higher plants is synthesized by cellulose synthase complexes (“rosettes”), which form 36 cellulose chains in parallel; these 36 chains have been proposed to aggregate together soon after synthesis to form the elementary microfibrils (Ding and Himmel 2006). The strong hydrogen bonding in cellulose renders it insoluble in water with increasing DP. As discussed by Zhang and Lynd (2004), cello-oligomers with DP 2–6 are water soluble and DP 7–12 are partially soluble in hot water.

As reviewed by O’Sullivan (1997), cellulose has at least six polymorphs, commonly designated as cellulose I, II, III_{I}, III_{II} and IV_{I} and IV_{II}. In addition, cellulose I has been found to exist as two polymorphs, cellulose Iα and Iβ. Cellulose I is the native form of cellulose, whereas cellulose II can be obtained from cellulose I by regeneration or mercerization. Cellulose II is thermodynamically the most stable form of cellulose. The cellulose chains in cellulose I have a parallel chain direction, whereas the chain direction in cellulose II is antiparallel. The two cellulose I polymorphs, Iα and Iβ, have the same conformation in their general skeleton, but have different hydrogen bonding patterns. The non-crystalline part of cellulose is usually termed amorphous cellulose. Microcrystalline cellulose (MCC or Avicel) is an often employed crystalline cellulose model substrate. It is prepared by partial acid hydrolysis of wood pulp followed by spray-drying (Krassig 1993). Although the amorphous regions should be removed during the acid treatment, MCC has been found to contain a significant fraction (30–50%) of amorphous cellulose (Krassig 1993; Zhang and Lynd 2004) and also some residual hemicelluloses, which may have a significant influence on the substrate properties of MCC in enzymatic hydrolysis (Várnai, et al. 2010).

1.1.2 Enzymatic hydrolysis of cellulose

Cellulose is a very stable molecule and it has been calculated that the uncatalyzed half-life of cellulose through spontaneous hydrolysis would be 5 million years (Wolfenden, et al. 1998). Thus, efficient cellulose-degrading systems are needed for industrial cellulose hydrolysis and in nature to sustain the global carbon cycle. Elwyn Reese, a pioneer in studying the systems of cellulolytic enzymes, proposed a two-step mechanism for cellulose hydrolysis in 1950 (Reese, et al. 1950). In this model two different enzyme activities, C_1 and C_x, were suggested to act stepwise on cellulose. The model has been heavily revised since 1950 and cellulose hydrolysis is currently believed to take place in three simultaneous steps: 1) physical and chemical changes to the yet unhydrolysed solid substrate, 2) primary hydrolysis, in which soluble cello-oligomers are released from the solid cellulose to the hydrolysate, and 3) secondary hydrolysis, in which the dissolved oligomers are hydrolysed to glucose (Zhang and Lynd 2004). The enzymatic hydrolysis of cellulose is performed in synergy by different cellulolytic enzymes, known as endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases. In addition, it has recently been discovered that oxidative enzymes (lytic polysaccharide monooxygenases, LPMOs, some of which are classified as glycosyl hydrolase family GH61 cellulases) act on cellulose as auxiliaries to the hydrolytic cellulases, and cellulase dosage for total hydrolysis may be significantly reduced by addition of these oxidoreductase enzymes (Harris, et al. 2010; Langston, et al. 2011; Quinlan, et al. 2011; Vaaje-Kolstad, et al. 2010). The role and mechanism of LPMOs as auxiliaries in cellulose hydrolysis are still not very well known, but these enzymes appear to catalyze the oxidative cleavage of cellulose chains, in the process oxidizing the C1, C4 or C6 positions in the AGUs. Thus new cellulose chain ends are made available for the cellobiohydrolases. Other non-hydrolytic proteins may also play a role in enhancing cellulose hydrolysis, such as swollenins and expansins (Georgelis, et al. 2013; Gourlay, et al. 2013). The current system of naming and classifying carbohydrate active enzymes (including cellulases and hemicellulases) based on their structure family was introduced by Henrissat et al. (1998) and recently the classifications system for carbohydrate active enzymes has been enlarged with several new groups of auxiliary enzymes (Levasseur, et al. 2013). According to the enlargement of the classification system of the carbohydrate active enzymes, the LPMOs formerly known as GH61 enzymes should now be termed family AA9 enzymes, where AA denotes Auxiliary Activities (for plant cell wall degradation).

Cellulose-degrading enzymes are secreted by a large number of different microorganisms, such as fungi and different bacteria (Enari 1983). The most studied and efficient organism, in terms of cellulase secretion, is the filamentous fungus *Trichoderma reesei* (named in honour of Elwyn Reese). *T. reesei* is known to produce at least two cellobiohydrolases, Cel6A and Cel7A, and five endoglucanases (Cel5A, Cel7B, Cel12A, Cel45A and Cel61A) (Karlsson, et al. 2002) (Table 1). The main enzyme components of the *T. reesei* cellulase system are the

cellobiohydrolases Cel6A and Cel7A, corresponding to 20 and 60% of the total secreted cellulolytic protein, whereas Cel5A which constitutes ~12% of the secreted cellulases, is the main endoglucanase (Teeri 1997; Zhang and Lynd 2004). All *T. reesei* cellulases except Cel12A, are modular enzymes, *i.e.* they consist of a core domain (CD) connected to a carbohydrate-binding domain (CBM) through an *O*-glycosylated peptide linker.

Table 1. Cellulases produced by *Trichoderma reesei*.

Cellulase	Synonym	Modular	Ref.
Cel5A	Endoglucanase II	Yes	Saloheimo, et al. 1988
Cel6A	Cellobiohydrolase II	Yes	Teeri, et al. 1987
Cel7A	Cellobiohydrolase I	Yes	Shoemaker, et al. 1983a, b Teeri, et al. 1983
Cel7B	Endoglucanase I	Yes	Penttilä, et al. 1986
Cel12A	Endoglucanase III	No	Okada, et al. 1998
Cel45A	Endoglucanase V	Yes	Saloheimo, et al. 1994
Cel61A	Endoglucanase IV*	Yes	Saloheimo, et al. 1997

* Recently assigned as lytic polysaccharide mono-oxygenase (LPMO)

The *T. reesei* cellobiohydrolases Cel6A and Cel7A hydrolyse the cellulose chains exclusively from either the non-reducing or reducing chain end, respectively (Teeri 1997). Cellobiohydrolases have their active site in a tunnel through the CD, whereas the active sites of the endoglucanases are located in a cleft on the CD surface. The tunnel-shaped active site of *T. reesei* Cel7A was elucidated by Divne et al. (1994) and based on the tunnel structure, the enzyme was proposed to hydrolyse cellulose in a processive manner, *i.e.* without desorption of the cellulase from the cellulose between the catalytic events. In later studies the tunnel has been found to contain ten specific sites for cellulose binding (Divne, et al. 1998). Cel6A hydrolyses the cellulose from its non-reducing end (Chanzy and Henrissat 1985), but its action is not as processive as it is for Cel7A (Igarashi, et al. 2009). As discussed by Srisodsuk et al. (1998), some cellobiohydrolases have also been suggested to exhibit endoactivity, but it has been much under debate whether the observed endoactivity really is a property of the cellobiohydrolase or whether the studied enzyme preparations have contained endoglucanase as minor impurities. The catalytic mechanism can be either retaining or inverting, depending on whether the C1 anomeric centre at the hydrolysed AGU has its stereochemistry retained or inverted in the process. The hydrolytic cleavage of the glycosidic bond takes place *via* an acid catalysis mechanism in which two carboxylic acid groups act together, one activating the glycosyl bond while the other assists in the nucleophilic attack of water (Withers 2001).

The *T. reesei* endoglucanases are believed to work in a non-processive manner, probably through cycles of adsorption and desorption between the catalytic events (Linder and Teeri 1996). Endoglucanases can act on both unsubstituted

and substituted celluloses, *e.g.* on carboxymethylcellulose (CMC), hydroxyethyl-cellulose (HEC), cellodextrins and phosphoric acid swollen cellulose and are as such not very specific (Enari 1983). Endoglucanases are generally believed to attack only the amorphous regions of the cellulose. β-Glucosidases hydrolyse cellobiose and cello-oligosaccharides to glucose. Cellobiose causes strong end-product inhibition of the cellobiohydrolases, which is why cellobiose needs to be continuosly hydrolysed to glucose. A schematic overview of enzymatic hydrolysis of cellulose with the *T. reesei* cellulase system is presented in Figure 3.

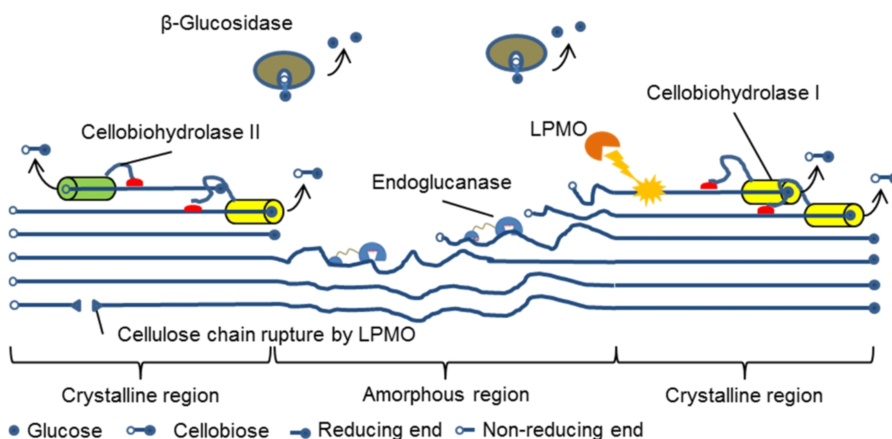

Figure 3. Schematic view of cellulose hydrolysis with the *Trichoderma reesei* cellulase system. LPMO = lytic polysaccharide mono-oxygenase enzyme.

T. reesei cellulases act synergistically, meaning that combining two or more cellulases produces more hydrolytic events together than the cumulative sum of the independent components. Most commonly synergism is exemplified by the endo/exo synergism, in which the endoactive cellulases hydrolyse intercrystal amorphous regions, thus providing the exoactive cellulases with new cellulose chain ends to work on (Srisodsuk, et al. 1998). In all, at least seven different modes of cellulase synergy have been described (Zhang and Lynd 2004).

The structure and composition of cellulosic substrates highly affect their enzymatic digestibility. Amorphous cellulose is typically hydrolysed much faster than crystalline cellulose by fungal cellulases. The high crystallinity of native cellulose hinders specifically the action of endoglucanases, whereas in regenerated cellulose (RC) more glycosidic bonds are available for scission (Dadi, et al. 2006). As reviewed by Zhang and Lynd (2004), amorphous cellulose may be hydrolysed with up to 30 times greater hydrolysis rates than crystalline cellulose, suggesting a cellulose hydrolysis model in which the amorphous regions are hydrolysed preferably before the crystalline regions. However, several studies have shown that crystallinity of the cellulosic substrate does not change during enzymatic hydrolysis (Kent, et al. 2010; Penttilä, et al. 2010; Puls and Wood 1991). Similarly, crystallite

size appears to remain constant during enzymatic cellulose hydrolysis (Penttilä, et al. 2010).

Cellulose crystallites in RC are usually in the form of cellulose II, which is more readily hydrolysed with enzymes than cellulose I (native cellulose) (Wada, et al. 2010). It has been suggested that van der Waals forces between the sheets of cellulose I would be stronger than in cellulose II, thus rendering cellulose I more recalcitrant towards hydrolysis (Wada, et al. 2010). Especially hydrated cellulose II has a higher hydrolysability than cellulose I. Cellulose crystallinity appears to impact more on initial hydrolysis rates than on maximum conversion (Zhu, et al. 2008). The mechanisms of enzymatic hydrolysis are partly different for regenerated substrates as compared to native lignocellulosic biomass. Especially the endoglucanase component of the cellulase cocktails has been reported to cause substantially more random hydrolysis, leading to DP reductions, on regenerated, more amorphous cellulose substrates (Engel, et al. 2012a). Recently ionic liquids (ILs) have been used to produce regenerated cellulose and lignocellulosics. For more efficient cellulase utilization, cellulase cocktails have been optimized for use on substrates regenerated from IL solution (Barr, et al. 2012; Engel, et al. 2012b).

The substrate DP is an important parameter for cellobiohydrolases, as a lower DP indicates relatively more chain ends for the cellobiohydrolases to work on (Zhang and Lynd 2004). Endoglucanase action is not known to be dependent on substrate DP. Cellulose accessibility both in terms of inner and outer surface area is important for cellulase adsorption and hydrolysis. In the total hydrolysis of lignocellulosic biomass, the accessibility of cellulose within the lignified and hemicellulose-containing matrix is especially important for efficient hydrolysis.

1.1.3 Role of the carbohydrate-binding module and cellulase substrate binding in cellulose hydrolysis

The presence and role of carbohydrate-binding modules (CBMs) in *T. reesei* cellulases were first reported for the cellobiohydrolase Cel7A (van Tilbeurgh, et al. 1986), and soon Cel6A was also reported to have a similar structure (Tomme, et al. 1988). Cleaving off the CBM from the *T. reesei* cellobiohydrolases resulted in the cellobiohydrolase core domains (CDs), which had 50–90% less hydrolytic activity on solid crystalline substrates, whereas the hydrolysis efficiency on small soluble substrates remained unchanged (Tomme, et al. 1988; van Tilbeurgh, et al. 1986). In the hydrolysis of amorphous cellulose, Cel6A CD exhibited a significant decrease in hydrolysis kinetics compared to the intact cellulase, whereas Cel7A and its CD had similar hydrolysis rates, indicating that the CBMs and CDs may have different binding and associated hydrolysis behaviour depending on the substrate morphology. At least three functions have been proposed for the CBMs: to increase the cellulase concentration close to the substrate through adsorption (Igarashi, et al. 2009; van Tilbeurgh, et al. 1986); to physically disrupt the cellulose prior to hydrolysis (amorphogenesis) (reviewed by Arantes and Saddler 2010; Din,

et al. 1994); and to target the cellulase towards specific regions on the substrate (Carrard, et al. 2000; Fox, et al. 2013).

Fungal CBMs (including those from the major cellobiohydrolases and endoglucanases of *T. reesei*) show great structural similarity and are all characterised as family 1 CBMs (Linder, et al. 1995b). The structure of these CBMs has been shown to be wedge-shaped with a rough and a flat face (Kraulis, et al. 1989). The flat face contains three aromatic amino acid residues by which the CBM interacts with the crystalline cellulose during binding. All three of these aromatic residues are tyrosines in the CBM of Cel7A, whereas one of the tyrosines is replaced by a tryptophan in the CBM of Cel5A (Linder, et al. 1995b). Replacement of a tyrosine with a tryptophan has been shown to greatly increase the cellulose binding affinity of the CBM (Linder, et al. 1995a). The spacing of the aromatic amino acid residues has been suggested to allow stacking with every second AGU of the bound cellulose chain (Tormo, et al. 1996). The flat face also contains some charged amino acid residues which are believed to participate in cellulose binding by forming hydrogen bonds (Linder, et al. 1995b).

Both the CBM and CD of *T. reesei* Cel7A bind to cellulose, but the binding of CBM is much tighter (Ståhlberg, et al. 1991). The presence of CBM does not appear to have any impact on the catalytic turnover number of the cellulases, which is linked to the sliding speed on the substrate. For both intact *T. reesei* Cel7A and its CD, this parameter was shown by high speed atomic force microscopy to be the same: 3.5 nm/s on cellulose chains (Igarashi, et al. 2009). Thus, the CBM of Cel7A has been concluded not to detach the cellulose chains from the crystalline regions and feed the chains into the catalytic tunnel, but that its role is mainly to increase the enzyme concentration close to the substrate. The hydrolysis rate per adsorbed cellulase unit has been observed to be the same on filter paper for both Cel7A and its CD, indicating that the CBM does not endow the intact enzyme with any extra catalytic properties (Nidetzky, et al. 1994). The role and structure of CBMs have been reviewed by *e.g.* Boraston et al. (2004) and Shoseyov et al. (2006).

The CBMs of *T. reesei* Cel6A and Cel7A are similar in structure and binding affinity, but whereas Cel7A CBM binds completely reversibly to cellulose (Linder and Teeri 1996), the CBM from Cel6A binds partially irreversibly (Carrard and Linder 1999). Addition of organic solvents, such as ethanol or dimethylsulphoxide (DMSO), results in decreased binding of both Cel6A and Cel7A CBMs, as is expected because substrate binding takes place mostly *via* hydrophobic interactions (Carrard and Linder 1999). The cellulose binding of *T. reesei* Cel6A and Cel7A CBMs is not pH sensitive, varying by less than 10% over a pH range of 2.5–11 (Carrard and Linder 1999). When the effect of temperature, ionic strength and pH on the binding of *T. reesei* cellulases was studied, temperature was observed to affect the binding significantly, with higher temperatures leading to decreased binding affinity, whereas the effects of ionic strength and pH varied between different enzymes (Kyriacou, et al. 1988).

A sequence comparison of known cellulases showed that the majority of the known putative cellulase genes actually do not contain a CBM (Várnai, et al. 2013). The CBM has been shown to be useful for *T. reesei* cellulases at low substrate

consistencies, whereas at high substrate consistency (20%), the CD and intact cellulases show very similar performance in cellulose hydrolysis. Screening for the most efficient cellulase candidates in diluted systems has apparently led to favouring the isolation of cellulases with CBM.

1.2 Ionic liquids in cellulose dissolution and lignocellulose pretreatment

1.2.1 Ionic liquids

Research into ionic liquids (ILs) is considered to have started with the synthesis of ethylammonium nitrate in 1914 (Walden 1914). Since a NATO Advanced Research Workshop in 2000, ILs have generally been defined as salts with melting points below 100 °C (Sun, et al. 2011). Much of the early work on ILs was carried out at the US Air Force Academy, where experiments were carried out with chloroaluminates for use in thermal batteries (Wilkes, et al. 2008). Soon, these were combined with imidazolium and pyridinium cations. The use of these ionic liquids was limited for a long time due to their reactivity with water. In the early 1990s water stable dialkylimidazolium ILs were introduced and IL chemistry as it is known today was born. Very few scientific articles dealing with ILs had appeared before 2000, whereafter the number of papers started growing rapidly (Seddon 2008). The number of potential ILs has been estimated to at least a million compounds, which means that the synthetic diversity is also great. The synthesis, purification and characterization of ILs have been reviewed by Clare et al. (2009).

ILs have some highly desirable properties. They are often called green solvents due to their very low vapour pressures, thus eliminating any emissions of volatile organic compounds (VOCs) (van Rantwijk and Sheldon 2007; Yang and Pan 2005). In addition, ILs are generally considered to be thermally and chemically stable, their properties are tunable (*e.g.* polarity, hydrophobicity) by making the correct choice of ions, and they have good solvent properties, making them good solvents for a wide range of different compounds. Due to their complex structures, ILs are able to interact with solutes through a great variety of different interactions, including dispersive, π - π, n - π, hydrogen bonding, dipolar and ionic/charge-charge interactions (Anderson, et al. 2002). ILs have low melting points, mainly due to their large and asymmetric cations, which give the salts low lattice energies for crystallisation (Huddleston, et al. 2001).

Depending on their ionic composition, ILs may be acidic, neutral or basic (MacFarlane, et al. 2006). The acid-base behaviour of different compounds may differ in ILs from those under aqueous conditions. Relatively weak acids may be very strong acids in ILs, as even weakly basic anions will shift the dissociation equilibrium strongly to the right for acid solutes because of the anion's high concentration. Acid-base conjugate ILs may be distillable if there is an equilibrium between the IL form and the unconjugated acid and base, *e.g.* in an IL with a weak basic anion and a cation with an exchangeable proton (MacFarlane, et al. 2006).

ILs have been distilled as intact ions or aggregates thereof (Earle, et al. 2006) or as anion-cation pairs (Leal, et al. 2007) at very low pressures. The distillation of 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO) and similar ILs has been patented (Massonne, et al. 2009). Acid-base conjugate ILs based on 1,1,3,3-tetramethylguanidium (TMG) or 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) are distillable under rather mild conditions (100–200 °C at ~1 mbar) as their unconjugated components (King, et al. 2011; Parviainen, et al. 2013).

IL polarity is not a simple concept and several different methods have been employed to determine the polarity of diverse ILs, usually giving rise to partially contradictory results (van Rantwijk and Sheldon 2007). ILs could be expected to be highly polar due to their ionic nature, but results have indicated that ILs have polarities similar to those of short chain alcohols and formaldehyde (Chen, et al. 2006c). IL polarity has little to do with the concept of hydrophobicity and hydrofilicity, and therefore predicting IL water miscibility is not straight forward (van Rantwijk and Sheldon 2007). ILs, even those labeled as hydrophobic, are at the same time hygroscopic and usually absorb some water if allowed to equilibrate with environmental moisture (Seddon, et al. 2000), which may be troublesome when carrying out reactions requiring dry conditions. Water and other solvents greatly decrease IL viscosity (Seddon, et al. 2000) and have a greater impact on the physical properties of hydrophilic ILs than on those of hydrophobic ILs (Huddleston, et al. 2001). The high viscosity of ILs may cause mass transfer problems and also causes problems in IL handling and unit operations.

ILs have a reputation of being thermostable, but studies give very different decomposition temperatures for different ILs (Huddleston, et al. 2001; Kosmulski, et al. 2004). Although ILs do not typically produce VOC emissions, their generical “greenness” has been much aquestioned. Some common IL ions are hydrolytically unstable, especially the fluorinated BF_4^- and PF_6^- anions, which may be hydrolysed to highly toxic HF (Swatloski, et al. 2003). ILs are not always inert solvents, as the C2 proton of the imidazolium ring may be deprotonated by a base to generate a highly nucleophilic carbene, which will react with most electrophiles (Aggarwal, et al. 2002). Many dialkylimidazolium ILs exhibit high ecotoxicity, which appears to increase with alkyl chain length (Docherty, et al. 2005). The biodegradability of many imidazolium-based ILs also appears to be negligible (Gathergood, et al. 2004). Other types of ILs, such as ILs based on cholinium or amino acids, are expected to be greener (van Rantwijk and Sheldon 2007). When registered for the European REACH (Registration, Evaluation, Authorisation and restriction of CHemicals) legislation, 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) was labeled toxic whereas [EMIM]AcO was labeled non-toxic (Sun, et al. 2011).

Depending on their properties and solvent miscibility, ILs may be used in many different modes of operation: as pure solvent, as co-solvent or in biphasic (and even triphasic) systems (Kragl, et al. 2002). Several examples of industrial applications of ILs already exist (Maase 2008). The most well-known example is the BASIL™ (Biphasic Acid Scavenging utilizing Ionic Liquids) process introduced in 2002 by BASF, in which an IL precursor (methylimidazole) is used as acid scavenger, forming an IL upon neutralization of an acid produced in the process. A

productivity increase of $8x10^4$ compared to the earlier process was achieved, demonstrating the potential of IL technology. PetroChina has introduced an industrial scale process for isobutane alkylation, in which an IL is used as catalyst (Liu, et al. 2006). Several other industrial applications of ILs have been proposed and some of the following examples have been tested in pilot scale: use of IL as entrainer in reactive distillation, use of ILs in chlorination, ether cleavage, olefin oligomerization, hydrosilylation and fluorination reactions (Maase 2008). Further applications include electroplating processes, additives to cleaning fuels, gas storage liquids etc. ILs have been considered to be expensive, but are expected to become cheaper with increasing use. It is predicted that on mid-term time scale many ILs will become available in multi-ton quantities for 25–50 $/kg (Gordon and Muldoon 2008); more optimistic price estimations go as far down as 2.2 $/kg (Reddy 2006).

1.2.2 Cellulose dissolution in ionic liquids

Native cellulose is not easily soluble in conventional solvents. Cellulose has traditionally been dissolved in both derivatizing and non-derivatizing solvents, which may be either aqueous or non-aqueous media (reviewed by Heinze and Liebert in 2001). Dissolution of cellulose was first reported by Swatloski *et al.* (2002) in the IL [BMIM]Cl. Soon afterwards, a new type of allyl-functionalized imidazolium-based IL, 1-allyl-3-methylimidazolium chloride ([AMIM]Cl), was introduced as a powerful cellulose solvent (Wu, et al. 2004; Zhang, et al. 2005). The patent of Graenacher from 1934 has been considered to be the first account of dissolving cellulose in an IL type of solution (Graenecher 1934), but the used solvent, water-free benzylpyridinium chloride ([BzPy]Cl), contained 1–2% dry pyridine and was as such not a pure salt, neither does the pure [BzPy]Cl fit the generally accepted IL definition of having a melting point below 100 °C. Some ILs have also been found to be good solvents for native lignocellulosic biomass and even the dissolution of wood has been reported (Kilpeläinen, et al. 2007). During the last decade, cellulose and wood dissolution in ILs and the underlying mechanisms have been extensively studied and reviewed (Mäki-Arvela, et al. 2010; Pinkert, et al. 2009; Zakrzewska, et al. 2010; Zhu, et al. 2006). Homogeneous functionalization and derivatization of cellulose in IL solution has been reviewed by Barthel and Heinze (2006) and by Liebert and Heinze (2008).

According to the reviews cellulose-dissolving ILs are based on imidazolium, pyridinium, pyrrolidinium, cholinium, tetrabutylammonium, tetrabutylphosphonium and alkylalkyloxyammonium cations as well as on protonated/alkylated polycyclic amidine bases, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and DBN (Figure 4). Reported anions are *e.g.* halogens, carboxylates, amides, imides, thiocyanates, phosphates, sulphates, sulphonates and dichloroaluminates. Cellulose solubility in an IL is clearly dependent on the type of both the cation and the anion. The cellulose dissolution is very sensitive to the presence of water (Swatloski, et al. 2002). Water is thus also an efficient anti-solvent for precipitating cellulose.

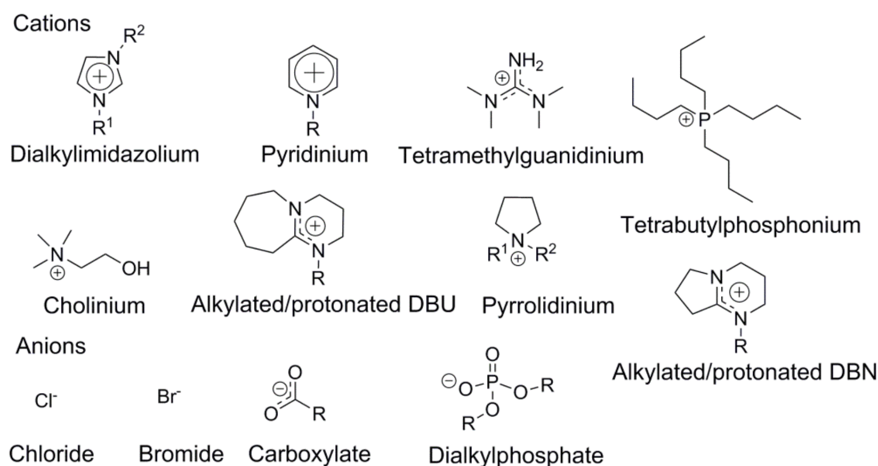

Figure 4. Different ions encountered in cellulose-dissolving ILs.

Halide-free dialkylimidazolium ILs are often encountered as cellulose solvents: dialkylphosphates (*e.g.* 1,3-dimethylimidazolium dimethylphosphate, [DMIM]DMP, reported as cellulose solvent by Mazza et al. in 2009) and acetates, of which [EMIM]AcO is a very powerful cellulose solvent (Zavrel, et al. 2009). The structures of the frequently used cellulose-dissolving ILs are presented in Figure 5. Recently, several new classes of ILs have been introduced as cellulose solvents in addition to the well-known imidazolium-based ILs. ILs consisting of conjugated acid-base pairs with cations based on the organic superbases TMG or DBN dissolve cellulose well and are reported to be distillable under rather mild conditions (King, et al. 2011; Parviainen, et al. 2013). Up to 20% (w/w) of cellulose could be dissolved in 5 min in an aqueous solution of 60% (w/w) tetrabutylphosphonium hydroxide ([P4444]OH) under very mild conditions (Abe, et al. 2012). Alkylalkyloxyammonium amino acid ILs were reported as cellulose solvents by Ohira et al. (2012a). 1-Hexylpyridinium chloride ([HPy]Cl) has also been shown to solubilize cellulose (Uju, et al. 2013). Very high cellulose dissolution rates have been reported in systems with cellulose-dissolving ILs diluted with aprotic co-solvents, such as 1,3-dimethyl-2-imidazolidinone (DMI), dimethylformamide (DMF) and DMSO (Ohira, et al. 2012b; Rinaldi 2011).

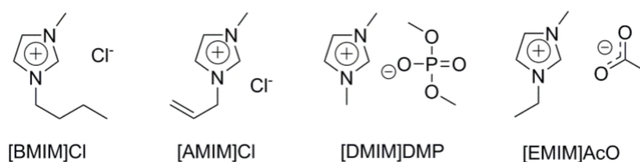

Figure 5. The most studied cellulose-dissolving imidazolium-based ILs: 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-allyl-3-methylimidazolium chloride ([AMIM]Cl), 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP) and 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO).

Imidazolium-, piperidinium- and ammonium-based ILs with polyethylene glycol (PEG) subsitutents have been introduced as especially designed enzyme-compatible cellulose solvents (Figure 6) (Tang, et al. 2012; Zhao, et al. 2008; Zhao, et al. 2009a; Zhao, et al. 2009b). The PEG substituents play a dual role in interacting with the dissolved cellulose and stabilising the enzyme through the oxygen atoms in the PEG, and the large size of the PEG-substituted cation dilutes the molar anion concentration, which has been suggested to be directly linked to enzyme inactivation (Tang, et al. 2012; Zhao, et al. 2008; Zhao, et al. 2009b). Another cellulose-dissolving enzyme-compatible IL, 1-hydroxyethyl-3-methylimidazolium acetate ([E(OH)MIM]AcO), which has the structure of [EMIM]AcO with a terminal hydroxyl group on the ethyl side chain of the cation, was reported by Li et al. (2012).

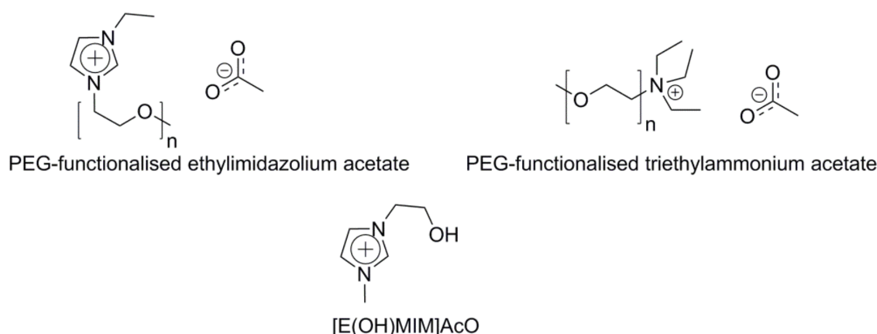

Figure 6. Tailor-made enzyme-compatible cellulose-dissolving ILs: polyethylene glycol (PEG) functionalised imidazolium and ammonium acetates and [E(OH)MIM]AcO (Li, et al. 2012; Tang, et al. 2012; Zhao, et al. 2008; Zhao, et al. 2009a; Zhao, et al. 2009b).

Cellulose-dissolving ILs are in general hydrophilic (Zhao, et al. 2008). The basicity of the anion of the ILs is generally regarded as a key property for cellulose dissolution, as the anion is believed to break up the hydrogen bond network which holds the cellulose chains together (Swatloski, et al. 2002; Tang, et al. 2012; Zhao, et al. 2009b).

Kamlet-Taft parameters can be used to predict cellulose solubillity in ILs (Doherty, et al. 2010). The Kamlet-Taft parameters are determined based on measuring solvatochromic effects (by UV-Vis) of the studied solvent on a set of dyes. Commonly used Kamlet-Taft parameters for ILs are hydrogen bond acidity (α), hydrogen bond basicity (β) and dipolarity/polarizability effects (π*) (Crowhurst, et al. 2003). Generally the β parameter (hydrogen bond basicity) efficiently predicts cellulose solubility (Doherty, et al. 2010), although recently the β-α parameter ("net basicity") has been proposed to predict cellulose solubility even more accurately (Hauru, et al. 2012). The presence of water in the solution directly decreases the β parameter, explaining the precipitation of cellulose in the waterbath regeneration process. The role of the IL cation in cellulose dissolution is somewhat under debate. Mostly, imidazolium-based cations have been studied. Already Swatloski et al. (2002) noticed that the structure of the cation also affects cellulose dissolution, as increasing the chain length of the alkyl substituents on the imidazolium led to sharply decreasing cellulose solubility. The imidazolium cation has been suggested to have hydrophobic interactions with the hydrophobic side of the cellulose (Liu, et al. 2010; Youngs, et al. 2007). The C2 proton on the imidazolium has also been proposed to interact as a weak hydrogen bond donor to the hydroxyl group oxygens in cellulose during dissolution (Youngs, et al. 2007). Recent results also suggest the cation acidity to be an important parameter for predicting cellulose solubility (Parviainen, et al. 2013). Cellulose solubility and dissolution rates are greatly affected by the IL's viscosity. Heating reduces the solution viscosity and increases cellulose solubility (Mazza, et al. 2009). Microwave irradiation can also be used to increase cellulose dissolution rates in ILs (Swatloski, et al. 2002).

1.2.3 Ionic liquid pretreatments of lignocellulosics for total enzymatic hydrolysis

Native lignocellulosic biomass is very recalcitrant towards enzymatic hydrolysis, which necessitates excessive substrate pretreatments before hydrolysis. The pretreatment process has been identified as a very expensive processing step in biorefineries and it is also decisive for designing down-stream processing (Wyman 2007). As reviewed by Chandra et al. (2007), substrate-related factors affecting the efficiency of enzymatic hydrolysis of lignocellulose are the presence and form of lignin and hemicelluloses, cellulose crystallinity, cellulose DP, and the accessible surface area of the substrate. Hemicelluloses and lignin impede cellulose hydrolysis by forming a physical barrier around the cellulose (Grethlein 1985; Mooney, et al. 1998) (Figure 1), and enzymes can also bind non-specifically to lignin (Sutcliffe and Saddler 1986). A good pretreatment process should have low capital and operating costs and be effective on a variety of different substrates, the generated side streams (hemicelluloses, lignin) should be easy to recover for further use and the formation of by-products, such as furfurals should be avoided, as these are known in many cases to inhibit down-stream processing (Chandra, et al. 2007). Furthermore, substrates which can be efficiently hydrolysed by enzymes at high substrate

loadings, with short residence times and with low enzyme concentrations should be produced (Shill, et al. 2011).

Conventional pretreatment processes are in general divided into physical, chemical and biological processes, or combinations thereof (Chandra, et al. 2007). Biological processes typically use wood degrading fungi, but the long treatment times (10–14 days) and large space requirements render this pretreatment option rather unattractive. Physical pretreatment methods involve different types of milling, reducing particle size and cellulose crystallinity while the surface area is increased. Benefits include good compatiblity with different substrate types, but high energy costs are a distinct drawback and lignin is not removed. Chemical pretreatment processes are based on both acid and alkali treatments, which primarily remove hemicelluloses and lignin. Organosolv treatments have similar lignin-removing effects. Cellulose crystallinity can be modified by treatments with cellulose swelling or dissolving solvents and reagents. Alkali- and acid-based pretreatments have the drawback of needing neutralization of the processing liquids, which in large scale can be problematical. Physicochemical pretreatment processes include steam explosion, ammonia freeze (or fibre) explosion (AFEX) and ammonia recycle percolation (ARP). In the current demonstration scale plants (*e.g.* Inbicon in Kalundborg, Denmark, SEKAB in Örnsköldsvik, Sweden and Abengoa in Salamanca, Spain) producing lignocellulosic ethanol steam/hydrothermal pretreatment is applied, in some cases combined with H_2SO_4 or SO_2 catalysis (Larsen, et al. 2012).

Certain ILs are promising in biomass pretreatment due to their unique ability to dissolve lignocellulosic biomass. The cellulosic component can be precipitated from IL solution by the addition of an anti-solvent, such as water or alcohol, and the precipitated cellulose fraction is very susceptible to subsequent enzymatic total hydrolysis (Dadi, et al. 2006). Kilpeläinen et al. (2007) were the first to demonstrate the complete dissolution of softwood in IL ([BMIM]Cl and [AMIM]Cl). In addition to softwood, hardwood has also been dissolved in ILs ([AMIM]Cl and [EMIM]AcO being the most efficient) (Zavrel, et al. 2009). Complete dissolution and regeneration of lignocellulosics is a moisture-sensitive process, as the presence of only 5–10% of water in the cellulose-dissolving imidazolium-ILs has been reported to lead to significantly decreased pretreatment efficiency (Doherty, et al. 2010). Cellulose regeneration from ILs for total hydrolysis necessitates a thorough washing of the regenerated substrate, as even residual amounts of many cellulose-dissolving ILs in the substrate have been shown to cause severe inactivation of the cellulases and inhibit down-stream microbial conversions (Hong, et al. 2012; Zhao, et al. 2009a). Whereas IL pretreatment prior to enzymatic total hydrolysis of lignocellulosics is a much studied topic, several groups have also been active in studying acid hydrolysis of plant cell wall polysaccharides dissolved in ILs (Binder and Raines, 2010; Sievers, et al. 2009; Sun, et al. 2013a). Fair hydrolysis yields have been reported for acid hydrolysis in ILs, but the by-product formation of furfurals, which are detrimental for down-stream microbial processes, could not fully be avoided in these systems.

Dadi et al. were the first to report that dissolution of MCC in IL ([BMIM]Cl or [AMIM]Cl) followed by regeneration dramatically accelerated the enzymatic hy-

drolysis rate (Dadi, et al. 2006; Dadi, et al. 2007). The increased digestibility of the regenerated cellulose (RC) was apparently due to decreased cellulose crystallinity. Thereafter, the number of articles about IL pretreatment has increased rapidly. Table 2 gives an overview of selected IL pretreatment articles published during recent years. The total number of articles in this field is over 400 (in mid-2013) and rapidly increasing. The table illustrates the variety of lignocellulosic substrates and ILs studied, as well as the main effects of the IL pretreatment on the substrate.

The most frequently used ILs in biomass pretreatment are [EMIM]AcO, [BMIM]Cl and [AMIM]Cl (Table 2). Dialkylphosphates, most notably [DMIM]DMP, and alkyloxyalkylsubstituted imidazolium ILs are also used. Recently cholinium-based ILs, as a completely new IL class, have also been of interest for pretreatment. MCC, as a highly crystalline cellulosic model compound, is the most studied hydrolysis substrate for pretreatment. During recent years, IL pretreatment has also been studied with more industrially relevant substrates, such as corn stover, rice and wheat straw, switchgrass and wood meal. The impact of the pretreatment depends on both the type of substrate (native lignocellulosic biomass *vs.* pure cellulose) and the type of IL. The IL pretreatments increase the enzymatic digestibility of the substrates through three main effects: 1) decrystallization or crystallinity transformation from cellulose I to cellulose II, 2) removal of hemicelluloses and lignin and 3) partial depolymerization of the cellulose. Reported crystallinity changes during pretreatment involve both decreasing total crystallinity and transitions from cellulose I to cellulose II (Bian, et al. 2014; Cheng, et al. 2011; Dadi, et al. 2006; Dadi, et al. 2007). Hemicellulose removal can be achieved by mildly treating the lignocellulose with diluted solutions of certain ILs (20 or 50%) (Hou, et al. 2012; Hou, et al. 2013a; Hou, et al. 2013b). IL pretreatment also appears to break up the LCCs (Singh, et al. 2009). Treatments leading to DP reductions are directly beneficial for enzymatic hydrolysis, as cellobiohydrolases are dependent on finding cellulose chain ends as starting points for hydrolysis (Zhang and Lynd 2004). DP decreases have been reported for IL pretreatments in several articles (Bian, et al. 2014; Uju, et al. 2013). IL pretreatments have also been carried out with very high solid loadings (Wu, et al. 2011), in sequential combination with other pretreatments (Geng and Henderson 2012) or in conjunction with microwave irradiation or sonication (Ha, et al. 2011; Li, et al. 2011; Liu and Chen 2006; Yang, et al. 2010). Research interest has partly moved from the complete dissolution of cellulose and biomass to lighter IL pretreatments, such as extracting the lignin from biomass. Pretreatments can be optimized either for increased hydrolysis kinetics or maximum digestibility of the substrate.

Table 2. Selected references of IL pretreatment of lignocellulosic biomass for total hydrolysis.
MCC = microcrystalline cellulose, LCC = lignin-carbohydrate complex, DP = degree of polymerization.

Substrate	IL(s)	Impact on substrate properties	Other comments	Ref.
MCC	[BMIM]Cl	Decrystallization	50-fold increase in hydrolysis kinetics	Dadi, et al. 2006
MCC	[BMIM]Cl, [AMIM]Cl	Decrystallization	90-fold increase in hydrolysis kinetics, optimized	Dadi, et al. 2007
MCC	Six dialkylphosphate imidazolium ILs	Decrystallization, decrease in DP	Ultrasonic treatment enhanced pretreatment efficiency	Yang, et al. 2010
MCC	[DMIM]DMP	Crystallinity changes	Decrystallization not the only effect on digestibility	Xie, et al. 2012
MCC	[BMIM]Cl	Decrystallization		Lozano, et al. 2012
MCC, filter paper, cotton	[BMIM]Cl, [AMIM]Cl, four alkyloxyalkyl substituted imidazolium and ammonium acetates	Decrystallization	Residual ILs potentially inactivated cellulases	Zhao, et al. 2009a
MCC, α-cellulose, Sigmacell	[EMIM]AcO	Increased porosity and accessibility		Engel, et al. 2012a
MCC, switchgrass, pine, eucalyptus	[EMIM]AcO	Crystallinity changes, swelling, potentially DP decreases	Different effects on crystallinity depending on original substrate	Cheng, et al. 2011
MCC, straw, willow, pledget	Large variety of different IL classes	Decrystallization		Li, et al. 2011
MCC, bagasse	[HPy]Cl, [EMIM]AcO	Delignification, crystallinity changes, DP reductions	[HPy]Cl caused significant release of cello-oligomers	Uju, et al. 2013
MCC, corn stover	[BMIM]Cl	Decrystallization	Comparison between [BMIM]Cl and H_3PO_4 treatments	Sathitsuksanoh, et al. 2012
MCC, filter paper, cotton	[EMIM]AcO, [BMIM]Cl	Drastic DP decreases, changes in crystallinity	Microwave irradiation significantly enhanced pretreatment efficiency	Ha, et al. 2011
MCC, rice straw	Cholinium glycine	Lignin and partial hemicellulose extraction		Liu, et al. 2012
Cotton	[BMIM]Cl	Decrystallization, crystallinity changes, DP decreases, increased surface area	Comparison between pretreatments with [BMIM]Cl, H_3PO_4, NaOH/urea and NMMO	Kuo and Lee 2009
Cotton waste textiles	[AMIM]Cl		Down-stream processing very sensitive to residual [AMIM]Cl	Hong, et al. 2012
Switchgrass	[EMIM]AcO	LCCs disrupted, lignin extraction		Singh, et al. 2009
Switchgrass	[BMIM]Cl	Decrease in DP due to added solid acid catalyst	Prehydrolysis with acid during IL pretreatment decreased enzyme requirement with 99%	Groff, et al. 2013

Table 2. (continued)

Substrate	IL(s)	Impact on substrate properties	Other comments	Ref.
Switchgrass, poplar	[EMIM]AcO	Decrystallization		Barr, et al. 2012
Corn stover	[BMIM]Cl	Lignin removal (by alkali extraction), decrystallization by IL treatment	Combined sequential treatment: alkali + IL	Geng and Henderson 2012
Corn stover	[EMIM]AcO	Decrystallization and lignin extraction	Very high (up to 50%) solid content in IL pretreatment step	Wu, et al. 2011
Corn cob	Nine imidazolium-based ILs	Decrystallization, increase in substrate surface area	Dialkylphosphate anions combine biomass solubility and enzyme compatibility	Li, et al. 2010
Wheat straw	[EMIM]DEP, [BMIM]Cl, [EMIM]AcO, [EMIM]DBP, [EMBy]DEP	Decrystallization ([EMIM]DEP studied in detail)		Li, et al. 2009
Wheat straw	[BMIM]Cl	Decrystallization, decrease in DP, increased substrate accessiblity		Liu and Chen 2006
Rice straw	Cholinium amino acids	Lignin removal		Hou, et al. 2012
Rice straw	Aqueous cholinium amino acids	Lignin removal	50% IL sufficient for delignification, reduced solution viscosity	Hou, et al. 2013a
Rice straw	20% cholinium lysine in water	Lignin removal	Process optimized for delignification without hemicellulose removal	Hou, et al. 2013b
Energy cane bagasse	[EMIM]AcO	Decrystallization, delignification		Qiu and Aita 2013
Cellulose from sugar cane bagasse	[EMIM]AcO	50% DP decrease and crystallinity change of cellulose, increased surface area		Bian, et al. 2014
Alkali extracted sugar cane bagasse	[EMIM]DEP	Disruption of cellulose structure	Low IL concentrations in pretreatment (0–20%)	Su, et al. 2012
Milled poplar wood	[EMIM]AcO	Crystallinity changes	Comparison with acid pretreatment included	Goshadrou, et al. 2013
Maple wood flour	[EMIM]AcO	Decrystallization, lignin extraction	Potential for lignin recovery for further use	Lee, et al. 2009
Alkaline treated eucalyptus	[AMIM]Cl, [BMIM]Ace, [BMIM]Cl, [EMIM]AcO	Various effects depending on IL	$MgCl_2$ or H_2SO_4 added as catalysts in pretreatment	Sun, et al. 2013b
Fibre sludge	[AMIM]Cl, [BMIM]Cl	Increased cellulose accessibility		Holm, et al. 2012
Poplar seed floss	[BMIM]Cl, [EMIM]Cl, [BMPy]Cl	Decrystallization		Bodirlau, et al. 2010
Kenaf core fibre	Cholinium mono- and dicarboxylates	Extraction of hemicelluloses and lignin	Cholinium carboxylates were found to have low toxicity compared to [EMIM]AcO	Ninomiya, et al. 2013

The current high price of especially imidazolium-based ILs sets high recovery targets for ILs in biorefinery applications. The extensive washing of regenerated substrates to remove trace amounts of IL prior to enzymatic hydrolysis leads to extremely diluted IL solutions. For recycling, it is very expensive to separate the water from the dilute IL solutions through evaporation or reverse osmosis, due to the high energy requirements (Park, et al. 2012). For IL recovery, the simplest suggested procedure has been to evaporate the anti-solvents from the ILs and directly re-use the ILs (Lee, et al. 2009; Li, et al. 2009; Lozano, et al. 2012; Qiu and Aita 2013; Wu, et al. 2011). Another alternative for IL recovery is the creation of aqueous-IL biphasic systems by mixing the IL phase with concentrated salt solutions (Gutowski, et al. 2003; He, et al. 2005; Li, et al. 2005). However, serious problems can be expected with lignin and other components accumulating into the IL (Shill, et al. 2011; Sun, et al. 2013a). A few techno-economic studies have been carried out regarding glucose production with IL pretreatments. Abels et al. (2013) reported the IL cost to be approximately 33% of the glucose price when IL was used for pretreatment and the polysaccharides were hydrolysed enzymatically. Sen et al. (2012) made a similar study on a process in which acid hydrolysis of corn stover was carried out in IL solution. In both cases, the IL was the dominant factor in the price of the glucose product, even if high IL recovery rates (99.75% and 98%) and an IL price of only 10 €/kg or $/kg were assumed. In these studies, the production price of glucose was evaluated to be at least five times higher than the current market price.

1.3 Enzyme action in ionic liquids

1.3.1 Enzymatic reactions in ionic liquids

The first study of using enzymes in (aqueous) IL was by Magnuson et al. (1984), who studied the stability of alkaline phosphatase in ethylammonium nitrate. Several reviews on both the fundamental science and applications of biocatalysis in ILs have been published (Kragl, et al. 2002; Moniruzzaman, et al. 2010a; Naushad, et al. 2012; van Rantwijk and Sheldon 2007; Yang and Pan 2005). ILs have been recognized as desirable media in enzyme-catalysed organic synthesis mainly due to their low volatility, thus mitigating VOC emissions, and their excellent solvent properties (van Rantwijk and Sheldon 2007). Most enzymes reported to be active in ILs are lipases, which work at water-oil interfaces in their natural role (Kragl, et al. 2002). In many cases, increased enzymatic activity, stability and selectivity have been observed in IL solutions (Yang and Pan 2005). Several interesting reports have been published concerning enzymatic polysaccharide acylations in IL solutions (Chen, et al. 2006a; Chen, et al. 2013; Zhao, et al. 2008).

Three different medium types should be considered when dealing with enzyme stability and action in ILs: anhydrous hydrophobic ILs, anhydrous hydrophilic ILs, and aqueous solutions of hydrophilic ILs. As a general rule hydrophilic ILs have been regarded as being destabilizing for enzymes, whereas hydrophobic ILs are

stabilizing (Zhao, et al. 2006b). Although hydrophobic ILs stabilize suspended enzymes, they do not dissolve carbohydrates to an appreciable extent (Zhao, et al. 2009b), and are thus of marginal interest for homogeneous polysaccharide modification. Enzyme-dissolving ILs usually also inactivate enzymes, with the exception of a few ILs such as cholinium $H_2PO_4^-$ (Fujita, et al. 2005). The inactivating effect of both hydrophilic organic solvents as well as ILs has been suggested to be caused by dehydration of essential water molecules from proteins (van Rantwijk and Sheldon 2007). Especially hydrophilic ILs effectively strip essential water from enzymes (Moniruzzaman, et al. 2010b). Protein denaturation is generally a two-step process, with unfolding as a first, reversible step and aggregation of the unfolded protein as the second, irreversible step (Constatinescu, et al. 2010). Both stabilizing and destabilizing ILs appear to prevent protein aggregation. ILs are not always inactivating, but may also promote the refolding of denaturated protein, as was demonstrated with hen egg white lysozome in ethylammonium nitrate (Summers and Flowers 2000). In the early studies of enzymes in ILs, diverse problems were encountered regarding IL purity, unexpected pH shifts and precipitation of buffer salts (Kragl, et al. 2002). Even low concentrations of chloride impurities may drastically decrease enzyme activity (Lee, et al. 2006), and IL purification has been shown to confer considerable benefits in retaining enzyme activity in these systems (Park and Kazlauskas 2001).

Enzyme stability in hydrophilic solvents may be predicted by the solution's Hildebrandt solubility parameter (δ), dielectric constant (ε), dipole moments (μ) or octanol-water partition coefficient, logP (Kaar, et al. 2003). According to the log P values, many common imidazolium-based ILs, including hydrophobic ILs with PF_6^- anions, should not support enzyme activity, although 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM]PF_6 has been shown not to inactivate enzymes to a significant degree. Thus, many usually applied measures for solvent compatibility with enzymes should be applied with great care in IL systems.

Pure IL media usually have high viscosities. In enzyme-mediated reactions, the high viscosity also decreases substrate diffusion rates and thereby reaction kinetics (Zhao, et al. 2006a). Dilution with low-viscosity organic solvents has in some cases been demonstrated to be advantageous (Chen, et al. 2006b). ILs are not generally neutral in pH, but have been shown to have even considerable effects on matrix pH values in buffer solutions (Engel, et al. 2010; Li, et al. 2012). Furthermore, ILs also increase the ionic strength of the matrix, which affects enzyme activity and stability. The effects of viscosity, ionic strength and pH cannot completely explain the enzyme inactivation in many ILs, suggesting that the IL-induced enzyme inactivation is also caused by other mechanisms (Engel, et al. 2010).

Cations and anions are characterized as kosmotropes or chaotropes, depending on their ability to promote or destroy water structure, respectively (Constantinescu, et al. 2007). Generally, kosmotropic anions stabilize proteins, whereas chaotropic cations have a destabilizing effect. In Zhao et al. (2006b), enzymes were found to be stabilized by kosmotropic anions and chaotropic cations, but destabilized by chaotropic anions and kosmotropic cations, partially contradicting the previously cited rule of thumb. The enzyme inactivation rates and inactivation reversibility or

irreversibility have been proposed to be mostly anion dependent (Kaar, et al. 2003). Nucleophilic anions are possibly able to coordinate with positively charged surface residues on the enzyme and cause conformational changes (Sheldon, et al. 2002), whereas large anions spread out their charge and thus have a weaker hydrogen bond basicity to interact with the enzymes and cause less disruption of the protein structure. Carbohydrate-dissolving ILs have anions with a high hydrogen bond basicity and thus also exert a denaturing effect on enzymes (Zhao 2010). In aqueous salt solutions, the protein stability can be related to the Hofmeister series for ions (originally introduced by Hofmeister in 1888). Several studies have indicated that the impact of ILs on enzyme action in aqueous IL solutions can generally also be predicted using the Hofmeister series (Constantinescu, et al. 2007; Lai, et al. 2011). In pure ILs the ions can be expected to affect the enzymes in a much more complex manner than in aqueous IL solutions. In fact, the Hofmeister series is applicable in IL environments only when the (hydrophilic) IL is sufficiently diluted for the ions to be dissociated from each other (Zhao, et al. 2006b). The destabilizing effect of imidazolium cations has been ordered to $[DMIM]^+ < [EMIM]^+ < [BMIM]^+$ (Lai, et al. 2011). Increasing cation hydrophobicity has been found to decrease enzyme stability (Constantinescu, et al. 2007).

1.3.2 Cellulase stability and activity in ionic liquids

The first study on *Trichoderma reesei* cellulase stability in a cellulose-dissolving IL ([BMIM]Cl) was published in 2003 (Turner, et al. 2003), indicating this IL to be very inactivating for the cellulases. The Cl^- ion was proposed to be responsible for the observed enzymatic inactivation. Water dilution of the IL matrix showed the unfolding to be reversible in [BMIM]Cl for the studied enzyme. In another early article on cellulase inactivation in IL matrices, *Humicola insolens* cellulase was found to be stable in $[BMIM]PF_6$ and 1-butyl-3-methylimidazolium tetrafluoroborate ($[BMIM]BF_4$), which do not dissolve cellulose, but the cellulase was inactivated in [BMIM]Cl (Paljevac, et al. 2006).

Many studies have been carried out with mesophilic cellulases from the well-characterized *T. reesei* and *Aspergillus niger* cellulolytic systems, either with the cellulases in a monocomponent form or as cellulase cocktails (Table 3). Most of the monocomponent cellulases studied in ILs have been endoglucanases. The IL tolerance of a cellobiohydrolase has only been reported in one study (Engel, et al. 2012b) and of monocomponent β-glucosidases in three studies (Engel, et al. 2012b; Thomas, et al. 2011; Wolski, et al. 2011). Recently, cellulases tolerant to cellulose-dissolving ILs have been screened from extremophilic sources, whereas much of the early work in this field was done with mesophilic cellulases. Carboxymethylcellulose (CMC) is the dominant substrate, especially in activity measurements, but the hydrolysis of different solid substrates has also been studied.

Table 3. Published studies on cellulase activity, stability and enzymatic cellulose hydrolysis in IL-containing matrices. RC = regenerated cellulose, CMC = carboxymethylcellulose, 4-MUC = 4-methylumbelliferyl-β-D-cellobioside, (A) = activity measurement, (H) = hydrolysis experiment.

Substrate	Cellulase	IL(s)	Comments	Ref
CMC	*Aspergillus niger* cellulase	[BMIM]Cl	High pressure during hydrolysis enhanced cellulase activity in the presence of IL (A)	Salvador, et al. 2010
CMC	*Humicola insolens* cellulase	[BMIM]Cl, [BMIM]BF_4, [BMIM]PF_6	Great excess of cellulase (H)	Paljevac, et al. 2006
CMC	Bacterial cellulases	6 different ILs	Cellulose-binding module (CBM) pivotal in cellulase IL tolerance (A)	Pottkämper, et al. 2009
CMC	Bacterial cellulases	6 different ILs	IL tolerance linked to thermo- and halophilicity (A)	Ilmberger, et al. 2012
CMC	*Thermoanaerobacter tengcongensis* endoglucanase	[BMIM]Cl, [AMIM]Cl	Thermophilic endoglucanases exhibited good IL tolerance (A)	Liang, et al. 2011
CMC	*Bacillus aquimaris* cellulase	[EMIM]$MeSO_4$, [EMIM]Br	Solvent- and alkali-tolerant cellulase exhibit good IL tolerance (A)	Trivedi, et al. 2011
CMC	*Halorhabdus utahensis* cellulase	[AMIM]Cl, [EMIM]AcO, [EMIM]Cl, [BMIM]Cl	Large number of charged surface groups on protein surface endows salt tolerance (A)	Zhang, et al. 2011
RC	*Trichoderma reesei* cellulase	[EMIM]DEP	*In situ* cellulose hydrolysis in IL introduced (H)	Kamiya, et al. 2008
RC	*T. reesei* cellulase, β-glucosidase	[DMIM]DMP, [EMIM]AcO, [EMIM]lactate	Green fluorescent protein screening for enzyme inactivation in IL (A&H)	Wolski, et al. 2011
Cellulose azure	*T. reesei* cellulase cocktail	8 different ILs	HEMA showed significant cellulase stabilization (H)	Bose, et al. 2010
Cellulose azure	*A. niger* endoglucanase	3 imidazolium ILs and HEMA	(A&H)	Bose, et al. 2012
Cellulose azure	*T. reesei* cellulase	[BMIM]Cl, [BMIM]BF_4	Comparison with LiCl/DMAc, NaCl and urea solutions (A)	Turner, et al. 2003
α-Cellulose, CMC, *para*-nitrophenyl-β-cellobioside	Celluclast® 1.5 L	[DMIM]DMP, [EMIM]AcO, [BMIM]Cl, [AMIM]Cl	Evaluation of IL matrix viscosity, ionic strength and pH cellulase activity (A&H)	Engel, et al. 2010
RC, cellobiose	*T. reesei* Cel7A and Cel7B, *A. niger* β-glucosidase	[DMIM]DMP	Optimization of cellulase cocktail for RC hydrolysis in the presence of IL (A&H)	Engel, et al. 2012b

Table 3. (continued)

Substrate	Cellulase	IL(s)	Comments	Ref
RC, CMC, regenerated yellow poplar	Celluclast® 1.5L and *A. niger* β-glucosidase	[EMIM]AcO	(A&H)	Wang, et al. 2011
Filter paper (regenerated from IL)	Liquid cellulase from Imperial Jade Bio-technology	[DMIM]DMP, [EMIM]DEP, [BMIM]DBP	(A&H)	Zhi, et al. 2012
Filter paper, CMC, xylan	*Penicillium janthinellum* mutant glycosyl hydrolases	[BMPy]Cl, [BMIM]Cl	(A&H)	Adsul, et al. 2009
CMC, pretreated corn stover, MCC	*Thermotoga maritima* and *Pyrococcus horikoshii* endoglucanases	[EMIM]AcO	Thermophilic endoglucanases exhibited good IL tolerance (A)	Datta, et al. 2010
IL pretreated switchgrass	Supernatants from thermophilic bacterial consortia	[EMIM]AcO	Thermophilic bacterial consortia adapted to switchgrass at 60 °C (A)	Gladden, et al. 2011
Azo-CMC, 4-MUC, MCC, tobacco cell wall polysaccharides	*Sulfolobus solfataricus* endoglucanase	[DMIM]DMP, [EMIM]AcO	Thermostable endoglucanase, shows high activity in 80% IL at 90 °C (A&H)	Klose, et al. 2012
IL pretreated switchgrass	Thermophilic cellulases	[EMIM]AcO	Cellulase cocktail optimized based on thermophilic cellulases (A&H)	Park, et al. 2012
CMC, RC, cotton linters, algal biomass	*Pseudoalteromonas* sp. cellulase	6 different ILs	Thermo-, halo- and alkali-tolerant cellulase exhibits good IL tolerance (A)	Trivedi, et al. 2013
MCC, RC	*T. reesei* cellulase	[EMIM]DEP	Immobilization of cellulase by glutaraldehyde cross-linking, low IL content (2%) (H)	Jones and Vasudevan 2010
CMC, MCC, straw, cotton, filter paper	Cellulase powder	[E(OH)MIM]AcO	Specially designed enzyme compatible and cellulose-dissolving IL (A&H)	Li, et al. 2012
Soluble *para*-nitrophenyl glycosides	β-glucosidases, xylanase, arabinofuranosidase	[DMIM]DMP, [EMIM]DMP, [EMIM]DEP [EMIM]AcO	(A)	Thomas, et al. 2011
Cellulose powder	*Trichoderma viride* cellulase	[BMIM]Cl	Cellulase stabilized in liposomes (H)	Yoshimoto, et al. 2013

Out of eight different ILs studied by Bose et al. (2010), tris-(2-hydroxyethyl) methylammonium methylsulphate (HEMA) was found to stabilize a *T. reesei* cellulase cocktail in temperatures up to 115 °C. Although HEMA is a promising IL in view of its cellulase compatibility, cellulose solubility in this ammonium-based IL is only ~1% which limits its applicability. In Zhi et al. (2012), cellulase stability was also studied in a series of dialkylphosphate ILs with increasing alkyl substituent size ([DMIM]DMP, 1-ethyl-3-methylimidazolium diethylphosphate [EMIM]DEP and 1-butyl-3-methylimidazolium dibutylphosphate [BMIM]DBP). [DMIM]DMP, with the smallest alkyl substituents, was found to be the least inactivating. In a comparison of the action of two β-glucosidases, a xylanase and two arabinofuranosidases in the presence of three dialkylphosphate ILs ([DMIM]DMP, 1-ethyl-3-methylimidazolium dimethylphosphate [EMIM]DMP and [EMIM]DEP) and [EMIM]AcO, [EMIM]DEP was found to be the most inactivating IL (Thomas, et al. 2011). *T. reesei* Cel7A (cellobiohydrolase) and Cel7B (endoglucanase) have been found to respond similarly to the presence of [DMIM]DMP, showing some residual activity in up to 30% (v/v) IL, whereas *A. niger* β-glucosidase was more IL sensitive and lost its activity already in 15% (v/v) of [DMIM]DMP (Engel, et al. 2012b). When Engel et al. (2010) compared the compatibility of the most common cellulose-dissolving ILs ([DMIM]DMP, [BMIM]Cl, [EMIM]AcO and [AMIM]Cl) with a commercial *T. reesei* cellulase cocktail, a general decrease of 70–85% in cellulase activity was observed in the presence of 10% (v/v) IL. Comparing the same IL from different manufacturers in some cases resulted in greater differences in relative cellulase activity than between the different IL types. However, [DMIM]DMP was concluded to be the least cellulase-inactivating of the studied ILs. The storage stability was examined in 10% (v/v) [DMIM]DMP, in which a rapid loss of activity (10–40% residual activity) was measured after an incubation time of one day, whereafter no further inactivation occured. The cellulase inactivation in aqueous [DMIM]DMP was shown to be reversible.

Wolski et al. (2011) developed a screening method in which green fluorescent protein is employed to determine protein stability in ILs, based on fluorescence measurements. With this method, [DMIM]DMP and [EMIM]lactate were identified as potentially enzyme-compatible ILs for *in situ* cellulose hydrolysis. In validating the screening results, *T. reesei* cellulases retained their activity in up to 40% [DMIM]DMP or [EMIM]lactate and *A. niger* β-glucosidase in up to 60% [DMIM]DMP. [DMIM]DMP was found to be more enzyme compatible than [EMIM]lactate. Inactivation of *A. niger* cellulase in [BMIM]Cl appears to correlate linearly with the water activity (Salvador, et al. 2010). Based on activity measurements on CMC after incubation in 10% [BMIM]Cl the cellulase regains activity upon dilution, supporting the earlier observations of Turner et al. (2003) concerning the reversibility of cellulase inactivation in aqueous [BMIM]Cl. Cellulase inactivation is temperature dependent, as a *T. reesei* cellulase mixture with *A. niger* β-glucosidase showed only minor activity losses at 4 °C during an incubation time of 1.5 h in up to 30% [EMIM]AcO, whereas inactivation proceeded much faster at 50 °C (Wang, et al. 2011).

Enzyme thermo- and halotolerance have been linked to IL tolerance, and IL-tolerant cellulases have been screened from different extremophilic and halophilic sources for better enzyme performance in the hydrophilic ILs used for cellulose

dissolution (Datta, et al. 2010; Gladden, et al. 2011; Ilmberger, et al. 2012; Klose, et al. 2012; Liang, et al. 2011). Increased IL tolerance has also been reported for enzymes active at high pH originating from solvent-tolerant bacteria (Trivedi, et al. 2011; Trivedi, et al. 2013). A cellulase from the haloalkaliphilic *Halorhabdus utahensis* has also been shown to have good IL tolerance (Zhang, et al. 2011), which was suggested to be due to the presence of a great number of negatively charged amino acid residues on the protein surface, low content of hydrophobic amino acids and a compact packing of the protein structure. The negative charge on the protein surface is anticipated to interact well with both water and high ion concentrations, as in an IL. Similar conclusions regarding the relationship between protein structure and IL tolerance were drawn by Karbalaei-Heidari et al. (2013).

Screening metagenomic libraries and mutation experiments have yielded encouraging results in finding cellulases with increased IL tolerance. Adsul et al. (2009) found several glycosyl hydrolases with improved ionic liquid tolerance from *Penicillium janthinellum* mutants. Pottkämper et al. (2009) screened the IL tolerance of 24 bacterial cellulases derived from metagenomic libraries in a number of different ILs. Most of the screened cellulases had very low IL tolerance and the most IL tolerant cellulases also displayed remarkably high halotolerance, which again would suggest that halotolerance and IL tolerance are correlated. Mutations in the CBM of the cellulases led to increased activity in the presence of ILs in some cases, which suggested that the CBM would play an important role in how cellulases are affected by IL.

Currently marketed commercial cellulose-degrading enzyme cocktails display low tolerance towards biomass-dissolving ILs, and some new cellulase mixtures have therefore been especially optimized for IL matrices. With an optimized cellulase cocktail based on thermophilic enzymes, over 50% of the original activity in aqueous conditions was reported to be retained in 20 (w/v) % [EMIM]AcO at 70 °C (Park, et al. 2012). Engel et al. (2012b) optimized a cellulase cocktail for the hydrolysis of RC in the presence of 10% (v/v) [DMIM]DMP based on inactivation data obtained for different cellulase components (*T. reesei* Cel7A and Cel7B, *A. niger* β-glucosidase) in this IL.

Some efforts have been made in designing cellulase-compatible ILs (Section 1.2.2 and Figure 6). Li et al. (2012) demonstrated the successful hydrolysis of straw, cotton and filter paper with cellulase powder in 15% (w/v) of a specially designed IL, [E(OH)MIM]AcO (see Section 1.2.2). The cellulase displayed good residual activities (over 50% of original activity) in up to 25% of this IL after a one day incubation and the unfolding temperature of the cellulase was found to increase in the presence of [E(OH)MIM]AcO. The presence of this IL caused the pH of the hydrolysis medium to increase which was suggested to be a major factor causing loss of cellulase activity in the studied system.

Different stabilization techniques have been applied to obtain better cellulase performance in ILs. Jones and Vasudevan (2010) reported cellulase cross-linking with glutaraldehyde and the use of this catalyst in media containing low concentrations (2% v/v) of the IL [EMIM]DEP. Cellulases have effectively been stabilised for better perfromance in ILs by either lyophilizing the enzyme together with PEG

(Turner, et al. 2003) or by covalently attaching PEG chains to the N-terminal end of the enzyme (Li, et al. 2013). The PEG chain is suggested to form a protective, hydrophilic region around the cellulase, which protects the cellulase from the IL and may also increase interactions between the modified cellulase and cellulose. Lozano et al. (2011) immobilized a commercial cellulase on Amberlite XAD4, a polymeric support, and coated the particles with a hydrophobic IL, which was observed to stabilize the cellulase against thermal inactivation. When coated by stabilizing IL, the immobilized enzyme showed a better stability in the very inactivating IL [BMIM]Cl. Stabilisation of *Trichoderma viride* cellulase as liposomes has also been reported to be a successful strategy for increasing the IL tolerance of enzymes (Yoshimoto, et al. 2013). Interestingly, cellulases have been found to show increased thermal stability in the presence of RC, indicating that inactivation kinetics measured in IL solutions without substrate may not give the complete picture of cellulase inactivation (Zhao, et al. 2009a). Enzyme stabilization in ILs has been reviewed by Moniruzzaman et al. (2010b) and by Zhao (2010).

1.3.3 Enzymatic hydrolysis of cellulose in cellulose-dissolving ionic liquids

The fact that cellulose is soluble in certain ILs opens up interesting possibilities for enzymatic hydrolysis of dissolved or regenerated cellulose. Regenerating lignocellulosic biomass from IL solution is known to be an efficient pretreatment prior to enzymatic hydrolysis (Section 1.2.3), but the high dilution ratio of the IL caused by the regeneration and subsequent washing presents challenges to economical IL recycling (Park, et al. 2012). Some residual IL is trapped inside the regenerated substrate, potentially causing enzyme inactivation during the subsequent hydrolysis (Hong, et al. 2012; Zhao, et al. 2009a). Therefore, it is of considerable interest to study direct hydrolysis of the regenerated substrate without removing the IL in a separate step between pretreatment and hydrolysis. It is however known, that cellulose-dissolving ILs have anions with a high tendency to form hydrogen bonds and inactivate enzymes (Zhao 2010). A great variety of substrates, enzyme preparations, ILs and methods have been used to assess IL effects on cellulase stability, activity on soluble substrates and yields from the hydrolysis of solid cellulosic substrates (Table 3).

Kamiya et al. (2008) introduced the term *in situ* saccharification, which means a combined one-pot procedure in which the substrate is first dissolved in a cellulose-dissolving IL, whereafter the regeneration is done by adding buffer and the enzymatic hydrolysis is carried out in the same vessel without removing the IL. Some other interesting concepts for biomass hydrolysis in IL matrices have also been proposed. A hyperthermophilic endoglucanase gene has been added *in planta* (Klose, et al. 2012); the enzyme is inactive during plant growth conditions, but in pretreatment conditions at high temperatures (90 °C) it becomes active and degrades the plant cell wall from inside the plant. Furthermore this cellulase showed an extraordinary tolerance to [EMIM]AcO and [DMIM]DMP, which means that cellulose-dissolving ILs can be used in the pretreatment process. Nakashima et al.

(2011) proposed the combination of IL pretreatment, enzymatic hydrolysis and fermentation to ethanol in a one-pot procedure. The used yeast displayed cellulases on its cell surface and hydrolysis could be efficiently carried out when additional free cellulases were added. The yeast was shown to be capable of fermenting the liberated sugars to ethanol, but the yeast tolerated a maximum IL concentration of only 200 mM. Also other studies have indicated microbial fermentation to be sensitive to inhibition by low IL residuals in the hydrolysates (Ninomiya, et al. 2013; Hong, et al. 2012).

An almost complete conversion of RC has been achieved with a cellulase cocktail of *T. reesei* cellulases and β-glucosidase from *A. niger* in the presence of 15% (v/v) [EMIM]AcO (Wang, et al. 2011), whereas IL-pretreated yellow poplar had rather low hydrolysis conversion (33%) in the same conditions. Regenerated filter paper was enzymatically hydrolysed in the presence of [DMIM]DMP, which doubled the hydrolysis yield compared to untreated filter paper (Zhi, et al. 2012). Engel et al. (2010) compared the enzymatic hydrolysis of α-cellulose both untreated and regenerated from [DMIM]DMP in buffer and in 10, 20 and 30% (v/v) [DMIM]DMP. The initial hydrolysis rates were greater for RC even in 30% (v/v) [DMIM]DMP than for untreated α-cellulose in buffer. However, the increasing presence of this IL lowered the hydrolysis yields in prolonged hydrolyses. Wolski et al. (2011) compared the enzymatic hydrolysis of IL-preatreated *Miscanthus* with a commercial *T. reesei* cellulase cocktail in aqueous [DMIM]DMP and [EMIM]lactate and found [DMIM]DMP to be a significanlty more enzyme-compatible IL than [EMIM]lactate, as hydrolysis still took place in 50% (w/w) IL. The most IL-tolerant cellulase to date was reported by Klose et al. (2012): a hyperthermophilic and halophilic GH12 endoglucanase lacking CBM from *Sulfolobus solfataricus* hydrolysed dissolved or regenerated MCC well in 80% (v/v) [DMIM]DMP and [EMIM]AcO at 90 °C. Based on these results, efficient pretreatment and saccharification should be possible with this enzyme in a single stage.

Separation of the hydrolysis products from the IL-containing hydrolysates is a challenge. Boronate-saccharide complexation with subsequent extraction (Brennan, et al. 2010) and different large-scale chromatographic procedures have been proposed for this task (Binder and Raines 2010; Feng, et al. 2011). The one-pot or *in situ* hydrolysis is a relatively new concept and currently suffers from problems with enzyme performance in high concentrations of biomass-dissolving ILs, but in the light of recently reported highly IL-tolerant cellulases this concept can indeed be seen as a future alternative to the earlier proposed regeneration pathway.

1.4 Carbohydrate analysis in solutions containing ionic liquids

Carbohydrate analytics form an important part of the research both for understanding fundamental mechanisms of enzymatic hydrolysis and for optimizing total hydrolysis procedures. Reports of IL effects on carbohydrate analytics are scarce, but it has been reported in several references that ILs have disturbing effects on different analytical methods (Hyvärinen, et al. 2011; Klembt, et al. 2008). Typically,

spectrophotometric methods such as the 3,5-dinitrosalicylic acid (DNS) (Sumner 1924) or *para*-hydroxybenzoic acid hydrazide (PAHBAH) (Lever 1973) assays are used for quantifying the total amount of reducing sugars, whereas high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE) techniques are used for analysing the identity of different mono- and oligosaccharides. Some references contain indications of ILs or impurities in ILs interfering with photometric assays or HPLC methods, typically used for enzyme kinetic and activity measurements (Klembt, et al. 2008). Analysis in IL matrices has been reported to be very challenging with HPLC and GC, as the columns tolerate only low amounts of salts (Hyvärinen, et al. 2011). In Hyvärinen et al. (2011) the chromatograms showed broad IL peaks which in some cases overlapped with the saccharide peaks, and the retention times of the IL peaks varied depending on the sample composition. The HPLC system was compatible with a maximum amount of 1.8% of IL in the sample.

ILs have been used as auxiliary substances in both chromatographic and electrophoretic methods (Stalcup and Cabovska 2005). In analytical applications ILs have been used as *e.g.* mobile phase additives, capillary wall coating agents, for covalent attachment to column and capillary walls etc. However, the usefullness of ILs in these applications cannot be compared to samples containing undefined amounts of different ILs, as in the case of typical hydrolysates from *in situ* saccharification. Vaher et al. (2011) used ILs as components in the background electrolyte (BGE) in capillary electrophoresis (CE). Many ILs are UV active and may as such be used as chromophores for indirect UV detection. Some ILs are suggested to act selectively with saccharides, which facilitates their analytic separation. The IL concentration must be kept low (below 20–30 mM) in these applications, as high contents of IL have been found to lead to baseline fluctuations.

Saccharide standard curves in the DNS spectrophotometric assay for samples containing 0–20% (v/v) [EMIM]AcO have been reported not to show any interference of the IL with the analysis (Wang, et al. 2011). DNS results were confirmed by HPLC for the hydrolysates, but no mention was made of IL effects on the chromatographic analysis.

Sugar derivatization with aromatic amines for saccharide analysis with HPLC in IL-containing hydrolysates has been reported in several studies. In Kamiya et al. (2008), glucose and cellobiose were analysed as 4-aminobenzoic acid ethyl ester (ABEE) derivatives in hydrolysates containing [EMIM]DEP by HPLC on a C18 column. However, no information was provided on the dilution factors or disturbing effects of the IL presence. Liquors containing ILs have also been analysed for carbohydrates with similar procedures in other studies, but nothing is generally commented about the final IL concentration in the samples or about the effect of IL on the analysis results (Sun, et al. 2013a; Uju, et al. 2013).

In addition to general harmful effects that ILs may have on analytical methods, they may also react with the saccharide analytes. Ebner et al. (2008) demonstrated that imidazolium-based ILs ([BMIM]Cl in this study) form carbenes at their C2 position in the imidazolium, which further react with the reducing ends of saccharides. Du and Qian (2011) further confirmed this reaction for other ILs, most notably

[EMIM]AcO, by quantum mechanical calculations. Currently, it is not known to what extent the formation of saccharide-imidazolium adducts affects saccharide analytics in IL matrices and whether this adduct formation is reversible during the preparation of samples for analysis.

2. Aims of the work

The overall goal of this work was to study the enzymatic hydrolysis of cellulose in aqueous ionic liquids (ILs). Mainly, cellulases from the mesophilic fungus *Trichoderma reesei* and some commercial cellulases were used. Monocomponent cellulase preparations were used to study how different enzymes respond to the presence of cellulose-dissolving ILs. Principally, the model substrates microcrystalline cellulose (MCC) and carboxymethylcellulose (CMC) were employed.

More specifically, the aims of this work were:

- To study the effect of both known imidazolium-based and newly introduced cellulose-dissolving IL classes on the hydrolysis of cellulose by *T. reesei* cellulases and commercial cellulases (hydrolysis yield, product distribution in the hydrolysates, molecular mass changes in the cellulose) (I–IV)
- To study *T. reesei* cellulase inactivation rates and the reversibility/irreversibility of cellulase inactivation in the presence of cellulose-dissolving ILs, as well as reasons for IL-induced cellulase inactivation (I–V)
- To study the effect of ILs on *T. reesei* cellulase binding to cellulose with intact modular cellulases and their corresponding core domains (IV)
- To develop analytical methods allowing the necessary mono- and oligosaccharide analyses in the presence of ILs (II)

3. Materials and methods

This section presents the main experimental techniques and materials used in this work. More detailed information can be found in the original papers I–V.

3.1 Cellulosic substrates

The cellulosic substrates used in this work are listed in Table 4. Carboxymethylcellulose (CMC) was used as dissolved substrate in the endoglucanase activity assays; the other substrates were used in suspension in the hydrolysis experiments. Regenerated cellulose (RC) was prepared by dissolving microcrystalline cellulose (MCC) in [EMIM]AcO overnight at 80 °C and precipitating the dissolved cellulose by adding water. The RC was washed with water until the washing liquid was colourless and then dried at 13 mbar at room temperature (RT) overnight to yield a brownish brittle solid. The weight average molecular mass (M_w) did not decrease due to the regeneration procedure (I, Table 3).

Table 4. Cellulosic substrates used in this work. MCC = microcrystalline cellulose, PHK DP = pre-hydrolysis kraft dissolving pulp, SDP = sulphite dissolving pulp, CMC = carboxymethylcellulose.

Substrate	Supplier	Specifications	Used in
MCC	Serva Gmbh	Particle size 0.020 mm	I, II, III, IV
Eucalyptus PHK DP	Bahia Specialty Cellulose	2.5% xylan	III
Beech SDP	Lenzing AG	3.2% xylan, 0.2% mannan	V
CMC	Sigma	Low viscosity CMC	I, II, III, IV, V

3.2 Enzymes and enzyme assays

Pure monocomponent preparations of *Trichoderma reesei* cellulases were mainly used in this work. In addition, some commercial endoglucanase preparations were used. Table 5 presents the enzyme preparations used, together with their supplier or production procedure if produced at VTT, and additional comments about the enzymes and the original article in which they were used.

Table 5. Cellulase preparations used in this work. *T. reesei* = *Trichoderma reesei*, *T. maritima* = *Thermotoga maritima*, *A. niger* = *Aspergillus niger*, EG = endoglucanase, CBH = cellobiohydrolase, CD = core domain.

Enzyme	Supplier / production procedure	Comments	Used in
T. reesei Cel5A	Suurnäkki, et al. 2000	Formerly EGII	I, II, III, IV
T. reesei Cel5A CD	Suurnäkki, et al. 2000	Formerly EGII CD	I, IV
T. reesei Cel7A	Rahikainen, et al. 2013	Formerly CBHI	IV
T. reesei Cel7A CD	Suurnäkki, et al. 2000	Formerly CBHI CD	IV
T. reesei Cel7B	Suurnäkki, et al. 2000	Formerly EGI	I
T. reesei cellulase	Sigma-Aldrich	Commercial cellulase cocktail	V
T. maritima Cel5A	Megazyme International	Thermophilic endoglucanase preparation	III
Puradax HA® 1200E	DuPont Industrial Biosciences	Endoglucanase preparation used in washing liquids	III
IndiAge® ONE	DuPont Industrial Biosciences	Endoglucanase preparation used in denim finishing	III
A. niger β-glucosidase	Novozymes	Crude β-glucosidase preparation	II

Endoglucanase activity was determined using a 1% (w/v) CMC solution as substrate for 10 min at 50 °C and quantifying the amount of reducing chain ends produced by the DNS assay (Section 3.6, assay conducted according to Ghose (1987) with the DNS reagent solution prepared as in Sumner (1924). Cellobiohydrolase activity for Cel7A and Cel7A core domain (CD) was determined on 4-methylumbelliferyl-β-D-lactoside (4-MUL) with an assay described in (van Tilbeurgh, et al. 1988). β-Glucosidase activity was determined on 4-nitrophenyl-β-D-glucopyranoside as described in (Bailey and Linko 1990) and xylanase activity on birch glucuronoxylan as described in (Bailey, et al. 1992). All activities were expressed as nanokatals (nkat), with 1 nkat defined as the number of catalysed reactions (mol) per time unit (s) (Dykbaer 2001).

The protein content of the enzyme preparations was detemined (in I, II, III) with a commercial kit based on the Lowry method (Lowry, et al. 1951), with bovine serum albumine (BSA) as protein standard. The molar protein concentrations of pure *T. reesei* monocomponent cellulase preparations were determined on the

basis of their absorption at 280 nm in IV. Protein purity was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), in which protein visualization is based on a UV-light driven reaction of tryptophan residues in the presence of trichlorocompounds (Kazmin, et al. 2002) with a Criterion stain-free imaging system.

For determining residual activities after different treatments, endoglucanase samples were incubated in matrices containing IL for different time periods in hydrolysis conditions (V). Thereafter, samples were withdrawn and their residual endoglucanase activity was determined on CMC. Inactivation time curves were determined on the basis of the residual endoglucanase activities. Endoglucanase samples were also boiled for 10 or 20 min after incubation in buffer and IL matrices under hydrolysis conditions in order to determine the efficiency of boiling for enzyme inactivation and the protecting effect of the ILs towards boiling (I and unpublished results).

3.3 Ionic liquids

The ILs employed in this study are presented in Table 6 and their structures in Figure 7. The nomenclature for the tetra-alkylphosphonium ILs is based on their alkyl substituents: the cations have four alkyl subsituents and the four numbers after the P denotes the length of the individual alkyl chains.

Table 6. Ionic liquids used in this work, their supplier/method of preparation and aggregation state at room temperature (RT) or melting point (mp).

IL	Supplier / preparation	Melting point	Used in
[DMIM]DMP	IoLiTec	Liquid at RT	IV
	Bradaric, et al. 2003		I, II
[EMIM]AcO	IoLiTec	Liquid at RT	I, II, III, IV
	BASF (Basionic TM BC 01)		V
[TMGH]COO	King, et al. 2011	78–83 °C	III
[TMGH]AcO	King, et al. 2011	90–97 °C	III
[TMGH]EtCOO	King, et al. 2011	62 °C	III
[TMGH]*n*-PrCOO	King, et al. 2011	67 °C	III
[DBNMe]DMP	Parviainen, et al. 2013	Liquid at RT	III
[DBNH]AcO	Parviainen, et al. 2013	Solid at RT, mp < 80 °C	III
[DBNH]EtCOO	Parviainen, et al. 2013	Liquid at RT	III
[P4444]OH	ABCR	Provided in 40% aqueous solution	Unpublished
[P8881]AcO	King, et al. 2013	Liquid at RT	Unpublished

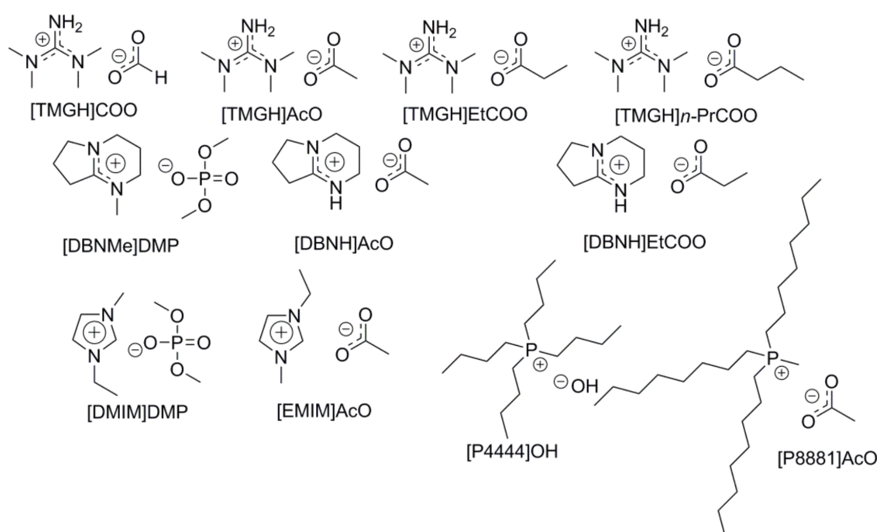

Figure 7. Structures of ILs employed in this work.

3.4 Hydrolysis of solid polymeric substrates in aqueous IL solutions

Enzymatic hydrolysis of solid cellulose was carried out with IL dosages of 0, 20, 40, 60, 80 and 90% (v/v in I, II or w/w in III, IV) in buffer (0.050 M citrate, pH 5.0 or 0.100 M phosphate, pH 6.0). 30 mg (dry weight) of substrate was weighed into a test tube, the defined amount of buffer was added and the mixture was stirred to homogeneity (Figure 8). The defined volume of IL was added to the mixture, and the mixture was temperated to the hydrolysis temperature (45 °C). Those ILs which were not liquid at RT were melted in a heated oil bath before addition to the hydrolysis mixture. The enzyme preparation was added with the cellulase dosage corresponding to an endoglucanase activity (on CMC) of 2000 nkat/g cellulose in I and II, or to a protein content of 1 mg/g of substrate (III). In IV, the enzyme dosage was based on cellulase concentration, which was 400 nM in the final hydrolysis mixture. The total hydrolysis sample volume was 3 mL (in I, II) or had a total weight of 3 g (in III, IV). The hydrolysis was carried out at 45 °C in closed test tubes in a water bath with continuous magnetic stirring for the specified hydrolysis time.

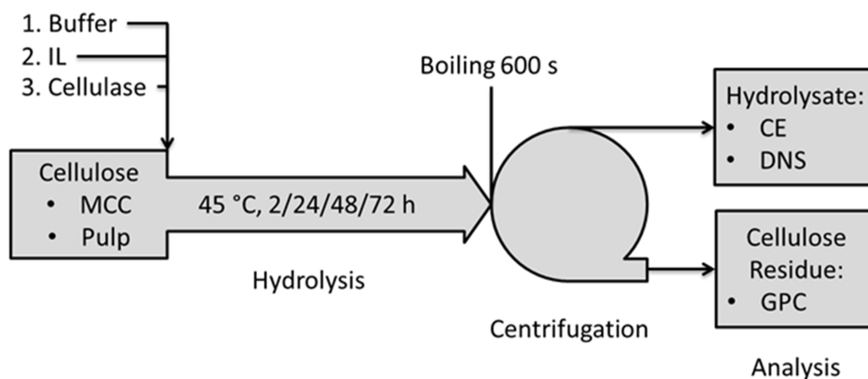

Figure 8. Flow chart for the hydrolysis experiment with product separation and analysis. CE = capillary electrophoresis, DNS = 3,5-dinitrosalicylic acid assay, GPC = gel permeation chromatography.

The hydrolysis was terminated by boiling the sample for 600 s. After cooling to RT, the reaction tube was centrifuged at 3000 rpm for 10 min and the clear supernatant was separated from the solid cellulose residue. In the high-IL hydrolysis samples the cellulose was (partly) dissolved and thus needed to be regenerated. This was done by adding 3 mL or g of distilled water after boiling, followed by vigorous mixing before centrifugation. When using RC as substrate (I), the procedure as described above was followed with the exception that the substrate was stirred and swollen in buffer overnight before the addition of enzyme. All experiments were carried out in triplicate. Reference samples were treated in the corresponding conditions without addition of enzyme.

3.5 Hydrolysis of cello-oligomers with β-glucosidases in IL solutions

Enzymatic hydrolysis of cello-oligomers (in the range of cellobiose to cellohexaose) to glucose with *A. niger* β-glucosidase was carried out in buffer (0.050 M citrate, pH 5.0 or 0.100 M phosphate, pH 6.0) in the presence of [DMIM]DMP and [EMIM]AcO, as described in II. The hydrolysis temperature was 45 °C and the hydrolysis time 20 h. The hydrolysate cello-oligomer composition was analysed by CE with pre-column derivatization as described in Section 3.6 and in II.

3.6 Saccharide analysis in hydrolysates

The saccharides solubilized in the enzymatic hydrolysis of solid substrates were analysed by DNS assay (III, IV and V) or by a CE method (I, II, III and V), which was especially optimised for saccharide analysis in IL matrices (II). DNS assay was carried out according to the procedure described in Ghose (1987) with the

DNS reagent solution prepared as described in Sumner (1924). When hydrolysates containing IL were analysed with the DNS assay, it was important to correct the background with the reference samples. Saccharide quantification was also carried out using the PAHBAH assay as described in Lever (1973) in III. In order to have a comparable quantification of reducing saccharides in hydrolysates with different distributions of oligosaccharides, a secondary acid hydrolysis was performed to degrade all oligosaccharides to monosaccharides. For acid hydrolysis, the sample (1 mL, pH adjusted to below 7) was mixed with 0.05 mL 70% H_2SO_4, autoclaved for 1 h at 120 °C and then diluted to 2.5 mL with milli-Q water.

CE with pre-column derivatization was employed for analysing hydrolysis products (mono- and oligosaccharides) in the hydrolysates. The method development and applicability are described in Section 4.1.2 and in more detail in II. The hydrolysate saccharides with galactose added as internal standard were derivatized by 4-aminobenzonitrile (ABN, in buffer and [DMIM]DMP matrices) or 4-aminobenzoic acid ethyl ester (ABEE, in [EMIM]AcO and [TMGH]carboxylates) through reductive amination. After centrifugation, aliquots of the sample liquid were analysed by CE. Saccharide quantitation was carried out against standard curves acquired for each analyte in the different matrices. Full technical details of this method can be found in II.

3.7 Analysis of solid cellulose hydrolysis residues

The molecular mass distribution of the solid cellulose residues after enzymatic hydrolysis and regeneration was determined by gel permeation chromatography (GPC) as described in I. The samples were washed to remove any residual IL (dry samples were activated by mixing with water) and a solvent exchange procedure was carried out in which the cellulose samples were treated three times with ethanol and three times with dimethylacetamide (DMAc), before dissolution in a dry solution of 8% (w/v) LiCl in DMAc. After complete cellulose dissolution, the sample was diluted, filtered and subjected to GPC analysis on the HPLC system described in I. The molecular mass distribution was determined by comparison to pullullan standards.

Fourier transform infrared (FTIR) spectroscopy with photoacoustic detection was applied to study crystallinity changes in the solid hydrolysis residues after enzymatic treatments in IL matrices (I). Cellulose crystallinity indices can be calculated from the FTIR spectra peaks. In this work, the lateral order index (LOI) was calculated as the peak absorption ratio between the peaks located at 1437 cm^{-1} and 899 cm^{-1} (Hurtubise and Krassig 1960) and the total crystallinity index (TCI) as the ratio between the 1378 cm^{-1} and 2900 cm^{-1} peak absorptions (Nelson and O'Connor 1964).

3.8 Cellulase substrate binding experiments with ^{3}H-labeled *Trichoderma reesei* cellulases

The binding of *T. reesei* cellulases and their core domains (CDs) to MCC in the presence of selected ILs was elucidated with labeled proteins (IV). *T. reesei* Cel5A, Cel5A CD, Cel7A and Cel7A CD were labeled with ^{3}H through reductive amination of free amines (lysine residues and N-terminus) with formaldehyde as methyl group donor and [^{3}H]$NaBH_4$ as reducing agent, as described in detail in IV. The ^{3}H-labeled protein preparations were characterized by activity assays and SDS-PAGE (IV, supplementary material), according to which no protein degradation or any reduction of specific enzyme activity had occurred during the labeling reaction. Binding experiments were carried out at 4 °C in 1% (w/w) MCC dispersion in 0.050 M citrate buffer (pH 5.0) containing [DMIM]DMP or [EMIM]AcO (0, 20 and 40% w/w) and initial cellulase concentrations of 0.1–10 µM. An equilibration time of 4 h was used, which was sufficient for equilibration in all the studied matrices at 4 °C. Measurements were carried out at equilibrated binding in order to ensure that the binding results were comparable with each other and not time dependent. 4 °C was chosen as binding temperature in order to suppress enzymatic hydrolysis of the MCC. The cellulase binding was calculated on the basis of the amount of unbound cellulase in the supernatant as quantified by Liquid Scintillation Counting (LSC) by comparing samples with MCC to reference samples without MCC. The presence of IL in the samples did not cause any observable interference with the LSC analysis. Isotherms were plotted based on the calculated bound enzyme per gram of MCC against the concentration of free enzyme at equilibrium.

4. Results and discussion

4.1 Analytical considerations and development of ionic liquid-compatible analytical methods (II)

4.1.1 Effects of ionic liquids on common carbohydrate analysis methods

In this work, the DNS assay was used for quantifying the total amount of reducing sugars in the hydrolysates (III, IV, V), or as a complimentary method to CE analysis (I, II, III). In general, ILs were observed to give a background absorption in the spectrophotometric detection (II). Some of the used ILs were coloured and additional colour formation usually took place during the heating phase of the DNS assay, suggesting that the DNS reagent solution reacted with the IL or some impurity in it. Thus it was of great importance to carefully correct the DNS result for any sample with the corresponding reference samples as background. The studied imidazolium-based ILs, [DMIM]DMP and [EMIM]AcO, were in all concentrations miscible with the DNS reagent solution, whereas 1,1,3,3-tetramethylguanidinium acetate ([TMGH]AcO) caused precipitation in concentrations greater than 20% (w/w). DNS analysis could be carried out for samples containing 20% (w/w) concentrations of [TMGH]carboxylates and DBN-based ILs (unpublished results). For the phosphonium-based ILs (tetrabutylphosphonium hydroxide [P4444]OH and trioctylmethylphosphonium acetate [P8881]AcO), precipitates were formed even with low concentrations of IL in the DNS assay. The PAHBAH assay could be used for analysis of samples containing [P4444]OH, but not [P8881]AcO. It appears that the IL compatibility of the DNS and PAHBAH assays must be evaluated experimentally for each IL separately. The limit of quantification (LOQ) for the DNS assay was 0.1 mg/mL for glucose in the experimental setup used in this work (*i.e.* 1% substrate consistency in the hydrolysis experiments), corresponding to a hydrolysis degree of 1% of the dry matter (III, IV).

The application of a secondary acid hydrolysis of cellulose hydrolysates containing the ILs [DMIM]DMP and [EMIM]AcO was studied with the aim of converting cello-oligomers to glucose, to ensure that the hydrolysis yield quantification as total amount of reducing saccharides was comparable as glucose equivalents. It was observed that the higher the concentration of IL, the less of the cello-

oligomers were hydrolysed to glucose (unpublished results). In Table 7, the hydrolysis yield (72 h MCC hydrolysis with *T. reesei* Cel7A in 0.050 M citrate buffer, pH 5.0 at 45 °C), as determined by DNS assay, of the crude hydrolysate is compared to the hydrolysis yield of the same samples after secondary acid hydrolysis. An increase factor was calculated to describe how much the secondary acid hydrolysis increased the analysed yield. A falling trend in this increase factor can be seen in the hydrolysates with increasing concentrations of both [DMIM]DMP and [EMIM]AcO. It was not studied whether any acid degradation of the saccharides in the sample solutions took place, in addition to the hydrolysis of the glycosidic bonds in the cello-oligomers. The acid hydrolysis was problematic when working with low hydrolysis yields, because the procedure further diluted the samples by a factor of 2.5. At least one reason for the problems encountered appeared to be the unpredictable pH shifts caused by the presence of ILs. Acid hydrolysis was not used in this work, but possibly this method could be optimized to work for samples containing IL in the future.

Table 7. Hydrolysis yields from 72 h MCC (1% w/w) hydrolysis with *Trichoderma reesei* Cel7A (400 nM) at 45 °C in 0.050 M citrate buffer (pH 5.0). The yields were determined before and after secondary acid hydrolysis by DNS assay. The increase factor illustrates how much the apparent yield increased as a result of the acid treatment before DNS assay. LOQ = limit of quantification.

Matrix	Crude Yield	StDev	Yield AH	StDev	Increase factor
Buffer pH 5.0	9.2	0.4	14.9	0.1	1.62
10% [DMIM]DMP	5.1	0.5	8.1	0.1	1.58
20% [DMIM]DMP	5.1	0.1	7.2	0.6	1.42
30% [DMIM]DMP	4.5	0.3	5.1	0.7	1.13
40% [DMIM]DMP	2.5	0.1	2.6	0.1	1.04
50% [DMIM]DMP		< LOQ			NA
10% [EMIM]AcO	6.8	0.5	8.2	0.1	1.20
20% [EMIM]AcO	4.2	0.3	4.6	0.3	1.09
30% [EMIM]AcO		< LOQ			NA
30% [EMIM]AcO		< LOQ			NA
40% [EMIM]AcO		< LOQ			NA
50% [EMIM]AcO		< LOQ			NA

Because the distribution of soluble oligosaccharides in the hydrolysates was of interest in this work, the ability of different high performance methods to characterize the various oligosaccharides in IL matrices was tested. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described in Tenkanen and Siika-aho (2000) allows the quantitative analysis of linear cello-, manno- and xylo-oligomers up to DP 6 in aqueous solutions.

However, in the presence of IL (tested with [DMIM]DMP), the saccharide standards could not be identified at all in the chromatograms (II). CE methods with direct (Rovio, et al. 2008) and indirect (Soga and Ross 1999) detection were also assessed for saccharide analysis in [EMIM]AcO and [DMIM]DMP matrices, but the saccharide standards did not give any identifiable signals in the test runs (unpublished results).

It is known that imidazolium-based ILs may be deprotonated at their C2 position to yield highly nucleophilic carbenes, which in turn react with the reducing ends of saccharides (see Section 1.4). Currently it is not known to what extent this reaction affects the saccharide quantification analytics. It can be concluded that ILs interfere with both quantitative and qualitative methods generally used for saccharide analysis in aqueous solutions. There is thus a clear need to further develop both robust and sensitive IL-compatible saccharide analytics in the future.

4.1.2 Development of a capillary electrophoresis method for carbohydrate analysis in aqueous ionic liquid matrices

Inspired by the work of Kamiya et al. (2008), in which hydrolysate saccharides were analysed with HPLC as ABEE derivatives in matrices containing the IL [EMIM]DEP, a new CE method to analyse saccharides in the presence of ILs was developed (II). In this method, reducing saccharides were derivatized with aromatic amines through reductive amination (Figure 9) prior to CE analysis (II). The CE method developed in this work was based on analysis conditions described in Sartori et al. (2003), with saccharide derivatization carried out as in Dahlman et al. (2000). It was found that the presence of ILs did not prevent the derivatization reaction from taking place (II). Depending on the type of IL present in the sample, better detector responses were obtained by using either ABEE or ABN as derivatization reagent. Tests were also made with 6-aminoquinoline, but this derivatization reagent was found to give many interfering peaks of unknown origin in the electropherogram. Generally, the benefits of derivatization are higher detection sensitivity and selectivity.

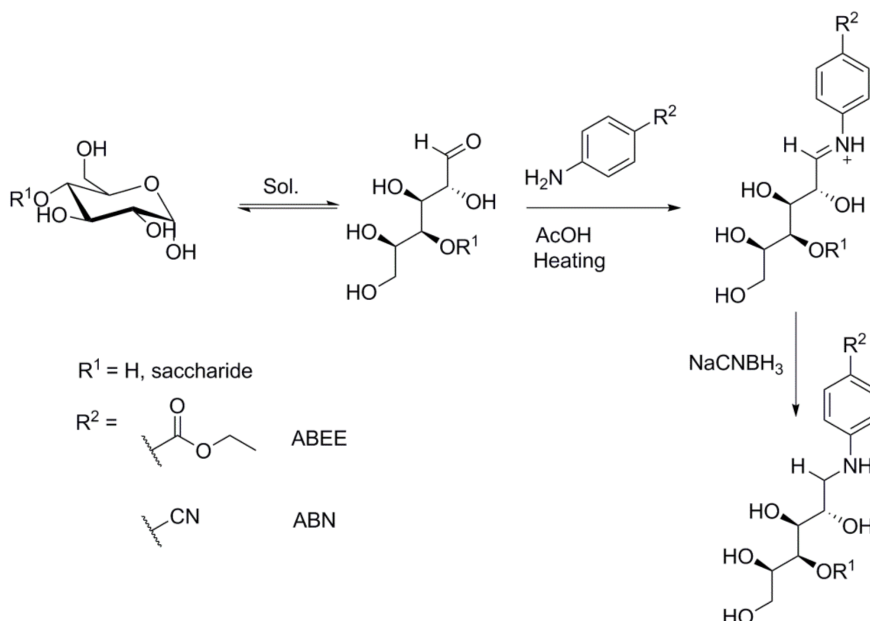

Figure 9. Saccharide labeling with aromatic amines through reductive amination.

Analysis conditions for labeled saccharides were optimized for CE (II). Increasing the analysis temperature from 15 °C (Sartori, et al. 2003) to 30 °C greatly improved the detector response and analyte peak shapes, possibly due to decreased sample viscosity. When optimizing the borate concentration of the background electrolyte (BGE), the optimum concentration was found to be the same (450 mM) in both aqueous and IL-containing samples, although the high ion content of the IL matrices was anticipated to seriously change the optimal BGE constitution. Borate ions in the BGE forms complexes with the saccharides, thus inducing charge to the otherwise neutral saccharides (Dahlman, et al. 2000; Hoffstetter-Kuhn, et al. 1991; Sjöberg, et al. 2004). The BGE composition was also optimized by leaving out the organic components used in (Sartori, et al. 2003), as these were found to offer no benefits in the presence of ILs. The electrophoresis was carried out in reverse polarity mode, which was highly beneficial because the saccharides could thus be detected before the very large peak of excess derivatization reagent, which seriously affected the baseline at higher migration times. The sample and BGE together form a very complex system, necessitating excessive capillary rinsing to minimize capillary blocking due to precipitations.

The applicability of the optimized CE method was studied with the monosaccharides galacturonic acid, glucose, mannose, arabinose and xylose, and cello-, manno- and xylo-oligomers in the range of monosaccharide to hexasaccharide. Of the monosaccharides, the mannose, glucose and arabinose peaks partially overlapped (II, Figure 2), whereas the others were well separated. Cello- and xylo-

oligomers were mainly separated, although some of the longer oligomers overlapped (II, Figure 3). Manno-oligomers could not be separated from each other with this method, as they migrated in the same peak (mannobiose to mannohexaose). Further details concerning the detection and quantification limits can be found in II.

In addition to the imidazolium-based ILs [DMIM]DMP and [EMIM]AcO, the CE method was also found to be applicable to samples containing other hydrophilic and cellulose-dissolving ILs: [TMGH]AcO and all three of the DBN-based ILs studied in this work (III and unpublished results). Depending on the type of IL, the method was compatible with 20 to 40% (v/v) of IL in the sample matrix. To further improve the method, it could be fruitful to study the use of fluorescing tags such as 8-aminopyrene-1,3,6-trisulfonic (APTS) or 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) for derivatising the analyte saccharides. Labeling the saccharides with fluorescing tags would potentially greatly improve the sensitivity of the analysis.

4.2 Dissolution of cellulosic substrates in aqueous ionic liquid solutions (I, III)

In this work it was important to know the conditions in which partial dissolution of cellulose takes place, as the dissolved fraction (either in solution or partially regenerated) can be expected to have very different substrate properties from the native cellulose in enzymatic hydrolysis (Dadi, et al. 2006; Dadi, et al. 2007; Goshadrou, et al. 2013). The dissolution of the cellulosic substrates under enzymatic hydrolysis conditions in IL solutions was followed by visual inspection, light microscopy and light transmission measurements (I). Based on previously published studies, cellulose was expected to dissolve only in IL matrices with low water content (Doherty, et al. 2010; Swatloski, et al. 2002). With the low substrate consistency of 1% (w/v) used in the hydrolysis experiments, partial or complete cellulose dissolution was observed when the IL concentration was higher than 80% (v/v). Based on the light transmission measurements, [EMIM]AcO was a much more efficient cellulose (MCC) solvent than [DMIM]DMP (I, Figure 2), with virtually full MCC dissolution in 90% (v/v) [EMIM]AcO. MCC did not completely dissolve even in 100% [DMIM]DMP under the applied mild conditions (45 °C, 1 d magnetic mixing). Partial dissolution was observed as increasing light transmission when the [DMIM]DMP content was above 90% (v/v). On the basis of visual inspection, [TMGH]AcO appeared to be between [EMIM]AcO and [DMIM]DMP in dissolving power for MCC. The dissolution of MCC under hydrolysis conditions as a function of [EMIM]AcO concentration in buffer is shown in Figure 10. In 90% [EMIM]AcO, the substrate was dissolved to give a completely transparent solution. In the experiments with eucalyptus pre-hydrolysis kraft dissolving pulp, the pulp was observed not to dissolve as well as the MCC (III). The less efficient dissolution of dissolving pulp was probably due to two factors: 1) the pulp had a higher molecular weight (M_w) of 300 000 g/mol (III, Figure 7) than MCC with a M_w of 50 000 g/mol (I, Table 3) and 2) the ground dissolving pulp was found to cluster to

some extent with concomitant mixing problems in both aqueous and IL containing hydrolysis matrices, whereas the mixing for the MCC was close to ideal due to its fine and even particle size (III).

Figure 10. MCC (1% w/v) dissolution in [EMIM]AcO/buffer (45 °C, 1 d mixing) as a function of [EMIM]AcO concentration. From the left: buffer, 20, 60, 80 and 90% (v/v) [EMIMAcO in buffer. Note that the bottles also contain a magnetic stirring bar.

The degree of dissolution of cellulose in ILs was anticipated to lead to crystallinity changes in the regenerated cellulose residues after hydrolysis, and therefore the crystallinity changes in the hydrolysis residues were assessed by FTIR spectroscopy. The total crystallinity index (TCI) and the lateral order index (LOI) were calculated from the FTIR spectra as explained in Section 3.7. TCI is a measure of the overall degree of order in the cellulose, whereas LOI is a measure of the ordered regions perpendicular to the chain direction and is greatly influenced by cellulose processing (Široky, et al. 2012). As expected, the crystallinity of dissolved and subsequently regenerated MCC samples was signicantly lower than the crystallinity of the original MCC (Table 8). The LOI values corresponded well with the expected crystallinity changes, whereas the TCI values did not correlate with the LOI values or with the expected crystallinity changes. Similar observations of the applicability of TCI and LOI were recently made by Zhao et al. (2009a). Clearly, samples treated in [EMIM]AcO had much lower crystallinity than samples treated in [DMIM]DMP, correlating with the superior dissolving power of [EMIM]AcO. The samples treated in [DMIM]DMP had a slightly lower LOI value than that of the original MCC. Interestingly, the RC did not show the lowest LOI value, but this was found in the hydrolysis samples treated in 90% [EMIM]AcO. This might be due to different regeneration conditions.

Table 8. Lateral order index (LOI) for MCC samples treated in [DMIM]DMP and [EMIM]AcO under hydrolysis conditions (I and unpublished results).

Sample	LOI
MCC, untreated	1.098
Regenerated MCC (RC)	0.415
RC, 72 h hydrolysis, Cel7B, buffer	0.458
MCC, 72 h, 90% [DMIM]DMP + buffer (no enzyme)	0.816
MCC, 72 h hydrolysis, Cel7B, 90% [DMIM]DMP + buffer	0.697
MCC, 72 h hydrolysis, Cel5A, 90% [DMIM]DMP + buffer	0.871
MCC, 48 h, 90% [EMIM]AcO + buffer (no enzyme)	0.124
MCC, 48 h hydrolysis, Cel7B, 90% [EMIM]AcO + buffer	0.015
MCC, 48 h hydrolysis, Cel5A, 90% [EMIM]AcO + buffer	0.242

4.3 Action of *Trichoderma reesei* cellulases in aqueous ionic liquid solutions (I, II, III, IV, V)

In this work, the action of the *T. reesei* endoglucanases Cel5A and Cel7B on MCC was studied in matrices containing 0–90% (v/v) [EMIM]AcO and [DMIM]DMP (I, II) and the *T. reesei* cellobiohydrolase Cel7A (IV) was studied in 0–50% (w/w) solutions of these two ILs. In addition, the action of *T. reesei* Cel5A on MCC and a eucalyptus pre-hydrolysis dissolving grade pulp was studied in matrices containing two novel classes of cellulose-dissolving ILs, based on the organic superbases TMG and DBN (III).

4.3.1 Effect of cellulose-dissolving ILs on cellulase activity (I, V)

Inactivation kinetics of the endoglucanase activity of a commercial *T. reesei* cellulase (lyophilized cellulase powder from Sigma) in 90 and 100% [EMIM]AcO (V) and 100% [DMIM]DMP (unpublished results) was studied in the absence of substrate. The cellulase was incubated in IL at 40 °C and its residual endoglucanase activity on CMC was measured at given time points (Section 3.2). The results show a distinct difference in inactivation rate (Figure 11). When incubated in [EMIM]AcO, the cellulase lost its endoglucanase activity completely (to less than 1% of the original activity) in 4 h. The inactivation rates were similar in both 90 and 100% (v/v) [EMIM]AcO. In 100% (v/v) [DMIM]DMP, on the other hand, the cellulase appeared to be rather stable and only a very slow gradual loss of endoglucanase activity was measured during a 72 h incubation period. Based on this experiment, [EMIM]AcO is a highly inactivating IL whereas [DMIM]DMP supports cellulase activity for prolonged periods. Several other recent studies also indicate

[DMIM]DMP to be comparably less inactivating for cellulases than most other cellulose-dissolving ILs (Engel, et al. 2010; Wolski, et al. 2011).

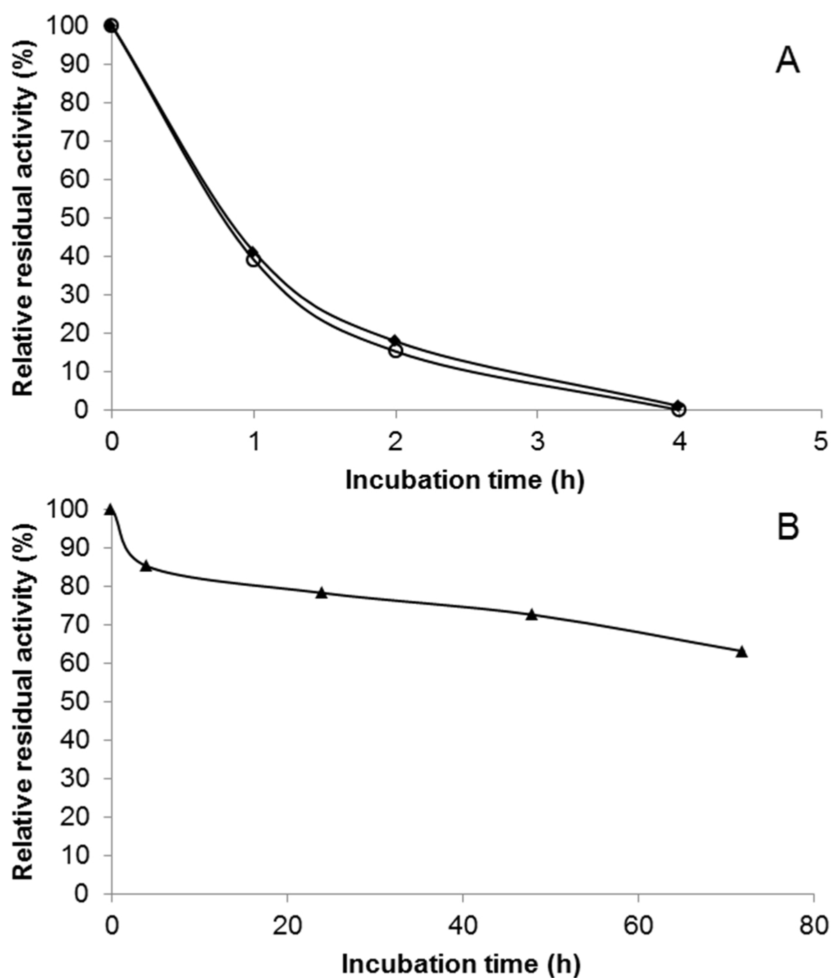

Figure 11. Inactivation of a commercial *Trichoderma reesei* cellulase powder during incubation in (A) [EMIM]AcO or (B) [DMIM]DMP. Legend: ○ 90% (v/v) [EMIM]AcO, ♦ 100% [EMIM]AcO, ▲ 100% [DMIM]DMP. The 100% activity level was measured as endoglucanase activity in 0.050 M citrate buffer (pH 5.0).

The reversibility of the inactivation of the commercial *T. reesei* cellulase was studied by diluting the samples incubated in [EMIM]AcO with buffer to very low final IL concentrations (below 4% v/v IL). No regeneration of activity was observed, suggesting that the inactivation of the enzyme in [EMIM]AcO was irreversible, or at least that the inactivated cellulase was not able to regain its activity in the pres-

ence of even as low [EMIM]AcO concentrations as 3% (w/w). In V, the residual cellulase activity after incubation in [EMIM]AcO was further studied by following the enzymatic effect on the molecular mass distribution of regenerated pulp. According to the results obtained by this alternative activity measurement method, some residual cellulase activity was left after incubation in [EMIM]AcO for even 10–11 h. These results indicate the endoglucanase activity measurement on CMC used in this work to be insufficient, and that alternative methods are needed to analyse the activity of cellulases in the presence of ILs. The studied cellulase did not regain its activity on CMC when diluting with buffer after incubation in [EMIM]AcO, but in the case in which the incubation in IL was done in the presence of pulp, the pulp may have had a protecting effect on the enzyme, possibly explaining the difference between the incubation times required for complete enzyme inactivation. Zhao et al. (2009a) reported an increase in the thermal stability of a commercial cellulase cocktail in the presence of regenerated cellulose, which was explained by a higher adsorption of the enzyme to the regenerated cellulose with high surface area. Possibly, similar effects led to the higher residual endoglucanase activity in the experiments, in which the cellulases were incubated together with pulp before activity assay.

Inactivation of *T. reesei* cellulases by boiling was studied to determine whether boiling was sufficient for complete enzyme inactivation and whether ILs protected the enzymes during boiling. *T. reesei* Cel5A was preincubated for different times (0.25, 2 and 72 h) in matrices containing [DMIM]DMP and thereafter boiled for 10 or 20 min. Boiling for 10 min reduced Cel5A activity on CMC by approximately 50% when preincubation (0.25 h) was performed in buffer. When incubated in 20–90% (v/v) [DMIM]DMP and boiled for 10 and 20 min, the residual endoglucanase activity was 20–60% of the original activity (unpublished results). The 20 min boiling clearly reduced the endoglucanase activity more than 10 min boiling. The presence of [DMIM]DMP appeared to protect the *T. reesei* Cel5A from inactivation during boiling (I and unpublished results). The data obtained in this work does not give any indication on how [DMIM]DMP could protect the cellulases during boiling, but it has been described in several articles how ILs can be used to stabilize enzymes under inactivating conditions *e.g.* by coating the enzymes with suitable ILs (Lozano, et al. 2011; Monhemi, et al. 2012). Based on these experiments, significant endoglucanase activity was present in the hydrolysates after boiling and it cannot be excluded that the residual active cellulase could be able to hydrolyse the regenerated cellulose during washing or regeneration after hydrolysis. The effect of [EMIM]AcO during boiling was not studied as this IL had been found to inactivate *T. reesei* cellulases irreversibly in 4 h (Figure 11).

4.3.2 Effect of ILs on yield and products in enzymatic cellulose hydrolysis (I, II, III, IV)

The effect of several different classes of cellulose-dissolving ILs (Figure 7) on the yields in cellulose hydrolysis with *T. reesei* cellulases was studied. [EMIM]AcO

was found to be more harmful than [DMIM]DMP in MCC hydrolysis with both *T. reesei* Cel5A (I, II, IV), Cel7B (I) and Cel7A (IV) (Table 9). No clear differences were observed in the effect of ILs on the hydrolysis yields of the two endoglucanases Cel5A and Cel7B. The action of cellobiohydrolase Cel7A was clearly less affected than that of the endoglucanases by the presence of IL. Although Cel5A was more efficient in the beginning of the hydrolysis (2 h timepoint), Cel7A gave higher yields both in buffer and in IL matrices (IV, Figures 1A and 1B). No soluble saccharides were released by the endoglucanases when the IL concentrations increased above 40–50% (w/w), whereas the cellobiohydrolase Cel7A was able to produce low amounts of saccharides from MCC even in 50% (w/w) of both ILs, as determined by the DNS assay. In Kamiya et al. (2008) it was also observed that a *T. reesei* cellulase cocktail was almost completely ineffective in cellulose hydrolysis when the IL concentration in the hydrolysis matrix increased above 40% (v/v). Some differences in hydrolysis yield were also observed depending on enzyme dosage and analysis method (Table 9). No cellulose dissolution occurred in these IL concentrations (see Section 4.2).

Table 9. Hydrolysis yields (in % of substrate dry weight) in 72 h MCC hydrolysis with *T. reesei* cellulases at 45 °C. Yields were determined by CE analysis, the IL concentration was in % (v/v) and the enzyme dosage was 2000 nkat/g of MCC, with the activity measured on CMC. In the entries marked with *, the yields were determined with the DNS assay, the IL concentration was in % (w/w) and the enzyme concentration was 400 nM. The error values are based on the standard deviation of three parallel samples.

Cellulase	Buffer	[DMIM]DMP		[EMIM]AcO		
		20% IL	40% IL	20% IL	40% IL	Ref.
Cel5A	8.8 ± 1.0	2.0 ± 0.3	Traces	0.4 ± 0.0	0.0	I
Cel5A*	9.5 ± 0.7	2.7 ± 0.5	1.1 ± 0.0	2.4 ± 0.4	Traces	IV
Cel7A*	9.2 ± 0.4	5.1 ± 0.1	2.5 ± 0.1	4.2 ± 0.3	Traces	IV
Cel7B	5.6 ± 0.5	1.0 ± 0.3	Traces	Traces	0.0	I

A distinct difference in the hydrolysis yields of *T. reesei* Cel5A as a function of time was observed in [DMIM]DMP and [EMIM]AcO matrices (II, Figure 5). The presence of [DMIM]DMP (20 and 40% v/v) slowed down the enzymatic hydrolysis rather than completely preventing it and the maximum hydrolysis level was not attained in 72 h. In 20% (v/v) of [EMIM]AcO the hydrolysis yield was very low with Cel5A and did not increase after the first hours of hydrolysis, suggesting that the cellulase was no longer able to hydrolyse MCC. This observation of the different effects of these two ILs on the hydrolysis time curves correlates well with the inactivation measurement data presented in Section 4.3.1. Taking into account how differently Cel5A is inactivated in [DMIM]DMP and [EMIM]AcO, the differences in 72 h hydrolysis yields are surprisingly small, suggesting that direct inactivation by

the ILs may not be the only reason for the observed low hydrolysis yields in IL matrices. There is a certain discrepancy between the hydrolysis yields obtained in [EMIM]AcO matrices in the different experimental series, the reason of which is unknown (I, II, III and IV). To some extent the differences might be explained by the use of two different analysis methods, CE with pre-column derivatization and DNS assay. With other ILs, of which the most studied was [DMIM]DMP, the results were more consistent.

T. reesei Cel5A was used as reference cellulase to compare the effects of [EMIM]AcO, [DMIM]DMP, tetra-alkylphosphonium-, TMG- and DBN-based ILs on the enzymatic hydrolysis of MCC (III and unpublished results). Four cellulose-dissolving [TMGH]carboxylates and three DBN-based ILs were included in the study: 1,1,3,3-tetramethylguanidinium formiate ([TMGH]COO), [TMGH]AcO, 1,1,3,3-tetramethylguanidinium propionate ([TMGH]EtCOO), 1,1,3,3-tetramethylguanidinium butyrate ([TMGH]*n*-PrCOO), 1,5-diazabicyclo[4.3.0]non-5-enium acetate ([DBNH]AcO), 1,5-diazabicyclo[4.3.0]non-5-enium propionate ([DBNH]EtCOO) and 1-methyl-1,5-diazabicyclo[4.3.0]non-5-enium dimethylphoshate ([DBNMe]DMP) (Figure 7), as well as [P4444]OH and [P8881]AcO. The compatibility screening was made at 20% (w/w) IL concentration for 72 h at 45 °C. In buffer solution (pH 5.0) the hydrolysis yield was 7.7%, whereas the hydrolysis yield was decreased by 70% or more in all the IL matrices. Thus, it was clear that all of the studied ILs were very harmful for the action of *T. reesei* Cel5A (Figure 12). In most cases, the difference between the effects of ILs were rather small and even within the error limits. The DBN-based ILs were the most detrimental to Cel5A action. The DBN-based cation is probably not very cellulase-compatible, as the other used acetates and dimethylphosphates were less inhibiting for the cellulase. Of the TMG-based ILs, the acetate was the most cellulase-compatible. Samples containing [P8881]AcO could not be analysed in this work because this IL precipitated with both the DNS and PAHBAH reagent solutions. The [P4444]OH samples were analysed with the PAHBAH assay and this IL was clearly very harmful to the action of *T. reesei* Cel5A. Based on this experiment it is clear that the new cellulose-dissolving IL classes studied here are even less cellulase-compatible than the imidazolium-based ILs, even though the new ILs have other desirable properties, such as distillability and a low price of starting materials.

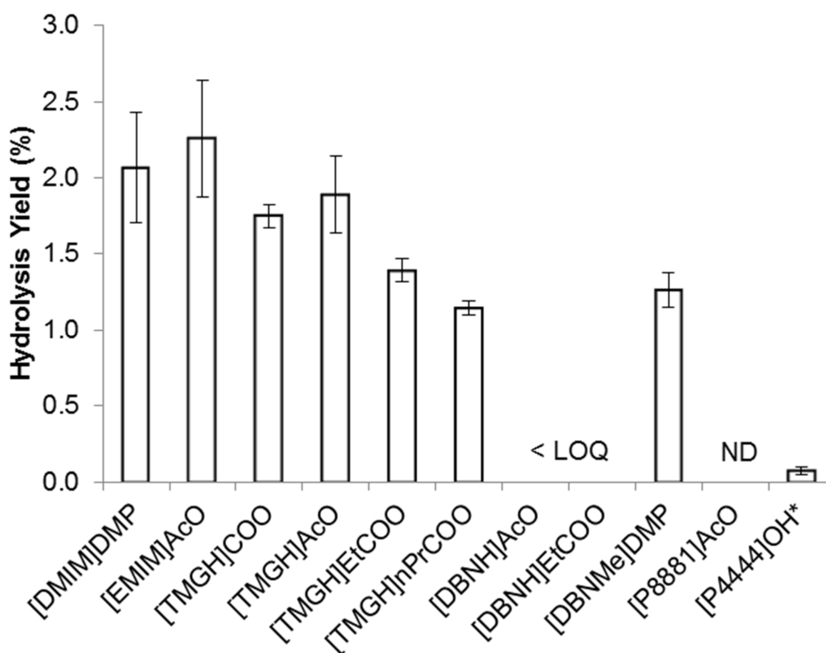

Figure 12. Comparison of the effect of 20% (w/w) of different cellulose-dissolving ILs on 72 h MCC hydrolysis yields (% of dry weight) with *Trichoderma reesei* Cel5A. The enzyme dosage was 2000 nkat/g MCC based on activity on CMC. Hydrolysis yields were determined by the DNS assay, except for samples containing [P4444]OH (marked with *), for which the yield was determined by the PAHBAH assay. The limit of quantification (LOQ) with the DNS assay was 1% yield, for PAHBAH assay the LOQ was below 0.1%. ND denotes that neither DNS nor PAHBAH assays were applicable. Error bars are based on the standard deviation for three replicate samples. The hydrolysis yield of the buffer reference was 7.7%.

In addition to the hydrolysis yields, the cello-oligomer distribution of the saccharides released from MCC by *T. reesei* Cel5A and Cel7B in the different IL matrices (I, III and V) were also determined using the CE method described in II and in Section 4.1.2. *T. reesei* Cel5A has been reported to produce glucose, cellobiose and cellotriose in MCC hydrolysis in buffer at pH 5.0, whereas the main products of Cel7B are glucose and cellobiose (Karlsson, et al. 2002). The same product distribution was observed in buffer (pH 5.0) in this work (I, Tables 1 and 2). The presence of IL in the hydrolysis matrix shifted the cello-oligomer distribution from glucose towards cellobiose and cellotriose for both Cel5A and Cel7B. No longer cello-oligomers were observed. The same shift in the cello-oligomer distribution was observed when the hydrolysis was carried out in phosphate buffer at pH 7.0 instead of in citrate buffer at pH 5.0. Thus, it cannot be ruled out that the shift to longer cello-oligomers in the product distribution could be a pH effect, as the IL did

cause the hydrolysis matrix pH to shift to more alkaline values (I, Figure 3). In (III) a similar shift was also observed in the product distributions of *T. reesei* Cel5A and IndiAge® ONE, but not in the product distribution of Puradax® HA 1200E, when these enzymes were studied in [EMIM]AcO and [TMGH]AcO (III, Table 2). Obviously, the shift to longer cello-oligomers is not only dependent on the IL, its concentration and the pH of the matrix, but also on the studied cellulase. The shift towards longer cello-oligomers in hydrolysates containing IL could also have an effect on any potential end-product inhibition, but in this work such an effect was considered negligible due to the very low concentrations of cello-oligomers in the final hydrolysates and the shift in the product distribution being rather modest.

4.3.3 Effect of IL on the molecular mass distribution of cellulose in enzymatic hydrolysis (I, III)

The solid cellulose residues from the partial hydrolysis of MCC with the *T. reesei* endoglucanases Cel5A and Cel7B in the presence of ILs were analyzed by GPC in order to study the changes in the molecular mass distribution. The ILs did not cause any complications in this analysis, as they could be washed off from the residual cellulose before cellulose dissolution for GPC analysis.

The weight average molecular mass (M_w) of MCC was not affected by the IL or endoglucanase treatments in buffer or in IL-containing matrices in which cellulose dissolution did not occur (Section 4.2 and I, Table 3). A significant reduction of the M_w from 50 000 g/mol to 35 000–36 000 g/mol was observed for MCC treated with Cel5A or Cel7B in 90% (v/v) [DMIM]DMP. The same was not observed in any of the [EMIM]AcO matrices, including those in which MCC had clearly decreased crystallinity after complete dissolution in aqueous [EMIM]AcO (Section 4.2 and Table 8). The M_w reduction appears to be limited by the substrate properties rather than by the used cellulase, as the M_w reduction as a function of hydrolysis time was almost identical for Cel5A and Cel7B and doubling the cellulase dosage did not lead to increased M_w reduction (I, Table 3). On the other hand, with the core domain (CD) of *T. reesei* Cel5A (lacking the carbohydrate-binding module, CBM), the M_w reduction did not reach the same level as with the intact Cel5A. The M_w reduction was not accompanied with any detected formation of soluble saccharides in MCC hydrolysis with *T. reesei* Cel5A and Cel7B (I), suggesting a different mode of endoglucanase action on the partially dissolved cellulose. The endoglucanases may have a preference for random chain scission of the dissolved cellulose molecules instead of producing soluble cello-oligomers, as during the hydrolysis of undissolved MCC. This mode of action is possibly coupled with low cellulase adsorption to the substrate in high IL concentrations (see Section 4.4.2). Based on the M_w reductions there appears to be a fundamental difference between how the studied endoglucanases were able to act in concentrated [DMIM]DMP and [EMIM]AcO solutions, correlating with the residual activity measurements after incubation in these two ILs (Section 4.3.1).

Two alternatives are possible for the observed molecular mass reductions in the residual cellulose: 1) the *T. reesei* endoglucanases Cel5A and Cel7B hydrolysed the dissolved cellulose chains in the aqueous 90% (v/v) [DMIM]DMP solution or 2) the endoglucanases were inactive in 90% (v/v) [DMIM]DMP throughout the hydrolysis time but regained their activity during regeneration and hydrolysed the amorphous regions of the partly regenerated MCC in the diluted IL. The M_w reduction was clearly dependent on the hydrolysis time for both Cel5A and Cel7B (I, Table 3), which would support alternative 1. Alternative 2 is supported by the observation that endoglucanases may retain significant residual activity during prolonged incubation times in [DMIM]DMP (Section 4.3.1 and Figure 11). As discussed in Section 4.3.1, boiling was not sufficient to completely inactivate the used endoglucanases, which means that significant endoglucanase activities were still present in the hydrolysis mixtures after the hydrolysis had been terminated. Furthermore, [DMIM]DMP appears to inactivate cellulase reversibly (Engel, et al. 2010), making it possible that the cellulases inactivated in highly concentrated IL could regain their activity when the IL matrix was diluted with water *e.g.* during regeneration of dissolved MCC or during washing of the MCC. In future work, other inactivation methods (*e.g.* chemical inhibition) should be considered to avoid having residual endoglucanase activities in the regeneration mixtures.

In order to determine whether post-hydrolysis (alternative 2) of residual cellulose can take place during the after-treatment of the hydrolysis mixtures, a 30 min hydrolysis of cellulose regenerated from partial dissolution in 90% (v/v) [DMIM]DMP for 72 h was carried out either in buffer or in 45% (v/v) [DMIM]DMP with fresh *T. reesei* endoglucanase using a dosage corresponding to the endoglucanase activity measured in the hydrolysates after boiling for 10 min (unpublished results). 45% (v/v) [DMIM]DMP corresponds to the hydrolysis matrix in the potential post-hydrolysis, as regeneration after hydrolysis was always carried out by adding one part water to one part of hydrolysis mixture. The residual cellulose samples treated in buffer with cellulase showed a clear decrease in M_w corresponding to the M_w reduction observed for samples treated with endoglucanases in 90% (v/v) [DMIM]DMP. However, the samples treated in 45% (v/v) [DMIM]DMP showed no decrease in M_w, indicating that the *T. reesei* endoglucanases were unable to hydrolyse the solid, partially regenerated residual cellulose under the conditions of the hydrolysis mixture after-treatment in this high content of IL. Based on this experiment, alternative 1 would be supported, *i.e.* the dissolved fraction of the cellulose was hydrolysed in 90% (v/v) [DMIM]DMP, in which the endoglucanases also exhibited a certain degree of activity. At the same time it also became clear that the partially regenerated cellulose could easily and rapidly be degraded by the endoglucanases in buffer. This experiment confirmed that no cellulose degradation due to adsorbed cellulases being released to the washing liquor occurred during washing of the cellulose residues. Endoglucanase denaturation and the possible regeneration of activity after incubation in highly concentrated IL is a topic which clearly requires further investigation.

Whereas the studied *T. reesei* endoglucanases were not able to reduce the M_w of MCC in buffer, even when 8.8% or 5.6% of the cellulose was solubilised by

Cel5A and Cel7B, respectively (I, Tables 1 and 2), the M_w of RC was considerably reduced during hydrolysis (I, Table 3). The reduction of M_w of RC was clearly different for the two cellulases. Cel5A reduced the M_w from 47 000 g/mol to 12 000 g/mol, whereas with Cel7B the M_w decreased from 47 000 g/mol to 32 000 g/mol. The M_w reductions were paired with approximately doubled yields of solubilized cello-oligomers (I, Tables 1 and 2) when RC was compared to untreated MCC as substrate. These results support the previous findings that endoglucanases play a more significant role in the hydrolysis of regenerated, more amorphous cellulose than in the hydrolysis of highly crystalline substrates (Engel, et al. 2012a). Furthermore, there appear to be great differences between the actions of different *T. reesei* endoglucanases on regenerated substrates.

4.4 Ionic liquid effects on hydrolysis and cellulose binding of *T. reesei* cellulases and their core domains (IV)

4.4.1 MCC hydrolysis with *T. reesei* Cel5A and Cel7A in ionic liquid matrices: comparison between intact and core domain cellulases

In this work, the role of the carbohydrate-binding module (CBM) in enzymatic cellulose hydrolysis and cellulase binding to MCC in IL matrices was studied systematically for the first time. In (I) the action of the *T. reesei* endoglucanase Cel5A and its core domain (CD) on MCC was compared in buffer and in 20% (v/v) [DMIM]DMP. In buffer, the intact Cel5A was manyfold more effective in MCC hydrolysis than its CD (I, Table 2), as was expected from previous studies (Tomme, et al. 1988). When 20% of [DMIM]DMP was present in the matrix, the intact Cel5A showed a 52% decrease in the 72 h hydrolysis yield, whereas surprisingly the hydrolysis yield of Cel5A CD was not affected by the presence of IL. In (IV), similar results were obtained when the hydrolysis of MCC with the *T. reesei* endoglucanase Cel5A, cellobiohydrolase Cel7A and their CDs was further studied at 45 °C in buffer and 10–50% (w/w) of [DMIM]DMP and [EMIM]AcO (IV, Figures 1A and 1B). Increasing IL concentrations affected the hydrolysis yields of all four enzymes negatively. From the hydrolysis results, it is clear that [EMIM]AcO was more harmful to the cellulase performance than [DMIM]DMP, as was observed thoughout this work.

Some interesting differences were observed in how the hydrolysis yields of MCC with intact and CD cellulases changed with the introduction of IL. When adding [DMIM]DMP (10% w/w) to the MCC hydrolysis matrices, the hydrolysis yields of the intact cellulases decreased to approximately the level of their corresponding CDs, suggesting that the CBM was not able to promote cellulose hydrolysis in [DMIM]DMP matrices. With [EMIM]AcO the same was observed for Cel5A but not for Cel7A, suggesting this effect to be potentially IL- and enzyme-dependent. After the drastic initial decrease in the hydrolysis yields of the intact cellulases caused by introducing IL into the hydrolysis matrix, both the intact and CD cellulases showed evenly decreasing yields with increasing IL concentrations.

4.4.2 Substrate binding of *T. reesei* Cel5A, Cel7A and their core domains in the presence of ionic liquids

Binding of ^{3}H-labeled *T. reesei* Cel5A, Cel7A and their respective CDs to MCC was studied in buffer and in the presence of 20 and 40% (w/w) of [DMIM]DMP and [EMIM]AcO. The low binding temperature (4 °C) was chosen in order to suppress cellulose hydrolysis, which would make comparing the binding results difficult.

In buffer, *T. reesei* Cel5A and Cel7A bound to MCC with similar binding isotherms (IV, Figure 2). Cel7A CD also bound relatively well to MCC, probably by using the substrate binding zone in its active site tunnel, as has been suggested in previous studies (Kotiranta, et al. 1999; Linder and Teeri 1996). Cel5A CD, on the other hand, exhibited extremely low binding to MCC in buffer, indicating that the CBM had an important role in the substrate binding of the intact *T. reesei* Cel5A. The difference in the binding ability of Cel5A CD and Cel7A CD is apparently due to the different structures of the substrate binding zones close to the catalytic site, which in cellobiohydrolases is a tunnel and in endoglucanases a cleft on the protein surface (Divne, et al. 1994; Teeri 1997).

The substrate binding of the studied cellulases decreased with increasing IL concentration (IV, Figures 3A, 3B and 3C). [EMIM]AcO had a stronger negative influence on substrate binding than [DMIM]DMP, similarly to its effect on the hydrolysis yields (IV, Figures 1A and 1B). However, the binding was more affected by ILs for *T. reesei* Cel5A than for Cel7A and its CD. In 40% (w/w) [DMIM]DMP the intact *T. reesei* Cel5A showed some binding, whereas no binding was observed in 40% (w/w) [EMIM]AcO. Cel7A could still bind to the substrate to some extent even in 40% (w/w) of the two ILs (IV, Figure 3B). The binding of Cel7A CD was the least affected by the presence of IL (IV, Figure 3C). The binding of Cel5A and Cel5A CD could not be compared in IL matrices, as Cel5A CD displayed extremely low binding in all of the studied matrices.

The negative influence of the ILs on the substrate binding of the studied cellulases could be due to different reasons. One possibility is that the IL causes conformational changes to the structure of the CBM, decreasing or completely annihilating its binding affinity, similarly to what has been proposed for the inactivation of intact enzymes in ILs (Sheldon, et al. 2002). In previous studies, the addition of organic solvents has also been observed to be detrimental to cellulase substrate binding (Carrard and Linder 1999). Solvents have been proposed to interfere with the hydrophobic interactions which play a major role in the cellulase binding to cellulose. As the ILs employed in this study have a significant organic character, similar interference with the hydrophobic interactions dominating substrate binding cannot be ruled out. Further studies are needed to understand the precise manner in which ILs affect the substrate binding of cellulases.

The effects of IL on the hydrolytic action and cellulose binding of the studied cellulases are compared in Table 10. On the basis of the results, there is not always a clear correlation between substrate binding and hydrolysis and the effects of ILs on these parameters. Cellulase substrate binding is known to be tempera-

ture-sensitive, with higher temperatures usually leading to decreased substrate binding (Carrard and Linder 1999; Kyriacou, et al. 1988; Linder and Teeri 1996). Therefore, it could be expected that enzyme binding in the presence of ILs is even more affected at hydrolysis temperatures than at 4 °C, which was used in these binding experiments. Significant substrate binding is apparently not needed in all cases for hydrolysis to take place, as was demonstrated with Cel5A CD in buffer, so not all of the negative IL effects on enzymatic hydrolysis can be attributed only to interference with binding.

Table 10. Comparison of the impact of [DMIM]DMP and [EMIM]AcO on the hydrolytic action and cellulose binding of *Trichoderma reesei* Cel5A, Cel7A and their core domains. The "+" sign indicates the degree to which the cellulase hydrolysis or binding was reduced by IL.

Cellulase	Reduction of hydrolysis yield vs buffer		Reduction of binding to MCC	
	[DMIM]DMP	[EMIM]AcO[1]	[DMIM]DMP	[EMIM]AcO
Cel5A	+++	+++	++	+++
Cel5A CD	+	+	ND	ND
Cel7A	++	+	+	++
Cel7A CD	+	+	+	+

[1]In high IL concentrations (>20% w/w), [EMIM]AcO was clearly more harmful than [DMIM]DMP in hydrolysis for all cellulases.

4.5 Study of the effect of ILs on commercial cellulases

4.5.1 IL effects on the action of commercial alkali- and thermostable cellulases (III)

The link between cellulase alkali- and thermostability and IL tolerance was studied in this work using three commercial thermo- or alkali-stable cellulase preparations: a thermophilic cellulase (Cel5A) from *Thermotoga maritima* and Puradax® HA 1200E and IndiAge® ONE (III). Puradax® HA 1200E is produced for detergent applications and IndiAge® ONE for denim finishing of textiles. *T. reesei* Cel5A was included in the study for comparison. The commercial cellulase preparations were characterized by SDS-PAGE, which showed them to be rather pure monocomponent preparations (III, Figure 1). Both MCC and eucalyptus pre-hydrolysis kraft dissolving pulp were used as substrates in the hydrolysis experiments. Two ILs were employed: [EMIM]AcO, and [TMGH]AcO, which, based on the IL screening described in Section 4.3.2, was chosen as a representative of the new cellulose-dissolving superbase ILs.

IndiAge® ONE was the most efficient cellulase in the hydrolysis of solid cellulose both in buffer and in the presence of IL (III, Figure 5), although Puradax® HA

1200E showed much higher specific activity on dissolved CMC (III, Figure 2). In the presence of ILs, the enzymatic hydrolysis yields were on the same level for MCC and dissolving pulp (III, Figures 5A and 5B). IndiAge® ONE was the most IL-tolerant cellulase, with some hydrolytic action even in 40% (w/w) IL, and its hydrolysis yields decreased linearly with the IL concentration. Puradax® HA 1200E was affected differently by ILs, as the hydrolysis yield was almost the same in 20% (w/w) IL as in buffer, but then rapidly decreased in higher IL concentrations. By comparison, *T. reesei* Cel5A was much more IL-sensitive than the commercial cellulase preparations. The differences between the impact of [EMIM]AcO and [TMGH]AcO on the hydrolysis yields were rather small. *T. maritima* Cel5A was found to give very poor hydrolysis yields on MCC both at 45 °C and at its optimum temperature (80 °C) in both buffer and IL matrices, so its IL tolerance could not be determined (unpublished results). The presence of ILs was observed to shift the product distribution to longer cello-oligomers for IndiAge® ONE but not for Puradax® HA 1200E (III, Table 2). The same effect was also noticed for *T. reesei* Cel5A and Cel7B (I, Tables 1 and 2) and appears to depend on the specific cellulases rather than on the IL. The cellulases were able to reduce the molecular mass of pulp but not of MCC in buffer and in some IL matrices (III, Figure 7).

The activity in high pH, thermostability, activity on CMC and hydrolysis yields on solid substrates in buffer and in the presence of ILs of the studied cellulases are presented in Table 11. As can be seen be seen from this table, enzyme activity in high pH did not give any direct benefits in cellulose hydrolysis in the presence of ILs. Activity on CMC correlated poorly with the hydrolysis of solid cellulose, suggesting that activity on soluble substrates is a poor indicator of action on solid substrates in hydrolysis experiments. Thermostability appeared to correlate better with IL tolerance, as was exemplified by IndiAge® ONE, which was more thermostable than Puradax® HA 1200E and *T. reesei* Cel5A (III, Figures 6A, 6B and 6C) and was also the most IL-tolerant cellulase. In order to obtain conclusive correlations between enzyme properties such as thermostability and IL tolerance a larger library of enzymes with various properties should be screened in selected ILs in the future.

Table 11. Comparison of the hydrolytic performance on solid and soluble substrates in IL matrices with alkali- and thermostability of cellulases (III). The “+” sign indicates the degree to which the specific property is assigned to the cellulase.

Cellulase	Activity in high pH	Thermostability	Activity on CMC	Solid hydrolysis in buffer	Solid hydrolysis in ILs
T. maritima Cel5A	+	+++[1]	+	-	-
Puradax® HA 1200E	+++	-	+++	+	++
IndiAge® ONE	+	++	+	+++	+++
T. reesei Cel5A	ND	+	+++[2]	++	+

[1] Based on manufacturers note
[2] Measured in I

4.5.2 Cello-oligomer hydrolysis with β-glucosidases in aqueous ionic liquid solutions (II)

The action of commercial *Aspergillus niger* β-glucosidase (Novozyme 188) on soluble cello-oligomers was studied in the presence of 20 and 40% (w/w) of [DMIM]DMP and [EMIM]AcO (II). Cello-oligomers in the range of cellobiose to cellohexaose were all completely hydrolysed to glucose in optimum conditions (buffer, pH 5.0), whereas increasing the pH to 6.0 partially impeded the hydrolysis of cellopentaose (II, Table 2), indicating the β-glucosidase to be pH sensitive. The presence of [DMIM]DMP and [EMIM]AcO led to lower hydrolysis degrees for both cellobiose and cellopentaose and the buffer pH also appeared to play a role in limiting the degree of hydrolysis. The β-glucosidase partially hydrolysed cello-oligomers even in 40% (w/w) [DMIM]DMP, whereas hydrolysis degrees were very low in the matrices containing [EMIM]AcO. This difference cannot be explained by pH effects. The pH value was the same (6.6) in 20% (w/w) [DMIM]DMP and 20% (w/w) [EMIM]AcO (with phosphate buffer in both), nevertheless the difference in hydrolysis degree was great. [EMIM]AcO is clearly more harmful for β-glucosidase action than [DMIM]DMP.

In this work, the crude β-glucosidase preparation was also found to contain some xylanase and endoglucanase activities which may have had minor effects on the cello-oligomer hydrolysis (II). Engel et al. (2012b) have previously reported that the β-glucosidase (Novozyme 188) did not exhibit any activity on cellobiose in matrices containing more than 15% (v/v) of [DMIM]DMP. Long hydrolysis experiments may clearly lead to different conclusions regarding IL-induced cellulase inactivation, as the β-glucosidase in this work was found still to hydrolyse cello-oligomers partially in up to 40% (w/w) [DMIM]DMP.

4.6 pH Effects on cellulase action in ionic liquid solutions (I, II, III)

Throughout this work, the pH values of the solution matrices in the hydrolysis experiments were monitored (I, II, III and unpublished results). The measured pH values of different IL matrices have been summarized in Table 12. Generally, all of the cellulose-dissolving ILs used in this work were basic in aqueous solution. This is not surprising, as the cellulose dissolution capacity is believed to be mainly dependent on the anion's capacity to accept H-bonds (*i.e.* basicity) (Doherty, et al. 2010; Tang, et al. 2012). However, pH values measured in high concentrations of IL should be regarded with a certain degree of caution, as the pH scale is defined for diluted water solutions. The basic IL shift could not be compensated by increasing the concentration of the acid component in the buffer (Table 12, rows 3 and 4). Based on the pH data in Table 12, not only the anion but also the cation plays a distinct role in the pH of aqueous IL matrices. In most cases, the added buffer (citrate at pH 5.0 or phosphate at pH 6.0) had only a minor impact on the matrix pH.

Table 12. pH of IL-containing hydrolysis matrices. C = 0.050 M citrate buffer (nominal pH 5.0), C* = 0.500 M citrate buffer (nominal pH 5.0); P = 0.100 M phosphate buffer (nominal pH 6.0). pH values in bold are from matrices in which IL and buffer were mixed on a volume basis (v/v); other pH values were measured from matrices in which IL and buffer were mixed on a weight basis (w/w).

		pH as function of IL concentration (%)						
IL	**Buffer**	**0**	**20**	**40**	**60**	**80**	**90**	**Ref**
[DMIM]DMP	C	**5.0**	**5.4**	**6.3**	**7.7**	**9.0**	**9.7**	I
[DMIM]DMP	P	6.0	6.6	7.2				II
[EMIM]AcO	C	**5.0**	**6.3**	**7.3**	**8.9**	**11.0**	**12.7**	I
[EMIM]AcO	C*	**5.0**	**6.2**	**7.2**	**8.8**	**11.1**	**12.3**	Unpublished results
[EMIM]AcO	P	6.0	6.5	7.4	8.8			II, III
[TMGH]COO	C	5.0	6.5					Unpublished results
[TMGH]AcO	C	5.0	5.6					Unpublished results
[TMGH]AcO	P	6.0	5.6	6.1	7.1			III
[TMGH]EtCOO	C	5.0	6.5					Unpublished results
[TMGH]*n*-PrCOO	C	5.0	6.4					Unpublished results
[DBNMe]DMP	P	6.0	6.8	8.1	10.5	12.6	13.6	III + unpublished results
[DBNH]AcO	P	6.0	7.1	7.9	9.7	11.2		Unpublished results
[DBNH]EtCOO	P	6.0	7.0	7.9	9.3	11.1	11.9	Unpublished results

In some recently published studies, the basicity of IL matrices has been attributed as a partial reason (Engel, et al. 2010) or even a dominating factor (Li, et al. 2012) for cellulase inactivation. The impact of IL basicity on enzyme action is probably very different for different enzymes depending on their pH activity curves. The β-glucosidase from *A. niger* studied in II was pH sensitive, but even in that case [EMIM]AcO was more harmful to the β-glucosidase action than [DMIM]DMP in matrices with practically the same pH, indicating that the ILs have other detrimental effects on the enzymes than basicity.

Comparison of the steeply decreasing hydrolysis yields of the *T. reesei* and commercial endoglucanases in increasing IL concentrations (Sections 4.3.2 and 4.5.1) with the IL matrix pH values displayed in Table 12 does not support the hypothesis that the poor cellulase performance in IL solutions would be explained only by a high matrix pH. At the same it was shown in hydrolysis experiments and activity measurements at different pH values in buffer that the studied enzymes

have considerable hydrolytic activity at pH 7 (I, Tables 1 and 2: MCC hydrolysis at pH 7.0 and III, Figure 2). In Section 4.5.1 it was clearly shown with the example of Puradax® HA 1200E that having activity in high pH does not necessarily lead to good performance on solid substrates in basic IL matrices. To conclude this discussion, the increase in pH value of the hydrolysis matrix is not the major reason for poor cellulase performance observed in this work in IL solutions. ILs probably have other harmful effects on the cellulases, such as structural changes in the protein structure leading to inactive protein conformations (Sheldon, et al. 2002). These effects need to be further studied for specific IL and enzyme combinations in the future.

5. Conclusions and future prospects

Two distinct process alternatives are available for the total enzymatic hydrolysis of lignocellulosics using ionic liquid pretreatment technologies: separate pretreatment with IL followed by washing off the IL before enzymatic hydrolysis (regeneration procedure), and the one-pot procedure in which the enzymatic hydrolysis is carried out in the same vessel as the pretreatment with the IL still present. The one-pot procedure offers several technical advantages such as simplified processes with less washing, separation and evaporation steps and easier IL recycling. However, the presence of high concentrations of cellulose-dissolving ILs effectively hinders the action of enzymes in hydrolysis. In this work, the effects of ILs on enzymatic hydrolysis of cellulose were elucidated. Some of the phenomena related to IL-induced cellulase inactivation and the effects of ILs on both the cellulases and the substrate in hydrolysis conditions were studied. Working with cellulose hydrolysis in IL solutions is challenging, as the ILs interfere with the commonly used analytical methods in carbohydrate chemistry. A CE method compatible with moderate amounts of ILs was successfully developed for the analysis of hydrolysates containing ILs. However, more work should be carried out in the future on developing saccharide analytics applicable in high salt concentrations for IL matrices.

ILs were confirmed to have a negative impact on the cellulose hydrolysis with mesophilic *Trichoderma reesei* cellulases, of which Cel5A, Cel7A and Cel7B were studied in detail. However, clear differences were observed regarding the inactivation rates and mechanisms of cellulases in ILs. [DMIM]DMP supported cellulase activity during prolonged incubation times, whereas enzyme inactivation in [EMIM]AcO was rapid and irreversible. Cellulase activity in ILs is apparently not the only factor affecting cellulose hydrolysis and possibly other factors, such as IL coating of the substrate, limit enzymatic hydrolysis. More studies are needed to elucidate the discrepancy between low hydrolysis yields and well-retained cellulase activity in ILs such as [DMIM]DMP. *T. reesei* endoglucanases were observed to cause reductions in the molecular weight of microcrystalline cellulose in 90% (v/v) of [DMIM]DMP, in which cellulose was partially dissolved. This observation was interesting as it suggested that cellulases may retain their activity even in very high concentrations of certain cellulose-dissolving ILs. The endoglucanases appeared to reduce the molecular weight without any detectable formation of cello-

oligomers, which is a different mode of action in comparison to the hydrolysis of undissolved MCC.

The results obtained in this work using commercial cellulases with increased thermo- and alkali-stability suggested that enzymes with increased IL tolerance may be found in already available enzyme products. Thermostablilty rather than activity in high pH appears to be linked to IL tolerance. In order to strengthen this hypothesis, wide screening studies with cellulases of different thermostability should be undertaken. The studied cellulose-dissolving ILs were all basic in aqueous solution to different degrees, but the basic impact was in this work generally determined not to be the main reason for the poor cellulase performance in ILs. Other IL properties affecting cellulase performance in hydrolysis, but which were not studied in this work, include high matrix viscosity, ionic strength and specific IL effects, which with some probability consist of protein unfolding and conformational changes to the active structure of the enzymes. The studies of cellulase binding and the role of the carbohydrate-binding module in ILs showed that cellulase substrate binding is probably severely hindered by the presence of ILs in the hydrolysis matrix. However, it was also shown that high substrate binding is not always a requirement for hydrolysis to take place.

If ILs are to become part of large-scale industrial biomass processing, such as polysaccharide hydrolysis and fermentation, in which cheap bulk products are produced, several challenges remain to be solved. ILs are still relatively expensive, even though their price is expected to decrease in the future. Virtually complete recyclability is needed. The distillable superbase ILs, which were also studied in this work, represent an interesting opportunity for good recyclability through distillation, and these ILs may be produced from cheaper starting materials than the conventional cellulose-dissolving imidazolium-based ILs. The biocompatibility, toxicology and biodegradability of ILs is also a topic which requires increased research efforts. This topic is not made any easier by the large number of different ILs available. Nevertheless, the large-scale use of ILs will not be possible until these issues have been sufficiently elucidated.

In the recent literature several breakthroughs have been made in designing enzyme-compatible IL systems for cellulose modification. Two main routes for tackling this problem are possible: designing cellulose-dissolving ILs which support enzyme activity or developing IL-tolerant enzymes. In the light of recently reported advances, it is reasonable to expect that the problems with enzyme inactivation in cellulose-dissolving ILs will have found a technical solution within next few years, although applying this solution to large-scale processing may not be straightforward. Cellulose hydrolysis is not the only interesting enzymatic modification of cellullose in ILs. Other potentially feasible enzymatic modifications of cellulose dissolved in IL solutions include *e.g.* oxidation, acylation and other derivatizations which could lead to new products or better production routes for existing industrial cellulose products.

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PUBLICATION I

Partial enzymatic hydrolysis of microcrystalline cellulose in ionic liquids by *Trichoderma reesei* endoglucanases

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PAPER

Partial enzymatic hydrolysis of microcrystalline cellulose in ionic liquids by *Trichoderma reesei* endoglucanases

Ronny Wahlström,* Stella Rovio and Anna Suurnäkki

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The enzymatic hydrolysis of cellulose in ionic liquid (IL) containing systems has recently received a lot of interest as a pretreatment in biomass conversion to liquid biofuels and chemicals. In this paper we present a study in which the activity and action of two *Trichoderma reesei* endoglucanases, Cel7B and Cel5A, were evaluated in aqueous solutions containing 0–90% (v/v) of the ionic liquids 1,3-dimethylimidazolium dimethylphosphate or 1-ethyl-3-methylimidazolium acetate, using microcrystalline cellulose (Avicell) as a model substrate. The degree of hydrolysis was analysed by capillary electrophoresis of the hydrolysates and gel permeation chromatography of the remaining cellulose residues. Both of the employed ionic liquids severely inactivated the *T. reesei* endoglucanases. Only traces of soluble oligosaccharides were present in hydrolysis mixtures containing 40% (v/v) or more of ionic liquids. The employed ILs were found to have a basic impact on the hydrolysis environment, but it could be concluded that the basicity of the ILs was not the only reason for the cellulase inactivation. The effect of an IL on the cellulose binding module in Cel5A was evaluated by comparing the hydrolysis yields of the intact Cel5A and the Cel5A core lacking the cellulose binding module. In this study the cellulose binding module was found to be the most ionic liquid sensitive part of the enzymes used. Comparative data from the partial hydrolysis of an ionic liquid regenerated cellulose is also reported.

Introduction

Cellulose is the world's most common renewable biopolymer, with an annual growth rate estimated at 90×10^9 tons per year.[1] The high biosphere production of cellulose makes it an interesting candidate as a raw material for any application, where it could be used to replace fossil resources. Being composed purely of anhydroglucose units, cellulose may be broken down into glucose by chemical (mineral acid) or enzymatic hydrolysis.[2] Glucose is a platform chemical, both for the production of fuel ethanol through fermentation, and for many other chemical products *via* either chemical or biochemical transformations.[3] Partial hydrolysis of cellulosic material, yielding water soluble cellooligomers, is another interesting research topic. Cellooligomers could find use as biologically active dietary additives[4] and as complex chemical building blocks and model compounds.[5]

Natural cellulose is a semicrystalline polymer consisting of crystalline and amorphous regions.[1] It is very recalcitrant towards enzymatic hydrolysis due to its high degree of crystallinity, low surface area for enzyme binding,[6] and its general insolubility.[2] In lignocellulosics, the presence of other biomass components further shelter the cellulose from hydrolysis.[6] A great variety of chemical, physical and biological pretreatment methods have been proposed for lignocellulosic biomass for use prior to enzymatic total or partial hydrolysis.[6,7] Typical to all of them is a high consumption of energy and often undesirable by-product formation,[8] facts that render them economically and/or environmentally unfeasible for total hydrolysis.

During the last decade a growing interest in the dissolution of cellulose in ionic liquids (ILs), followed by subsequent homogeneous modification, or regeneration by the addition of a counter-solvent, has arisen. ILs are defined as salts with melting points below 100 °C.[9] These compounds possess some very interesting solvent properties due to their dual ionic and organic nature. ILs have generally been considered as green solvents, mainly due to their thermal stability and negligible vapour pressure, which eliminates any VOC (volatile organic compound) emissions.[10] As early as 1934 Graenacher received a patent on dissolving and processing cellulose in benzylpyridinium chloride.[11] However, modifying cellulose in ionic liquids started receiving interest only after Swatloski *et al.* reported the dissolution of cellulose in 1-*n*-butyl-3-methylimidazolium chloride [BMIM]Cl in 2002.[12] It has also been shown that even wood, in the form of saw dust or wood chips, can dissolve in some ionic liquids.[13]

Cellulases are the main enzymes for the enzymatic hydrolysis of cellulose. These enzymes have been studied in cellulose hydrolysis both as complex mixtures and in the monocomponent

VTT Technical Research Center of Finland, P.O. Box 1000, FI-02044 VTT, Espoo, Finland. E-mail: ronny.wahlstrom@vtt.fi; Fax: +358 20 722 7071; Tel: +358 40 02 54 073

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form. Cellulases may be divided into three types of functionalities, which work together synergistically: endoglucanases, which randomly hydrolyse the cellulose chain in its amorphous regions producing cellooligomers, exoglucanases, which hydrolyse the cellulose chains from either the reducing or the non-reducing end producing mainly cellobiose and β-glucosidases, which cleave the resulting cellobiose units produced by the glucanases.[14]

One of the earliest studies on the enzymatic hydrolysis of cellulose in aqueous ILs was published by Turner *et al.* in 2003.[15] This study clearly demonstrated that ILs greatly inactivate cellulase enzymes. Dadi *et al.* showed that the enzymatic hydrolysis of cellulose might be greatly enhanced by IL pretreatments of the substrates.[16] In this process concept, the enzymatic hydrolysis takes place in a separate process step after the regeneration of cellulose from the IL solution. The increase in reaction rates was attributed to the lower degree of crystallinity in the regenerated cellulose (RC). Kamiya *et al.* introduced the term "*in situ* saccharification", where the regeneration of cellulose and the subsequent enzymatic hydrolysis are carried out in a one-pot procedure.[17] The presence of 1,3-dimethylimidazolium diethylphosphate was reported to inactivate the cellulases, with very little enzymatic activity in IL contents over 40% (v/v). Several studies elucidating cellulase inactivation in ILs have been published since, employing different hydrolysis conditions, enzyme cocktails, ILs and substrates.[8,18–27]

Various factors affecting cellulase inactivation in ionic liquids have been proposed. Basic anions such as Cl^-, Br^-, NO_3^- and $CF_3SO_3^-$ in ILs seem to be strongly inactivating as they interfere with the hydrogen bond network keeping the enzyme together.[28] Fluorinated anions, such as the BF_4^- and PF_6^-, have, on the other hand, been shown to be more compatible with enzymes in some cases. The "enzyme-friendliness" of ILs has been defined in terms of their chao- and chosmotropicity, as well as by using the Hofmeister series for predicting the effects of anions on enzymatic stability.[29] The high viscosity of IL solutions also play a significant role in slowing down enzymatic reactions[8,28] due to mass transfer constraints. It also remains unclear whether an enzyme is irreversibly denatured or simply inhibited by the presence of ILs.

In this paper we report how the presence of two hydrophilic, cellulose dissolving room-temperature ionic liquids, 1,3-dimethylimidazolium dimethylphosphate [DMIM]DMP and 1-ethyl-3-methylimidazolium acetate [EMIM]AcO affects the partial hydrolysis of microcrystalline cellulose (MCC) by two purified *Trichoderma reesei* endoglucanases, Cel7B (EGI) and Cel5A (EGII). These two ILs were chosen for this work because they are both hydrophilic, dissolve cellulose, and have been pointed out to be enzyme compatible to a certain degree.[24,30] [DMIM]DMP is one of the most studied ILs in enzymatic hydrolysis. It has been found to be a strong candidate for use in biorefinery applications, as it combines good biomass dissolving capability with a certain degree of "enzyme-friendliness".[24] A major advantage is the fact that [DMIM]DMP may be produced on an industrial scale in a one-pot procedure without considerable by-product formation.[31] [EMIM]AcO is known as a powerful cellulose solvent, but the enzyme compatibility of this IL has not been clarified conclusively. The enzymatic hydrolysis of MCC was carried out in the presence of 0–90% (v/v) of IL. After hydrolysis, the solid cellulose residue was analysed by gel-permeation chromatography (GPC) for changes in the molecular weight, and the soluble cellooligosaccharides were analysed using capillary electrophoresis (CE) with pre-column derivatization. Based on the results, the two ILs are compared both in terms of their interactions with the substrate and their effect on enzymatic activity.

Experimental

Materials

[DMIM]DMP was prepared as described in the literature.[31] [EMIM]AcO (purity > 98%) was purchased from Ionic Liquid Technologies (Heilbronn) and used without further purification. The halide content of the [EMIM]AcO determined by ion chromatography was: chloride < 100 ppm and bromide < 50 ppm. The dry weight of the cellulose substrate (MCC, Serva, research grade, particle size 0.020 mm) was determined as the average mass loss for three parallel samples by keeping the cellulose at 105 °C for 14 h. The cellulase preparations of *Trichoderma reesei* Cel7B, Cel5A and the core domain of Cel5A were produced, isolated and purified at VTT according to Suurnäkki *et al.*[32] The Cel7B activity was 12 700 nkat mL^{-1} (specific activity 3050 nkat mg^{-1} protein) as determined by the HEC (hydroxyethylcellulose) assay[33] where the activity measurement was done in a 1% (w/w) carboxymethylcellulose (CMC) substrate solution in 50 mM sodium citrate buffer (pH 5.0) at 50 °C. The activity measurement time was 10 min and the measurement was terminated by adding a DNS reagent solution to the samples and boiling for 5 min. The endoglucanase activity was determined by measuring the absorption of the boiled samples at 540 nm and comparing the absorption to that of glucose standards treated in the same way as the enzyme samples. The Cel5A activity was 17 900 nkat mL^{-1} (specific activity 2030 nkat mg^{-1} protein), whereas the Cel5A core domain activity was 13 800 nkat mL^{-1} (specific activity 3730 nkat mg^{-1} protein). The unit katal (kat) is defined by the International Union for Pure and Applied Chemistry (IUPAC) as the number of catalysed reactions per time with the unit of mol s^{-1}.[34]

Hydrolysis

Hydrolysis mixtures were prepared with 20, 40, 60, 80 and 90% (v/v) IL dosage in sodium citrate buffer (50 mM, pH = 5.0). IL-free hydrolysis mixtures were prepared in a sodium citrate buffer as well as in a sodium phosphate buffer (50 mM, pH = 7.0). 30 mg (dry weight) of microcrystalline cellulose was measured into a test tube, the defined amount of buffer was added and the mixture was stirred to homogeneity. The defined volume of IL was mixed into the mixture, before adding the enzyme preparation corresponding to a total activity of 2000 nkat g^{-1} cellulose. The total hydrolysis sample volume was 3 mL. The hydrolysis was carried out at 45 °C in closed test tubes in a water bath with continuous magnetic stirring. The hydrolysis time was 2, 24, 48 or 72 h. The hydrolysis was stopped by boiling the sample for 600 s to denature the enzyme. After cooling to room temperature, the reaction tube was centrifuged at 3000 rpm for 10 min and the clear supernatant was separated from the solid cellulose residue. In the 90% (v/v) IL hydrolysis samples the cellulose was partly dissolved and thus needed to be regenerated. This was achieved by adding 3 mL of distilled water after enzyme denaturation by boiling, followed by vigorous mixing before centrifugation. When using regenerated cellulose (RC) as the

substrate, the above described procedure was followed with the exception that the substrate was stirred and swollen in buffer overnight before the addition of enzyme. All experiments were carried out in triplicates. Reference samples were treated in the corresponding conditions without the addition of enzyme. Protein containing reference samples were prepared with the inactivated cellulase or the corresponding amount of bovine serum albumin (BSA, model protein). pH-values were followed during hydrolysis with a Knick pH meter 766 Calimatic equipped with a Mettler-Toledo Inlab Semi-Micro electrode (pH range 0–12).

Determination of residual enzymatic activity

The enzyme preparations were first incubated in 20, 40, 60, 80, or 90% (v/v) solutions of [DMIM]DMP or [EMIM]AcO in the sodium citrate buffer (50 mM, pH = 5.0) for a specific time at 45 °C. Reference samples were incubated accordingly in pure sodium citrate buffer. 100 μL of the pre-incubated enzyme sample was transferred to the test tubes where the enzyme residual activity measurement was done. The enzyme residual activity was measured as HEC activity as described above. The final IL content was fixed at 9% (v/v) in all cases during the enzyme residual activity measurement and the CMC concentration was kept constant to ensure similar incubation conditions for all samples. The measurement was done in triplicates.

Regeneration of microcrystalline cellulose

MCC (4.09 g) was dissolved in [EMIM]AcO (45 mL), by stirring and heating to 80 °C overnight, yielding a 9% (w/v) solution. The cellulose was precipitated by adding de-ionized water (100 mL), stirring and vacuum filtering the cellulose. The cellulose was washed three times by this procedure and finally dried under reduced pressure (13 mbar) overnight. The final product was a brownish brittle solid. The dry weight of the regenerated cellulose was 90.6 w%, determined as the average mass loss for three parallel samples by keeping the cellulose at 105 °C for 14 h.

Analyses

In most of the reported hydrolysis studies, the yield of the reducing sugars has been determined by a dinitrosalicylic acid (DNS) photometric assay.[35] It has previously been pointed out that ILs may interfere with both photometric assays and HPLC techniques.[36] We found the DNS reagent to produce colour together with high contents of ILs (both [DMIM]DMP and [EMIM]AcO). For low concentrations of ILs, the DNS method has been shown to be reliable.[21] In our work, CE was found to be a practical technique to separate the derivatized cellooligomers as sugar–borate complexes under alkaline conditions in the presence of ILs. Although the ILs used proved to be very challenging matrices, the CE-method could be optimized allowing the quantification of the cellooligosaccharides in moderate IL concentrations (up to 40% (v/v)).

Determination of cellulose dissolution in aqueous ionic liquid solutions

Cellulose dissolution in the studied ILs was evaluated by measuring the light transmission through dispersions/solutions of MCC in aqueous IL solutions. Samples were prepared simulating the employed hydrolysis conditions. A 1% (w/v) MCC solution/dispersion was prepared in an aqueous IL solution and stirred for 1 day at 45 °C in sealed test tubes. The samples were shaken immediately before measuring and analysed at 45 °C with a Turbiscan Lab (Formulaction, France) device. Light transmission was monitored as a measure of the cellulose dissolution. Cellulose dissolution was also followed by visual evaluation and light microscopy. Light microscopy was performed with an Olympus BX61 microscope and digital image recording was performed with the Soft Imaging Systems analySIS® 3.2 software.

Capillary electrophoresis

The saccharides in the enzymatic hydrolysates were derivatized with 4-aminobenzonitrile (ABN, samples in aqueous solution or containing [DMIM]DMP) or 4-aminobenzoic acid ethyl ester (ABEE, samples containing [EMIM]AcO), according to a procedure previously described by Dahlman *et al.*[37] Capillary electrophoresis was carried out with a P/ACE MDQ capillary electrophoresis instrument (Beckman Coulter, Fullerton, CA, USA) equipped with a photodiode array (PDA) UV/vis detector. Running conditions were adapted from Sartori *et al.*,[38] but keeping the cartridge temperature at 30 °C and without any addition of organic modifier to the running electrolyte. Standard curves were acquired for glucose, cellobiose and cellotriose against galactose as the internal standard.

Gel-permeation chromatography

The solid cellulose residues were washed with distilled water and dissolved and treated as described in the literature.[39] The GPC analyses were carried out on a Waters HPLC system using 0.8% (w/v) LiCl/DMAc as eluent with a flow rate of 0.36 mL min^{-1}. The molecular size separation was achieved at 80 °C on two Agilent PL-gel 20 μm Minimix A columns in series preceded by an Agilent PL-gel 20 μm Minimix A guard column. A refractive index (RI) detector was used for monitoring the cellulose elution. The chromatograms were numerically evaluated using Waters Inc. Empower 2 software. The cellulose molecular weight distributions were calculated by comparison with a pullulan standard series with a linear range of 5900 g mol^{-1}–1 600 000 g mol^{-1} (R^2 = 0.996).

IR spectroscopy of cellulose residues

The cellulose samples were initially air dried and finally dried in a desiccator to remove any remaining free moisture. FTIR spectra of the MCC, RC and solid cellulose hydrolysis residues were measured using a Bio-Rad 6000 spectrometer equipped with a MTEC 300 photoacoustic (PA) detector. The PA cell was kept under an atmosphere of helium during the measurements. The cross-section of the beam inside the cell was $\sim$1 mm^2 and the optical velocity of the interferometer was 0.16 cm s^{-1}. The spectra were collected in the wavenumber region 400–4000 cm^{-1}, with a spectral resolution of 8 cm^{-1}. From the IR spectra the lateral order index (LOI) was calculated as the peak ratio between the absorption at 1437 cm^{-1} and 899 cm^{-1} ($\alpha_{1437}/\alpha_{899}$) as described in the literature.[40] The total crystallinity index (TCI) was likewise calculated as the peak ratio between the absorption

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at 1378 cm^{-1} and 2900 cm^{-1} ($\alpha_{1378}/\alpha_{2900}$) as described in the literature.[41]

Results and discussion

Ionic liquid interactions with microcrystalline cellulose

The interaction of ILs with microcrystalline cellulose (MCC) mixed in IL contents of 20, 40, 60, 80, and 90% (v/v) in buffer was studied. Low contents of ILs did not cause any detectable cellulose dissolution. A high IL content of 90% (v/v) in the mixture lead to visible interactions with the cellulose substrate and the hydrolysis mixture became less opaque, *i. e.* dissolution occurred. The effect was more pronounced with [EMIM]AcO whereas the changes were barely noticeable for [DMIM]DMP. In the case of [EMIM]AcO, similar interactions were visibly observed already at an IL content of 80% (v/v), suggesting that [EMIM]AcO is a more powerful cellulose solvent than [DMIM]DMP. This is in line with the previously reported comparisons between cellulose dissolution in these two ILs at 50 °C.[42] By light microscopy the aqueous cellulose mixtures of [EMIM]AcO were found to contain some cellulose crystals, indicating the dissolution to be partial (Fig. 1). The light transmission measurements showed an increase in the transmission for cellulose suspensions for both ILs already at 80% (v/v) concentration of IL (Fig. 2). The light transmission was stronger for the solutions of [EMIM]AcO than the corresponding solutions of [DMIM]DMP confirming the superior solvation capacity of [EMIM]AcO. The transmission values clearly indicate a change at a concentration of 85–90% (v/v) of [EMIM]AcO, whereas [DMIM]DMP seems to tolerate hardly any water in order to dissolve cellulose efficiently. At a 90% (v/v) [DMIM]DMP solution most of the cellulose was still not dissolved.

IR spectroscopy was used to determine the crystallinity of MCC after interactions with the different amounts of ILs in MCC–IL–buffer samples. The lateral order index (LOI), also known as the crystallinity index CrI,[43] was calculated as the peak ratio $\alpha_{1437}/\alpha_{899}$.[40] The LOI of commercial MCC was measured to be 1.1. As expected, the LOI of the regenerated cellulose (RC) was significantly lower with a value of 0.41–0.45. Surprisingly, the crystallinity of MCC that had been treated in 90% (v/v) [EMIM]AcO was even lower with values in the range 0.1–0.25. This difference in LOI values might be due to differences in the dissolution and regeneration conditions in preparing RC in pure [EMIM]AcO *vs.* the treatment of MCC during the hydrolyses. The LOI values of samples that had been treated in 90% (v/v) [DMIM]DMP were in the range 0.7–0.9, confirming that [DMIM]DMP is not capable of dissolving the MCC to a high extent nor in any other way to significantly alter the MCC structure under these conditions. Also the total crystallinity index (TCI) was calculated as the peak ratio $\alpha_{1378}/\alpha_{2900}$,[41] but the TCI values did not correlate with the LOI values or the expected changes in crystallinity. Recently, similar conclusions about the LOI and TCI values for regenerated cellulosic materials were published by Zhao *et al.*[26]

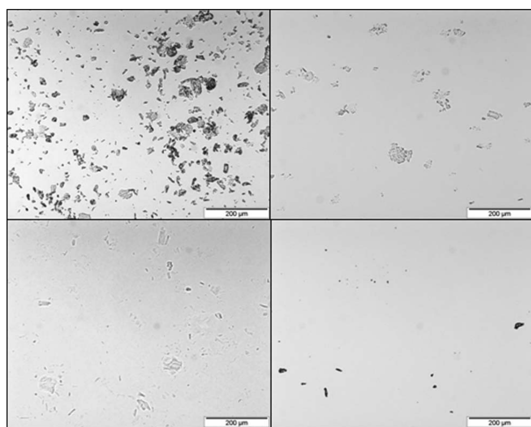

Fig. 1 Light microscopy pictures of microcrystalline cellulose suspended/dissolved in pure buffer (upper left), or 60 (upper right), 85 (lower left) or 90% (v/v) (lower right) [EMIM]AcO solutions in buffer. The magnification is 100.

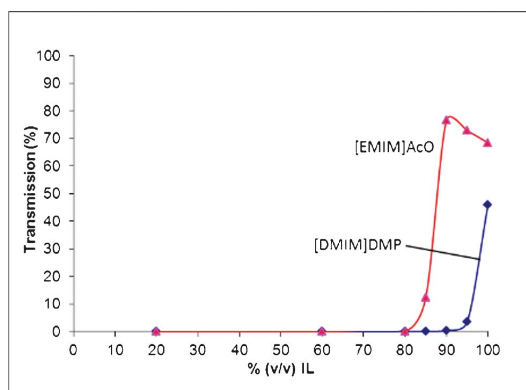

Fig. 2 Light transmission (T) for aqueous cellulose suspensions/solutions in [DMIM]DMP and [EMIM]AcO at 45 °C.

Effects of ILs on endoglucanase activity

The hydrolytic activity of cellulases has been reported to be negatively affected by ILs.[15,17] IL derived factors such as salt concentration,[15] ionic strength[8] and chao/chosmotropicity[29] have been proposed to be responsible for enzyme inactivation. We studied the effect of the pH, IL type and IL amount on the activity and action of two *T. reesei* main endoglucanases.

Effect of pH changes. The addition of IL to the buffer solution (pH = 5.0) caused a distinct basic shift (Fig. 3). [EMIM]AcO was found to be more basic than [DMIM]DMP. Both *T. reesei* Cel7B and *T. reesei* Cel5A have optima at pH 5.[44] Kragl *et al.* have previously reported the problems experienced with drifting pH values when using ILs in enzymatic reactions in general.[9] Engel *et al.* also measured basic shifts in the pH values of aqueous IL solutions and compensated the basic shift of ILs by adjusting the pH of the hydrolysis mixtures by adding H_2SO_4.[8]

To find out whether the pH drift is one of the major reasons for the previously reported enzyme inactivation,[8,15,17] a

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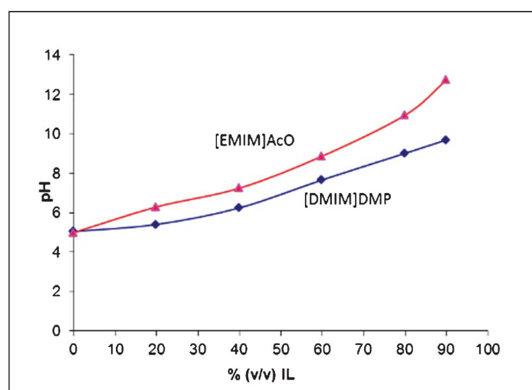

Fig. 3 Apparent pH values for ionic liquid in buffer solutions (pH 5.0).

hydrolysis experiment at pH 7 in buffer was carried out and the saccharide yield and composition was analysed by CE. The hydrolysis yield of soluble oligosaccharides at pH 7 was 50% and 20% for Cel5A and Cel7B, respectively, as compared to the respective yields in buffer at pH 5 (Table 1 and 2). The oligosaccharide distribution was to some extent altered by the change in pH, as measured after 72 h of hydrolysis. The oligosaccharides formed were glucose, cellobiose and cellotriose; no larger oligosaccharides were found with CE. At pH 7 the Cel7B produced traces of cellotetraose and cellopentaose (results not shown), as well as cellotriose, which is not a normal product pattern of Cel7B under optimum conditions[45] (Table 1). This would indicate a slowdown of the hydrolysis, yielding intermediary products, due to the deviation from the optimum pH. The fairly well retained hydrolytic activity of Cel5A at pH 7 (Table 2) is expected as this enzyme is known to be active (on β-glucan) over a wide pH window from at least pH 3 to pH 9.[45] Cel7B was clearly more sensitive to the deviations from its optimum pH.

According to the pH curves displayed in Fig. 3, a pH-value of 7.0 corresponds to roughly 60% (v/v) [DMIM]DMP or 40% (v/v) [EMIM]AcO in sodium citrate buffer. For neither Cel7B nor Cel5A no soluble oligosaccharides could be detected after 72 h of hydrolysis in the hydrolysis mixtures containing these amounts of [DMIM]DMP (results not shown) or [EMIM]AcO (Table 1 and 2). This clearly indicates, in the case of Cel5A, that the basic pH drift caused by the ILs is not the only reason for the enzyme's inactivation. Enzymatic activity decreases much faster with increasing IL content than the increase of pH gives reason to. This is also in line with the results of Engels *et al.*,[8] where the pH was adjusted in the IL solutions, yet enzyme inactivation was observed in the presence of ILs. In the case of Cel7B, a major decrease is recorded both at pH 7 as well as when ILs are present allowing no conclusion to be made as to whether the reason for the drop is the changed pH or other effects caused by the presence of ILs.

Residual endoglucanase activity in ILs. In order to establish whether the studied enzymes, *T. reesei* Cel7B and Cel5A, retained any of their hydrolytic activity in high concentrations of ILs, the enzymatic residual activities were measured in 80 and 90% (v/v) of [DMIM]DMP with an incubation time of 15 min. Both Cel7B and Cel5A retained roughly 50% of their hydrolytic activity on the carboxymethylcellulose substrate as compared to the activity measured under similar conditions after incubation in pure buffer at pH 5. Wang *et al.* have previously shown that commercial cellulase preparations retain their activity fairly well in 5–30% (v/v) of [EMIM]AcO in citrate buffer at both 4 and 50 °C for incubation times of up to 24 h.[21]

Enzyme inactivation for Cel5A was studied with prolonged incubation times of 2 h and 72 h in 90% (v/v) [DMIM]DMP in hydrolysis conditions. Interestingly, the measured residual endoglucanase activity was at approximately the same level as that measured after 15 min incubation. This would indicate a very fast decrease in enzymatic activity when the enzyme is mixed with the IL, with an equilibrium settling after which the enzyme activity stays rather stable. It was further noticed that 10 min boiling inactivated the enzyme in 90% (v/v) [DMIM]DMP solution hardly at all. These results were verified to be due to the enzymatic activity by varying the amount of enzyme in the measurements, as well as the incubation time in the residual activity measurement.

Effect of ILs on the cellulose binding module. Both Cel7B and Cel5A are modular enzymes, *i. e.* they have cellulose binding modules (CBMs) linked to the catalytic core domain by a peptide linker.[32,45] The CBM is used for substrate recognition and binding. The CBMs are especially important for the exoglucanase (cellobiohydrolase) type of cellulases, which act on crystalline cellulose. Previously it has been proposed that the CBMs in endoglucanases would be particularly sensitive to ILs.[46]

Table 1 The formation of soluble oligosaccharides and yield of the enzymatic hydrolysis of MCC or RC by Cel7B after 2 h and 72 h treatment at pH 5, pH 7 and in 20 and 40% (v/v) solutions of [DMIM]DMP (IL1) and [EMIM]AcO (IL2) at 45 °C as analysed by CE (Glc = glucose, CB = cellobiose, CTr = cellotriose, LOD = limit of detection, ND = not determined, MCC = microcrystalline cellulose, RC = regenerated cellulose)

Cel7B	Formed soluble oligosaccharides/yield							
	2 h				72 h			
Sample	Glc (mg L^{-1})	CB (mg L^{-1})	CTr (mg L^{-1})	Yield (%)	Glc (mg L^{-1})	CB (mg L^{-1})	CTr (mg L^{-1})	Yield (%)
MCC, buffer (pH 5)	29.7	112.3	< LOD	1.3	160.5	429.5	11.7	5.6
MCC, buffer (pH 7)	7.9	41.7	13.1	0.6	14.9	79.0	24.2	1.1
MCC, 20% IL1	2.2	20.1	< LOD	0.2	19.0	90.8	3.6	1.0
MCC, 40% IL1	< LOD	< LOD	< LOD	0.0	< LOD	6.5	< LOD	< 0.1
MCC, 20% IL2	< LOD	4.6	5.5	< 0.1	Traces	4.4	6.5	< 0.1
MCC, 40% IL2	< LOD	< LOD	< LOD	0.0	< LOD	< LOD	< LOD	0.0
RC, buffer (pH 5)	ND	ND	ND	ND	425.8	799.8	< LOD	11.4

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Table 2 The formation of soluble oligosaccharides and yield of the enzymatic hydrolysis of MCC or RC by Cel5A or Cel5A Core after 2 h and 72 h treatment at pH 5, pH 7 and in 20, 40 and 90% (v/v) solutions of [DMIM]DMP (IL1) and [EMIM]AcO (IL2) at 45 °C as analysed by CE (Glc = glucose, CB = cellobiose, CTr = cellotriose, LOD = limit of detection, ND = not determined, MCC = microcrystalline cellulose, RC = regenerated cellulose)

Cel5A	Formed soluble oligosaccharides/yield							
	2 h				72 h			
Sample	Glc (mg L^{-1})	CB (mg L^{-1})	CTr (mg L^{-1})	Yield (%)	Glc (mg L^{-1})	CB (mg L^{-1})	CTr (mg L^{-1})	Yield (%)
Buffer (pH 5)	25.8	86.6	87.1	1.9	256.2	458.9	219.4	8.8
Buffer (pH 7)	10.3	47.7	52.9	1.1	41.7	192.4	199.9	4.2
20% IL1	2.0	17.2	< LOD	0.3	25.4	94.1	89.4	2.0
40% IL1	Traces	Traces	< LOD	0.0	< LOD	3.3	4.8	0.1
90% IL1	< LOD	< LOD	< LOD	0.0	< LOD	< LOD	< LOD	0.0
20% IL2	< LOD	5.9	9.4	0.1	Traces	19.5	21.9	0.4
40% IL2	< LOD	< LOD	< LOD	0.0	< LOD	< LOD	< LOD	0.0
Cel5A Core, buffer pH 5	ND	ND	ND	ND	13.5	45.6	40.5	0.9
Cel5A Core, 20% IL1	ND	ND	ND	ND	8.3	48.1	47.9	1.0
Cel5A Core, 90% IL1	ND	ND	ND	ND	< LOD	< LOD	< LOD	0.0
RC, buffer (pH 5)	ND	ND	ND	ND	572.2	865.6	392.3	17.1

To explore the function of the CBM in the presence of ILs, hydrolysis of MCC was carried out in a buffer at pH 5 (optimum conditions) and in 20 and 90% (v/v) of [DMIM]DMP with both intact Cel5A containing CBM and Cel5A without CBM (Cel5A Core) (Table 2). The cellulose hydrolysis yield at optimum conditions with the Cel5A Core was considerably lower than that with the intact Cel5A, resulting in about 10% of the yield of the intact enzyme. Interestingly, the hydrolysis yield of the Cel5A Core was the same in 20% (v/v) of [DMIM]DMP as in the optimum conditions. The product distribution was, however, somewhat different with lower amounts of glucose and higher amounts of cellobiose and cellotriose after the IL containing hydrolysis as compared to that after the hydrolysis in pure buffer. The hydrolysis activity of the intact enzyme decreased, however, drastically in the presence of 20% (v/v) of [DMIM]DMP as compared with its activity in the buffer. According to these results, the action of CBM of Cel5A seems to be highly affected by the presence of [DMIM]DMP. The effect of this IL on the structure of the CBM and its substrate recognition ability, both potential factors affecting the CBM action on the cellulose, needs to be elucidated in further studies. In the high [DMIM]DMP concentration of 90% (v/v) no oligosaccharides could be detected in the hydrolysate for neither Cel5A nor Cel5A Core.

Enzymatic hydrolysis of MCC in the presence of ILs

Formation of soluble oligosaccharides. Both the *T. reesei* endoglucanases, Cel7B and Cel5A, produced glucose and cellobiose in the reference system comprising of 1% (w/v) MCC in sodium citrate buffer (pH = 5.0) (Table 1 and 2). For Cel5A cellotriose was also a major product. The product distribution is well in line with previously published data.[45] As could be expected from previously published studies,[16] the use of RC as substrate greatly increased the yield of soluble oligosaccharides (Table 1 and 2), as compared to MCC, in aqueous buffer. The yields were roughly doubled, but no significant changes were observed in the distribution of the oligomers, these being glucose and cellobiose for Cel7B and glucose, cellobiose and cellotriose for Cel5A.

Both [DMIM]DMP and [EMIM]AcO are very inactivating for the studied endoglucanases, [EMIM]AcO even more so than [DMIM]DMP. More cellobiose and cellotriose and less glucose was produced in the presence of both [DMIM]DMP and [EMIM]AcO as compared to the product distribution in the citrate buffer reference at pH 5 (Table 1 and 2). This suggests an overall slowing down of the hydrolysis. The total yield of solubilized saccharides was significantly decreased as the IL concentration increased. With only 20% (v/v) of [DMIM]DMP present in the hydrolysis mixture, the overall yield decreased to approximately 20% of the reference in 72 h of hydrolysis. 40% (v/v) of this IL only allowed very low hydrolysis rates for both the enzymes. In 20% (v/v) [EMIM]AcO extremely low concentrations of oligosaccharides were detected as compared to the amounts found in 20% (v/v) [DMIM]DMP. In 40% (v/v) of [EMIM]AcO neither of the studied enzymes were able to catalyse the formation of any oligosaccharides. Cel5A, being more active for MCC in the reference buffer system, also seemed to be slightly more tolerant towards the ILs, and especially against [EMIM]AcO, than Cel7B. In hydrolysis samples containing an IL in the range 60–90% (v/v) of IL no soluble oligosaccharides could be detected after enzymatic treatment.

The kinetics of oligosaccharide formation in MCC hydrolysis was different for the two enzymes when [EMIM]AcO was present. Cel7B produced a low amount of cellobiose and cellotriose during the first two hours and was then totally inactivated with no further saccharide formation (Table 1). Cel5A was still able to increase the amount of solubilized saccharides in these circumstances after a two hour hydrolysis (Table 2). In [DMIM]DMP this same difference between the enzymes was not observed, but both enzymes were able to produce saccharides during the course of the 72 h MCC hydrolysis also after the 2 h time point. It could be concluded that the inactivating effect of the ILs was different for the two *T. reesei* endoglucanases studied and that the inactivation is dependent on both the enzyme and the IL it is exposed to.

Molecular weight of the solid residue after enzymatic hydrolysis

The insoluble cellulose residues, separated from the hydrolysate by centrifugation after enzymatic hydrolysis, were subjected to GPC analysis. When the hydrolysis was carried out in a buffer solution at pH 5 or 7 with either Cel7B, Cel5A or Cel5A Core,

no changes in the molecular weight distributions were observed even after 72 h of treatment (Table 3). Endoglucanases are known to hydrolyse primarily the amorphous regions of cellulose.[14] A decrease in molecular weight was therefore expected in the aqueous conditions as yields of up to 8% of soluble oligosaccharides were observed (Tables 1 and 2). This conflict between the fairly high yield of saccharides and no decrease in the molecular weight may best be explained with a mechanism where the cellulases are able to hydrolyse only the outermost layer of the cellulose crystals, whereas the internal parts of the crystals remain intact. The same mechanism has previously been suggested based on observations that the crystallinity of MCC was not increased in partial hydrolysis.[47]

A decrease of 20–30% in the weight average molecular weight (M_w), as well as decreases in the number average molecular weight (M_n) for samples treated enzymatically in 90% (v/v) [DMIM]DMP, could be observed (Table 3). The changes in the molecular weight distribution can clearly be seen when comparing the distributions of the samples (Fig. 4). The decrease in M_w was observed for both Cel7B and Cel5A. Cel5A Core was also able to reduce the M_w, even if not to the same extent as the two intact endoglucanases. This effect was noticed to increase with increasing hydrolysis time. The M_w was not affected either by the presence of BSA or by addition of endoglucanase, which had been inactivated by boiling. It is possible that the 90% (v/v) [DMIM]DMP solution has sufficient dissolving power to alter the MCC structure during hydrolysis to a more accessible substrate for the enzyme, the conditions in this solution at the same time being such that the enzyme still retains some of its cellulose chain scission activity. The decrease in the molecular weight of cellulose was not observed in any other conditions than in the 90% (v/v) [DMIM]DMP solutions. It was shown by the residual activity measurements in 90% (v/v) [DMIM]DMP, that

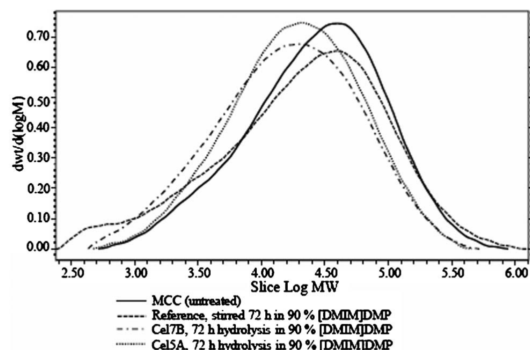

Fig. 4 Molecular weight distributions for MCC samples after 72 h enzymatic treatments in 90% (v/v) [DMIM]DMP.

both Cel7B and Cel5A retain about 50% of their activity at least for 15 min in this media.

Interestingly, the decrease in M_w for MCC noticed in 90% (v/v) [DMIM]DMP was not accompanied by any detectable formation of soluble cellooligomers. This may imply that the enzymes' activity is altered by the conditions applied or that the enzymes are able to carry out random chain scission on the substrate which is in a new, easier accessible form due to the interactions with the IL. Hydrolysis experiments were also carried out with a doubled enzyme dosage (Table 3). Increasing the enzyme dosage did not lead to an increased rate of cellulose chain scission. According to this result, the limiting factor is not the IL induced enzyme inactivation, but must rather be substrate dependent. The possibility that the cellulose could be enzymatically hydrolysed during its regeneration after the hydrolysis cannot be ruled out, especially as we have shown that 10 min

Table 3 Molecular weight and polydispersity for microcrystalline cellulose (MCC) or regenerated cellulose (RC) hydrolysis residues (M_n = number average molecular weight, M_w = weight average molecular weight, StDev = standard deviation)

Sample	Time (h)	M_n (g mol^{-1})	M_w (g mol^{-1})	StDev(M_w)	Polydispersity
MCC					
Reference (MCC, untreated)		10 000	50 000	5500	5.16
Cel7B, buffer pH 5	72	12 000	49 000	1600	4.02
Cel5A, buffer pH5	72	11 000	48 000	5000	4.33
Cel5A Core, buffer pH5	72	9000	49 000	3000	5.29
Cel7B, buffer pH 7	72	11 000	50 000	400	4.36
Cel5A, buffer pH 7	72	10 000	47 000	4300	4.89
Reference, buffer	72	10 000	47 000	3800	4.62
Cel7B, 90% [DMIM]DMP	2	10 000	45 000	4900	4.50
Cel7B, 90% [DMIM]DMP	24	7000	34 000	400	4.84
Cel7B, 90% [DMIM]DMP	72	8000	36 000	3200	4.66
Cel5A, 90% [DMIM]DMP	2	9000	41 000	1000	4.57
Cel5A, 90% [DMIM]DMP	24	7000	36 000	6000	4.95
Cel5A, 90% [DMIM]DMP	72	9000	35 000	1600	4.10
Reference, 90% [DMIM]DMP	2	10 000	48 000	4400	4.68
Reference, 90% [DMIM]DMP	24	8000	45 000	3600	5.68
Reference, 90% [DMIM]DMP	72	11 000	49 000	3500	4.56
Cel5A Core 90% [DMIM]DMP	2	9000	43 000	600	4.57
Cel5A Core 90% [DMIM]DMP	72	9000	40 000	1800	4.33
Cel7B double enzyme dosage, 90% [DMIM]DMP	72	8000	40 000	3700	4.75
Cel5A double enzyme dosage, 90% [DMIM]DMP	72	8000	34 000	2100	4.50
RC					
RC (untreated)		9000	47 000	4200	5.15
RC, Cel7B, buffer pH 5	72	7000	32 000	800	4.78
RC, Cel5A, buffer pH 5	72	5000	12 000	500	2.45
RC reference, buffer	72	9000	44 000	600	4.91

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boiling does not completely inactivate the enzymes. Further studies are needed to fully clarify the mode of action of *T. reesei* endoglucanases in the MCC in the presence of high concentrations of [DMIM]DMP.

In [EMIM]AcO, no decrease in M_w was noticed for any concentration of this IL in the hydrolysis mixture (results not shown). Although [EMIM]AcO is a much more powerful cellulose solvent than [DMIM]DMP, it is probable that this IL totally inactivates the studied endoglucanases, in contrast to [DMIM]DMP.

The RC was very efficiently hydrolysed by both Cel7B and Cel5A under optimum conditions at pH 5, both in terms of the produced cellooligomers (Table 1 and 2) and in terms of the decreased M_w (Table 3). The difference between the two endoglucanases was large: Cel7B was able to cause a decrease in M_w of 27% against the reference, whereas Cel5A caused a decrease in M_w of 73%, which is also clearly seen by comparing the molecular weight distributions (Fig. 5). This change in M_w was accompanied by a roughly doubled production of soluble oligosaccharides in the hydrolysate (Table 1 and 2).

Conclusions

The literature has little data regarding the effect of ILs on purified monocomponent cellulases. This paper provides results on how the activity of two endoglucanases from the *Trichoderma reesei* on microcrystalline cellulose is affected by the presence of ILs. Furthermore, the effect of the presence of IL in hydrolysis on the resulting cellooligomer product distribution and the molecular weight of the insoluble cellulose residue is elucidated. Both the employed ILs, [DMIM]DMP and [EMIM]AcO, were found to be severely inactivating for the enzymes used. [EMIM]AcO, being a more powerful cellulose solvent, was also more enzyme inactivating than [DMIM]DMP. The studied endoglucanases, *T. reesei* Cel7B and Cel5A, displayed some differences in IL tolerance. The product distribution of cellulose hydrolysis was similar under both optimum conditions and in the presence of ILs, though shifting the hydrolysis conditions away from the optimum usually caused the amount of cellobiose and cellotriose to grow at the expense of the glucose concentration. Based on the comparison of the effect of [DMIM]DMP on the cellulose hydrolysis yield by the native Cel5A and Cel5A Core it seems that the action of the cellulose binding module, CBM, present in the native *T. reesei* Cel5A is highly affected by the presence of this IL.

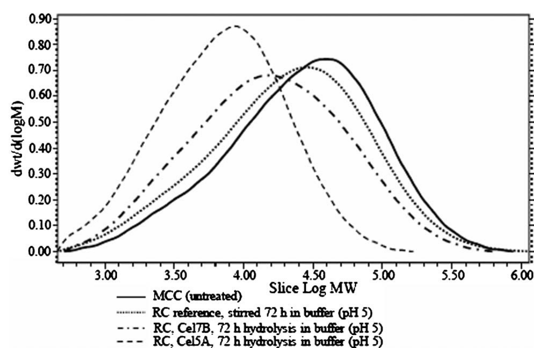

Fig. 5 Molecular weight distributions for cellulose regenerated in ionic liquid (RC) samples after 72 h enzymatic treatment in buffer at pH 5.

Soluble oligosaccharides were not produced in the enzymatic hydrolysis at IL concentrations of more than 40% (v/v). Surprisingly, both the studied endoglucanases appeared, however, to reduce the molecular weight of the solid cellulose residue in hydrolysis mixtures containing 90% (v/v) [DMIM]DMP. Similar phenomena were not observed in systems containing [EMIM]AcO nor in any other attempted conditions including the optimum conditions in buffer at pH 5. Further studies are needed to fully clarify the mode of action of *T. reesei* endoglucanases in the MCC in the presence of high concentrations of [DMIM]DMP and during the regeneration of the cellulose sample. IL pretreated, regenerated cellulose was shown to yield by far the best hydrolysis results both in terms of solubilized oligosaccharides and the decreased molecular weight of the cellulose residue.

Both [DMIM]DMP and [EMIM]AcO were found to cause a strong basic drift to the hydrolysis mixtures' pH values, but it could be established that the pH drift was not the main cause for the low observed enzymatic activities in the presence of these ILs. The IL containing matrices were also found to be challenging for the analytics. In particular, the DNS assay and HPLC techniques were disturbed when attempting to analyse samples with high IL concentrations. The cellooligomers could be quantified in the presence of ILs employing CE techniques with pre-column derivatization.

There is a great need to further develop IL compatible analytical methods, if accurate research results are to be obtained in this research field. There is also a clear need for new, more enzyme-friendly ILs, which combine properties such as low price, recyclability, non-toxicity and of course the ability to dissolve biomass. Stabilizing the cellulase enzymes in these systems as well as finding cost effective methods to separate the hydrolysis products from the IL containing hydrolysates are future challenges.

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PUBLICATION II

Analysis of mono- and oligosaccharides in ionic liquid containing matrices

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Analysis of mono- and oligosaccharides in ionic liquid containing matrices

Ronny Wahlström *, Stella Rovio, Anna Suurnäkki

VTT Technical Research Centre of Finland, PO Box 1000, FI-02044 VTT, Espoo, Finland

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ABSTRACT

Ionic liquids (ILs), that is, salts with melting points <100 °C, have recently attracted a lot of attention in biomass processing due to their ability to dissolve lignocellulosics. In this work, we studied how two imidazolium-based, hydrophilic, cellulose dissolving ionic liquids 1,3-dimethylimidazolium dimethylphosphate [DMIM]DMP and 1-ethyl-3-methylimidazolium acetate [EMIM]AcO affect the usually employed analytical methods for mono- and oligosaccharides, typical products from hydrolytic treatments of biomass. HPLC methods were severely hampered by the presence of ILs with loss of separation power and severe baseline problems, making their use for saccharide quantification extremely challenging. Problems in DNS photometric assay and chromatography were also encountered at high ionic liquid concentrations and many capillary electrophoresis (CE) methods did not allow an efficient analysis of saccharides in these matrices. In this paper we describe an optimized CE method with pre-column derivatization for the qualitative and quantitative analysis of mono- and oligosaccharides in sample matrices containing moderate (20–40% (v/v)) concentrations of ILs. The IL content and type in the sample matrix was found to affect both peak shape and quantification parameters. Generally, the presence of high IL concentrations (⩾20% (v/v)) had a dampening effect on the detection of the analytes. IL in lower concentrations of <20% (v/v) was, however, found to improve peak shape and/or separation in some cases. The optimized CE method has good sensitivity in moderate concentrations of the ionic liquids used, with limits of detection of 5 mg/L for cellooligomers up to the size of cellotetraose and 5–20 mg/L for cellopentaose and cellohexaose, depending on the matrix. The method was used for analysing the action of a commercial β-glucosidase in ILs and for analysing saccharides in the IL containing hydrolysates from the hydrolysis of microcrystalline cellulose with *Trichoderma reesei* endoglucanase Cel5A. According to the results, [DMIM]DMP and [EMIM]AcO] showed clear differences in enzyme inactivation.

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1. Introduction

Ionic liquids (ILs) have recently received a great deal of interest as a new media for biomass dissolution and chemical modification. ILs are defined as salts with melting points <100 °C.[1] In 2002, Swatloski et al. described the dissolution of cellulose in ionic liquids.[2] The dissolution of wood, in the form of saw dust, has also been demonstrated in ILs.[3] This dissolution ability of ILs can also be exploited in pre-treatment of lignocellulosics prior to enzymatic total hydrolysis and fermentation used in the production of ethanol from renewable feed stocks. Dadi et al.[4] described a pre-treatment process in which the biomass is first dissolved in IL and then precipitated by the addition of an anti-solvent such as water or alcohol. This pre-treatment process was shown to greatly enhance subsequent enzymatic hydrolysis rates. Kamiya et al.[5] reported an alternative process where the cellulosic substrate was first dissolved in ionic liquid and then enzymatically hydrolysed in the same vessel after addition of buffer. After the introduction of these two concepts a number of papers dealing with enzymatic hydrolysis of cellulosics in combination with IL treatments have been published.

Only a few reports have been published on the IL compatibility of analytical methods for saccharides. ILs have, however, been proposed to interact with photometric assays and high performance liquid chromatography (HPLC) methods,[6] which are generally used for analysing the soluble saccharides formed in enzymatic hydrolysis of lignocellulosics. Recently, Hyvärinen et al.[7] discussed difficulties caused by the high salt content in chromatographic saccharide analysis in IL containing sample matrices. On the other hand, ILs have previously been used as auxiliaries in both chromatographic and electrophoretic separation techniques, predominantly as column stationary phases or mobile phase additives (chromatography) or as organic modifiers in background electrolyte solutions (BGEs) and capillary coatings in capillary electrophoresis (CE).[8] Recently, Vaher et al. demonstrated that ILs in low concentrations could act as chromophores for indirect UV detection in the sensitive analysis of small saccharides.[9] According to Vaher et al. the

* Corresponding author. Tel.: +358 40 02 54 073; fax: +358 20 72 27 071.
E-mail address: ronny.wahlstrom@vtt.fi (R. Wahlström).

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presence of low amounts of ILs (10–50 mM) in the BGEs increased the resolution between the saccharide peaks, but increasing the content of IL further led to baseline fluctuation. Another observation was that ILs with long hydrocarbon chains may actually function as surfactants and reverse the electroosmotic flow. High sensitivity saccharide analytical methods in high content IL matrices have not been described previously.

Saccharides are non-ionic compounds in their natural state. To enable the resolution of neutral, non-derivatized saccharides by electric fields as in CE, alkaline borate buffers are used to form charged borate–saccharide complexes.[10–12] The detection (usually measured at 195 nm) is considerably improved by the formation of borate–saccharide complexes.[12] Also indirect detection of saccharides in CE analysis is possible. In this case, a UV absorbing compound (such as sorbic acid[13] or 2,6-pyridinedicarboxylic acid[14]) is added to the BGE and the saccharides are analysed under basic conditions.

The resolving power of CE with pre-column derivatization has previously been demonstrated for mixtures of monosaccharides, including uronic and hexenuronic acids, and small xylo- and cellooligomers, in aqueous solutions.[10,11,15] Maltooligosaccharides with degrees of polymerization (DP) of up to 13 have been separated employing CE techniques.[16] Good separation results for derivatized saccharides have also been obtained employing micellar electrokinetic capillary chromatography (MEKC).[17] CE has been used in the separation of monosaccharide mixtures in matrices containing *N*-methylmorpholine-*N*-oxide (NMMO) used as industrial cellulose solvent.[18] In this study, monosaccharides were analysed in aqueous matrices containing roughly 10% NMMO prior to derivatization and analysis. The presence of NMMO was reported to interfere neither with the derivatization reaction nor with analysis with CE.

Advantages of sample derivatization include a manifold increase in detectability. Usually derivatized saccharides have absorption maxima with wavelengths greater than those of underivatized analytes, which increases also the selectivity of detection. Commonly encountered carbohydrate derivatization reagents are for example, 4-aminobenzoic acid ethyl ester (ABEE),[10,11,15] 4-aminobenzonitrile (ABN),[15] 6-aminoquinoline (6-AQ)[19] and 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS).[16] The derivatization proceeds via reductive amination and needs a free reducing end of the analyte. Reductive amination works well for aldoses, but ketoses such as fructose are not well derivatized.[10,16] Great excesses of derivatization reagent are usually used. In the derivatization method described by Dahlberg et al.,[11] the derivatization reaction is quenched by addition of alkaline borate buffer, which is suggested to form highly water soluble saccharide–borate complexes at the same time as the excess ABEE reagent is precipitated. Alkaline borate buffers are generally employed as BGEs for the separation of ABEE, ABN and similar saccharide derivatives in CE, with some variations in the alkalinity and borate concentration. Occasionally, additives such as surfactants and alcohols are added to the BGEs to improve resolution between adjacent peaks.[15] Both normal[10] and reverse polarity[15] modes have been employed.

Our work on developing IL compatible analytics for saccharide identification and quantification was started to allow us to study the action of hydrolytic enzymes on cellulose in imidazolium-based ILs.[20] The ionic liquids studied were 1,3-dimethylimidazolium dimethylphosphate [DMIM]DMP and 1-ethyl-3-methylimidazolium acetate [EMIM]AcO. The primary aim was to find a method that allows the sensitive quantification of cellooligomers up to the size of cellohexaose in these IL containing matrices. In this paper, we discuss how the presence of [DMIM]DMP and[EMIM]AcO affect the routine methods, such as DNS assay, different chromatography methods and CE in saccharide analysis. We present an optimized method for cellooligomer analysis in significant contents of ILs (20–40% (v/v)) employing CE with pre-column derivatization. The separation power of this method is demonstrated for both mono- and oligosaccharides obtained from wood-derived biomass and results for the quantification of the water soluble cellooligomers glucose, cellobiose, cellotriose, cellotetraose, cellopentaose and cellohexaose in four different matrices are presented. The usefulness of the method is illustrated by two studies, in the first of which the action of a commercial β-glucosidase preparation is studied in [DMIM]DMP and [EMIM]AcO matrices on cellooligomeric substrates, and in the second of which the partial enzymatic hydrolysis of microcrystalline cellulose by an endoglucanase is followed for different time points in IL matrices.

2. Materials and methods

2.1. Chemicals

[DMIM]DMP was prepared as described in the literature.[21] [EMIM]AcO (purity >98%) was purchased from Ionic Liquid Technologies (Heilbronn, Germany) and used without further purifications. The halide content of the [EMIM]AcO determined by ion chromatography was: chloride <100 mg/kg and bromide <50 mg/kg.

Cello-, manno- and xylooligomers in the range of bioses to hexaoses were purchased from Megazyme International (Wicklow, Ireland). Boric acid, sodium hydroxide (NaOH), 1,5-dimethyl-1,5-diaza-undecamethylene polymethobromide (hexadimethrine bromide), xylose, galactose, mannose and arabinose were obtained from Sigma–Aldrich (Steinheim, Germany). Glucose was from VWR International (Leuven, Belgium), galacturonic acid (from citrus origin) was purchased from BDH Chemicals (Poole, UK). Water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). For the preparation of 3,5-dinitrosalicylic acid (DNS) reagent solution according to Sumner,[22] DNS and potassium sodium tartrate tetrahydrate were acquired from Merck (Darmstadt, Germany). All chemicals were used as received if not otherwise stated.

β-Glucosidase (Novozym 188) was obtained from Novozymes (Bagsvaerd, Denmark) and used as such. β-Glucosidase, xylanase and endoglucanase activities were measured for the crude β-glucosidase preparation and determined to be 5900, 2970 and 740 nkat/mL, respectively. The unit katal (kat) is defined by the International Union for Pure and Applied Chemistry (IUPAC) as the number of catalysed reactions per time unit as mol/s.[23] β-Glucosidase activity was measured according to Bailey and Linko[24] and xylanase activity according to Bailey et al.[25] but at pH 5.0. Endoglucanase activity measurements were carried out according to the HEC assay[26] but using carboxymethylcellulose (CMC) in buffer at pH 5.0.

2.2. Chromatography and DNS assay

Reversed-phase chromatography was carried out based on experimental conditions described by Yasuno et al.[27] Analyses were carried out on a Dionex Ultimate 3000 HPLC system equipped with a Phenomenex C-18 Gemini-NX 3 μm 110A 150 × 2 mm column and a diode array detector. The eluent was a 0.2 M potassium borate buffer at pH 9 with 5% MeOH.

DNS photometric assay was carried out according to the IUPAC standard procedure[26] with the DNS reagent solution prepared as described by Sumner[22] using a Hitachi U-2000 spectrophotometer for absorption measurements at 540 nm. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was done according to our previously published in-house method.[28]

2.3. Capillary electrophoresis

2.3.1. Derivatization prior to CE analysis

The saccharides were derivatized with 4-aminobenzonitrile (ABN, samples in aqueous solution or containing [DMIM]DMP)

or 4-aminobenzoic acid ethyl ester (ABEE, samples containing [EMIM]AcO), according to a modified procedure based on Dahlman et al.[11] The reagent solution was prepared by dissolving ABN (0.29 g) or ABEE (0.40 g) in 10 mL methanol containing acetic acid (AcOH, 1.0 g). Sodium cyanoborohydride ($NaCNBH_3$, 0.1 g) was added to the reagent solution immediately prior to derivatization. Samples containing >40% [DMIM]DMP were diluted with distilled water to a final concentration of 40% [DMIM]DMP and samples containing >20% [EMIM]AcO were diluted with distilled water to a final concentration of 20% [EMIM]AcO. The sample was mixed with a 1 g/L galactose solution (internal standard) to yield a sample with a final galactose concentration of 20 mg/L. The sample with internal standard (1 part) was mixed with derivatization reagent solution (1 part) in a sealable glass vial and the vial was kept at 80 °C for 60 min. When the reaction completed, 1 part of alkaline boric acid buffer (450 mM, pH 8.5) was added, the mixture was vigorously vortexed, transferred to plastic Eppendorf tubes and centrifuged (14,000 rpm, 5 min) in order to sediment any precipitation. Aliquots of the supernatant were then as such taken for injection to the CE.

2.3.2. CE running conditions

Capillary electrophoresis was carried out with a P/ACE MDQ capillary electrophoresis instrument (Beckman-Coulter, Fullerton, CA, USA) equipped with a photodiode array (PDA) UV/Vis detector. Absorption of derivatized saccharides was measured at wavelengths of 284 nm for ABN derivatives and 305 nm for ABEE derivatives. The employed fused silica capillary had a total length of 60 cm, an effective length of 50 cm to the detector and an inner diameter of 50 μm. The injection was performed applying a pressure of 0.5 psi (34.5 mbar) for 30 s. The applied voltage was 20 kV in reverse polarity mode and the capillary cartridge was kept at a temperature of 30 °C. Every run was preceded by rinsing the capillary 3 min with 0.1 M HCl, 1 min with 0.1 M NaOH and 5 min with the running electrolyte. The capillary was conditioned before running any samples by flushing 10 min with 0.1 M HCl, 10 min with 0.1 M NaOH and 10 min with water. All rinsing actions were carried out at a pressure of 20 psi (1380 mbar). As background electrolyte (BGE) solution was used a 438 mM sodium borate solution (pH 9.7), containing 0.001% (w/v) of 1,5-dimethyl-1,5-diaza-undecamethylene polymethobromide (hexadimethrine bromide). Each sample was analysed with two repetitions.

2.3.3. Quantitation

Standard curves were acquired for glucose, cellobiose, cellotriose, cellotetraose, cellopentaose and cellohexaose in the range of 5–200 mg/L (cellotetraose, cellopentaose, cellohexaose) or 5–300 mg/L (glucose, cellobiose, cellotriose), all the standards containing 20 mg/L galactose as internal standard. Galactose was chosen as internal standard in our work, as it migrates before the expected mono- and oligosaccharides in our samples and has a good detector response when derivatized. Every calibration measurement was repeated 6 times. The Beckman-Coulter 32 Karat Software version 8.0 was used for evaluation of migration times and peak areas. The peak areas were corrected by dividing the measured area by the migration time. For determining linearity ranges and response factors for the saccharides towards the internal standard (ISTD), x and y values were calculated according to:

$$x = \mathrm{Conc(Analyte)}/\mathrm{Conc(ISTD)}$$

$$y = \mathrm{Area(Analyte)}/\mathrm{Area(ISTD)}$$

where Conc(Analyte) is the concentration of the analyte of interest and Conc(ISTD) the concentration of the internal standard (20 mg/L), and Area(Analyte) denotes the corrected area of the peak of interest and Area(ISTD) the corrected area of the internal standard. By plotting y against x linearity zones and the response line equations could be determined. The concentration of cellooligomers could thus be evaluated using the above described relationship and solving it for Conc(Analyte), correcting with the sample dilution coefficients. Limits of detection (LOD) and limits of quantification (LOQ) were evaluated by comparing the signal to noise ratio (S/N) values of the calibration points' peak area to the limits being LOD = 3 (S/N), LOQ = 10 (S/N).

2.4. Hydrolysis of cellooligomers with β-glucosidase in aqueous ionic liquid solutions

Buffer (0.050 M citrate pH 5.0 or 0.1 M phosphate pH 6.0) and the cellooligomer solution (150 mg/L) were mixed in a 2 mL plastic Eppendorf tube. The defined amount of ionic liquid was added followed by the enzyme preparation as a 1:400 dilution of the commercial enzyme solution, the enzyme dosage thus corresponding to an activity of 1000 nkat/g of cellooligomer substrate. The hydrolysis was carried out in a FinePCR Thermo Micromixer (Mxi4t) at a shaking rate of 300 rpm in 45 °C for 20 h. The hydrolysis was stopped by heating the reaction tubes to 98 °C in a block heater for 10 min. The cellooligomer composition of the hydrolysates was analysed according to the CE method described in Section 2.3. All the hydrolysis experiments were carried out with two parallel samples and one reference where no enzyme was added. The pH values of the hydrolysis media were measured with a Knick pH meter 766 Calimatic equipped with a Mettler-Toledo 110 Inlab Semi-Micro electrode (pH range 0–12).

2.5. Hydrolysis of microcrystalline cellulose with *Trichoderma reesei* Cel5A in aqueous ionic liquid matrices

The cellulase preparation of *Trichoderma reesei* Cel5A was produced, isolated and purified at VTT according to Suurnäkki et al.[29] The Cel5A activity was determined by the standard HEC assay[26] but using carboxymethylcellulose (CMC) as substrate in buffer at pH 5.0. The enzyme dosage per gram of cellulose (dry weight) was 2000 nkatal. Hydrolysis mixtures were prepared with 0%, 20%, 40%, 60%, 80% and 90% (v/v) IL dosage in sodium citrate buffer (50 mM, pH 5.0), making the total sample volume 3 mL, and the hydrolysis time was 2, 24, 48 or 72 h. Thirty milligrams (dry weight) of microcrystalline cellulose was measured into a test tube, the defined amount of buffer was added and the mixture was stirred to homogeneity, followed by addition of the IL and enzyme. The hydrolysis was carried out at 45 °C in closed test tubes in a water bath with continuous magnetic stirring. The hydrolysis was stopped by boiling the sample for 600 s to denature the enzyme. After cooling to room temperature, the reaction tube was centrifuged at 3000 rpm for 10 min and the clear supernatant was separated from the solid cellulose residue and further prepared for CE analysis as described in Section 2.3.

3. Results and discussion

3.1. Effect of ionic liquid on DNS assay and chromatographic methods for saccharide analysis

DNS assay[22,30] is a well-known and often employed analytical method for the estimation of the total amount of reducing saccharides in aqueous solutions. This method has also been shown to be reliable in up to 20% (v/v) of [EMIM]AcO in the solution matrix.[31] In our experiments, it was, however, found that the commercial [EMIM]AcO used especially at high concentrations of over 40% (v/v) caused serious colouration which gave strong absorption at 540 nm, leading to problems in background calibration. It could not be concluded whether the colour formation was due to the

actual IL or impurities in it. [DMIM]DMP was also found to cause some colouration, but not to the same extent as [EMIM]AcO. DNS assay also has its limitations as although it gives an estimate of the total amount of reducing ends it is not able to distinguish different mono- and oligosaccharides from each other.

High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is known to be an excellent method for saccharide analysis in aqueous systems. Clear benefits of this method are the good sensitivity and the fact that there is no need to derivatize the samples.[16] In our experiments, saccharides like glucose, cellobiose and cellotriose could not be detected in the presence of even 20% (v/v) of [DMIM]DMP. It is probable that the high content of ions in the sample matrix interferes with the anion exchange capability of the column and furthermore the IL ions may have an impact on the detection at the PAD detector.

We tried the use of reversed-phase HPLC systems for samples with ABEE and ABN derivatized samples. In aqueous solutions the monosaccharides and some disaccharides could be separated but for larger oligosaccharides a good separation could not be achieved. When the experiment was repeated in matrices containing 40% (v/v) [DMIM]DMP or [EMIM]AcO, the monosaccharide peaks could not be detected anymore and severe baseline problems were encountered. Kamiya et al. quantified glucose and cellobiose as ABEE derivatives in IL containing hydrolysates with reverse phase HPLC,[5] according to a method of Yasuno et al.[27] The sample matrix contained 1,3-dimethylimidazolium diethylphosphate, an IL very similar to [DMIM]DMP in structure, but further details about the analysis and the actual IL content in the samples were not given.

3.2. Optimization of CE method and calibration for cellooligomer quantification in IL matrices

Both CE methods with direct and indirect detection modes were tested for saccharide analysis in the presence of two imidazolium-based ILs. The presence of significant amounts (>20–40% (v/v)) of [EMIM]AcO or [DMIM]DMP completely deleted the saccharide signals when CE analysis conditions known to be favourable for saccharide analysis in pure aqueous solutions were applied.[14,32] Derivatization of reducing saccharides with UV active tags proved to be a feasible method for improving saccharide detection in CE and a method employing pre-column derivatization was chosen for use in IL containing matrices. We optimized the CE running conditions for samples containing [DMIM]DMP and [EMIM]AcO based on a method previously described by Sartori et al.[15]

3.2.1. Optimization of derivatization conditions

The derivatization reaction was carried out with ABEE or ABN as described in Section 2.3.1. The method follows a general procedure for ABEE derivatization by Dahlman et al.,[11] with some modifications, for example, the method was found to be applicable for the ABN derivatization analogously. Experiments were carried out similarly with derivatization times of both 30 and 60 min at 80 °C for both glucose and cellobiose with ABEE. ABEE derivatization of samples containing glucose and galactose was carried out in 40% (v/v) [EMIM]AcO matrices with reaction times of 60 and 120 min, as well as with doubled amount of acetic acid in the derivatization reagent solution. [EMIM]AcO was in our experiments found to be very basic[20] which motivated the attempt to acidify the derivatization conditions. Extending the reaction time from 30 to 60 min and up to 120 min did not lead to an increase in the analyte peak areas and the use of double amounts of AcOH led to failure of the actual analysis with CE. The derivatization was complete already after 30 min for both glucose and cellobiose. No hydrolysis of cellobiose took place with 60 min reaction time which was confirmed by the absence of glucose peaks in the electropherograms of pure derivatized cellobiose standards. The possibility of partial hydrolysis of oligomeric polysaccharides during reductive amination has been proposed previously.[16] In our work no hydrolysis of any cellooligomers used was observed under the applied derivatization conditions.

The presence of [DMIM]DMP or [EMIM]AcO did not prevent the actual derivatization reaction from taking place. It was not, however, elucidated whether the saccharide conversion in the derivatization reaction is changed by the presence of ILs, as compared to the conversion in aqueous reaction matrices. The presence of ILs apparently increased the solubility of the reagents. The derivatization reaction was found to be rather robust and also the optimum conditions were similar in IL matrices to those earlier reported for aqueous solutions in terms of employed reagent solutions, reaction times and reaction temperatures.[11] We applied exactly the same derivatization protocol in derivatizing with ABN as with ABEE, but it was found that the alkaline borate buffer does not efficiently precipitate the excess ABN reagent in the quenching stage. This did, however, not have any detrimental effects on the CE analysis. Similar results have previously been reported for a method where the derivatization reaction is not quenched in any way, but the samples are simply cooled, filtered and injected into the CE for analysis.[15] The derivatized samples were found to be very stable. Properly stored (at −18 °C) we found the derivatives to give reproducible results even after months of storage. The derivatization procedure is easy to carry out in series, making it feasible for analysing large numbers of samples at once.

3.2.2. Optimization of background electrolyte

One reason for the general deterioration in saccharide derivative analysis results in IL matrices might be due to changes in the ion strength and thereby electrical properties of the BGE. Therefore, the effect of different borate concentrations on peak shape and separation was studied. The separation of saccharide derivatives in CE has earlier been shown to be better by employing concentrated borate buffers as BGEs.[11] In our work, BGEs with borate concentration in the range of 110–600 mM were studied. 450 mM borate buffer was found to be a good compromise between all the criteria for an optimized analysis. In both aqueous and [DMIM]DMP (40% (v/v)) containing matrices, peak areas generally decreased and monosaccharide and small oligosaccharide peaks suffered of increased tailing when applying low borate concentration BGEs. In some cases, the peak shapes of the larger oligosaccharides were better when applying low borate concentrations than at the relatively high 450 mM concentration. Using extremely concentrated borate buffers did not yield better analysis results neither in pure aqueous matrices nor in IL containing matrices. In the method described by Sartori et al. for CE analysis of saccharides in aqueous matrices the BGE contained 5% methanol and 5% 1-propanol.[15] We found, that in the presence of the ILs and especially in the presence of [EMIM]AcO, the analysis results were better when no alcohol was added to the BGE. The use of hexadimethrine bromide when reversing the separation polarity was found to improve the analysis whereas the presence of another surfactant, cetyltrimethylammonium bromide (CTAB) was noticed to lead to complete failure of the analysis. The BGE pH was kept at 9.7, as it has been shown that higher alkalinities may cause worse peak resolutions and longer separation times under similar conditions.[10]

3.2.3. Optimization of CE method

In the CE analysis, the ILs were found to greatly influence the analysis results (Fig. 1). At IL concentrations of 20–40% (v/v) in the sample matrix, the IL effect was mainly seen as peak deformation in the form of tailing or sometimes partial peak splitting. In addition, the presence of ILs in different concentrations seemed to influence the peak areas of the separate saccharides to different

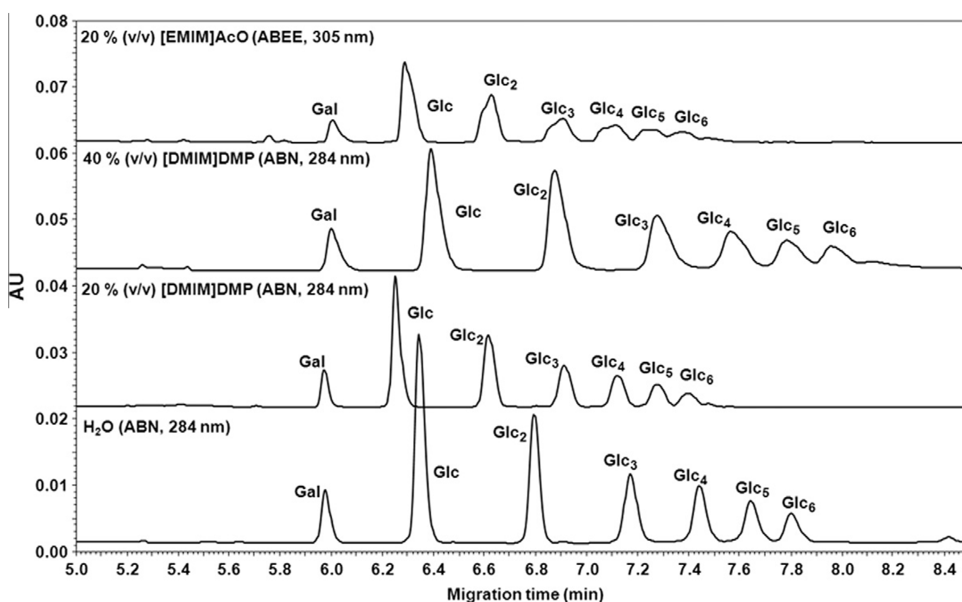

Figure 1. Separation of cellooligomers in aqueous and IL containing matrices. Comparison of electropherograms obtained for a standard mixture of ABN or ABEE derivatives of 20 mg/L galactose (Gal) and 100 mg/L of glucose (Glu), cellobiose (Glc_2), cellotriose (Glc_3), cellotetraose(Glc_4), cellopentaose (Glc_5) and cellohexaose (Glc_6). The electropherograms have been aligned on the Gal peak (internal standard). Experimental conditions were according to Section 2.3, with ABN derivatization (detection at 284 nm) for samples in aqueous and [DMIM]DMP containing matrices and ABEE derivatization (detection at 305 nm) for [EMIM]AcO containing matrices.

extents, necessitating calibration against the internal standard in every different sample matrix separately. [EMIM]AcO was found to affect CE analysis much more than [DMIM]DMP. Interestingly, there also seemed to be a difference in derivative to IL compatibility; ABN derivatization gave better analysis results in aqueous and [DMIM]DMP containing solutions whereas ABEE was to be preferred as derivatization reagent in samples containing [EMIM]AcO. The presence of high contents of ILs in the samples gradually led to peak dampening. Therefore, we studied the trade-off between diluting the samples and the response of the analytes in the IL containing samples. It was found, that samples containing more than 40% (v/v) of [DMIM]DMP gained in overall sensitivity by diluting the sample to a final IL strength of 40% (v/v). [EMIM]AcO had a much stronger dampening effect and any sample containing this IL was found to give the best overall sensitivity after diluting to a final IL content of 20% (v/v). It should be noted, that the derivatization procedure dilutes the sample to a third of its nominal analyte and IL concentrations as the used sample/derivatization reagent solution/quenching borate buffer volume ratio is 1:1:1.

The reason for the deleterious effects of ILs in the sample matrix in CE is not known. One possible explanation may be related to the coating of the capillary walls with the IL imidazolium cation, which has previously been described and exploited in the separation of polyphenols.[33] The presence of ILs did not always have a negative impact on the actual peak area. Actually, a 20% (v/v) concentration of [DMIM]DMP seemed to give a better response factor for the smaller of the studied cellooligomers (glucose, cellobiose, cellotriose) than was the case in pure aqueous solution. This result was not further developed in this study. The reviewed literature and our results show that under some circumstances, the ILs may in moderate contents work as organic modifiers with beneficial effects, such as improved peak shape.

The peaks migrated in the same order and roughly with the same migration times when both pure aqueous samples and IL containing samples were used, which makes it possible to use high quality electropherograms acquired from aqueous samples for the prediction of separation in IL containing matrices.

The running conditions were further developed based on a method published by Sartori et al.[15] In normal polarity mode the largest oligosaccharides migrate first, after the excess reagent peak, followed by the smaller in ascending order. By applying reverse polarity mode the migration order of the saccharides is reversed, but the most important feature is that the saccharides migrate before the large excess reagent peak, which was found to seriously affect the baseline and also the integration of peaks migrating after it. Great gain in terms of peak shape was acquired by raising the temperature to 30 °C, which is higher than in the previous methods cited in this paper. Further increasing the temperature led to a complete failure of the analysis. It was also found that analysis sensitivity could be increased by using a quite long injection time of 30 s, but any further increase was not beneficial. The voltage optimum was determined to be 20 kV. It was observed that the combination of fairly concentrated buffer together with the use of surfactant and the IL in the sample caused severe capillary fouling. Efficient washing procedures needed to be developed to guarantee an acceptable capillary life length. Washing with propanol was tried as a means of cleansing the capillary from organic precipitations but no clear benefit could be demonstrated. However, the combination of acidic (HCl) and alkaline (NaOH) rinsing cycles prior to sample injection was beneficial.

3.2.4. Qualitative and quantitative analysis of mono- and oligosaccharides in IL matrices

The optimized CE method was exploited in qualitative saccharide derivative analysis in IL containing aqueous solutions. It was shown that most common cellulose and hemicellulose derived mono- and oligosaccharides could be resolved as ABN or ABEE derivatives. The method was efficient for separating galacturonic acid, galactose and xylose (Fig. 2), but arabinose, mannose and glucose migrated partially together. As indicated earlier by Dahlman

et al. these monosaccharides can be separated by CE by carefully optimized conditions in aqueous solutions.[11] Also most of the studied xylo-and cellooligomers (Fig. 3) could be separated. Mannooligosaccharides could not be separated from each other. In our study, the presence of ILs in the matrix was not the reason for the weak separation between arabinose, mannose and glucose. It was found that the peak separation, especially for arabinose, mannose and glucose, was better in samples containing 20% (v/v) [DMIM]DMP than in purely aqueous sample matrices, which demonstrates that the presence of ILs in certain concentrations may indeed be beneficial for the CE analysis.

The CE method was calibrated for quantitative analysis of the cellooligomers in the range of glucose to cellohexaose in four different sample matrices: pure aqueous solution and aqueous solutions containing 20% and 40% (v/v) [DMIM]DMP and 20% (v/v) [EMIM]AcO. The regression data are given in Table 1. The regression data are somewhat different for the analysis in different media and therefore the calibration should be performed separately for each IL containing media for maximum accuracy. Linearity ranges were studied in the range of 5–300 mg/mL for glucose, cellobiose and cellotriose, and 5–200 mg/mL for cellotetraose, cellopentaose and cellohexaose. The limits of detection (LoD, S/N 3) and quantification (LoQ, S/N 10) determined represent well the same level of sensitivity as compared to those results previously reported in the literature for aqueous matrixes.[15] Also LoD and LoQ values calculated for the IL containing matrices show good sensitivities, considering the difficulty of the matrix. Because the detection is based on quantifying the reducing end groups as UV active derivatives, the different oligomers were detected corresponding to their molar concentration rather than their mass concentrations, which leads to higher detection and quantification limits expressed in mass per sample volume according to the molecular weight of the saccharide. The cellooligomers showed good linearity ranges in the studied concentration intervals. It needs to be stressed that the linearity ranges given in this paper do not represent the whole linearity range of the different analytes.

Reductive amination as well as other derivatization methods and also DNS assay require a free reducing end group on the saccharide. Ebner et al.[34] have shown that the imidazolium C2 carbon in 1-alkyl-3-methylimidazolium ILs may react to various degrees with the reducing end of saccharides in IL solutions. The reaction is proposed to proceed via the deprotonation of the acidic proton at C2 in the imidazolium ring, giving a highly nucleophilic carbene which reacts with the reducing end group of a saccharide.[35] In the case of glucose, glucose-imidazolium adducts have been reported to be formed in up to 15–20% yield of the glucose under fairly mild conditions and the addition of base has been found to greatly accelerate the reaction rate.[34] The reaction has also been concluded to be reversible, especially under basic conditions. Acetate ILs have been suggested to be especially susceptible for this kind of reaction due to their alkalinity.[35] Consequently, quantification results may be too low if this side-reaction takes place and if it is not reversed at some point during the sample treatment. Up to date, it has not to our knowledge been elucidated whether carbene formation of imidazolium ILs with subsequent addition to saccharide reducing ends takes place in aqueous solutions of ILs in the same manner and to the same extent as it has been shown to do in pure IL solutions. To assess the general accuracy of any saccharide analytics in IL matrices, this reaction should be studied in more detail.

3.3. Hydrolysis of cellooligomers by β-glucosidase in IL matrices

In the total hydrolysis of cellulose, the employed cellulase cocktails generally consist of three different types of cellulases; endoglucanases, exoglucanases and β-glucosidases, which catalyse the hydrolysis of the cellooligomers formed by the other enzymes to glucose. These enzymes work in synergy and their simultaneous function is vital for high hydrolysis yields.[36] Up to date, some studies have been published about the inactivating effects of ILs on endoglucanases,[20,37] whereas β-glucosidases have received relatively little attention in these matrices. Recently, Engel et al.[37] published inactivation data for a commercial β-glucosidase in

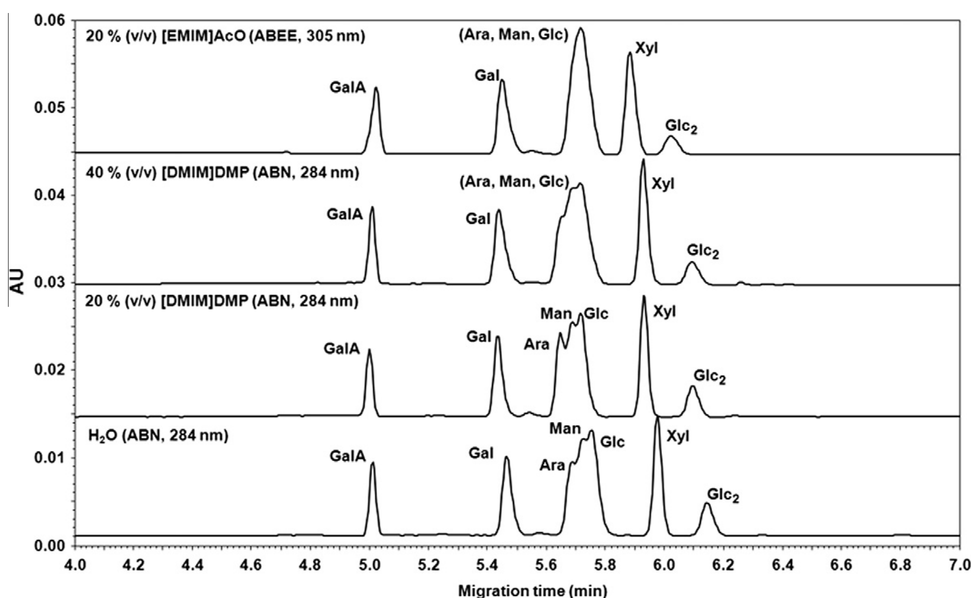

Figure 2. Separation of galacturonic acid (GalA), galactose (Gal), arabinose (Ara), mannose (Man), glucose (Glc), xylose (Xyl) and cellobiose (Glc_2) ABN (detection at 284 nm) or ABEE (detection at 305 nm) derivatives in four different matrices. Experimental conditions as described in Section 2.3. The electropherograms have been aligned on the GalA peak.

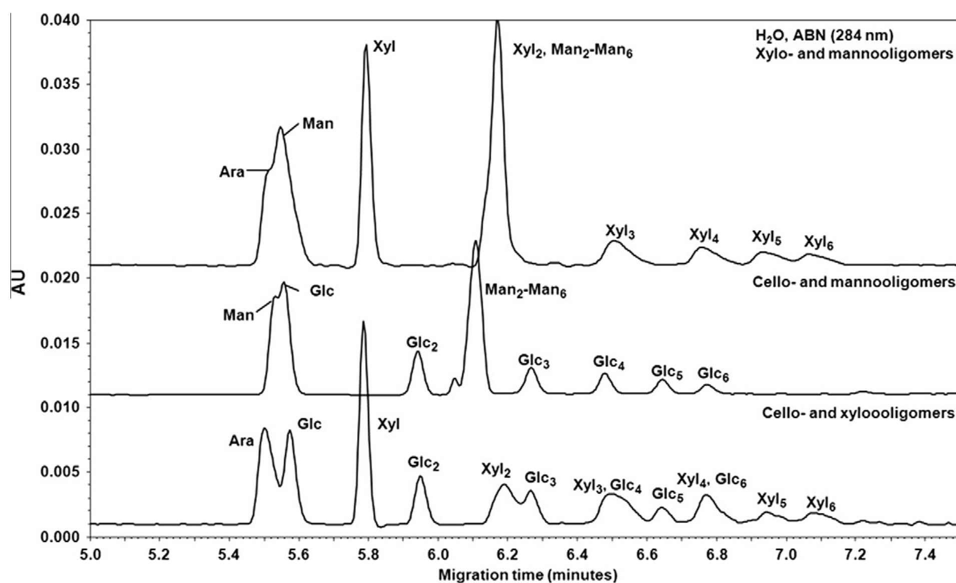

Figure 3. Separation of cello-, xylo- and mannooligomers (ranging from monosaccharide to hexasaccharide) as ABN derivatives (detection at 284 nm) in aqueous solution. The electropherograms have been aligned on the Xyl or Glc_2 peaks. Each saccharide standard had a concentration of 100 mg/L, experimental conditions were according to Section 2.3. Peak labelling is analogous to that introduced in the caption of Figure 1.

Table 1
Quantification and regression data for cellooligomers ranging from glucose to cellohexaose (saccharide labelling as in Fig. 1) in four different matrices

Saccharide/matrix	LOD (mg/L)	LR (mg/L)	RSD(low) %	RSD(high) %	Equation	R^2
Water						
Glc	<5	5–150	2.38	1.45	$y = 0.0429x - 0.0301$	0.997
Glc_2	<5	5–300	2.14	0.87	$y = 0.0261x - 0.0479$	0.999
Glc_3	5	10–300	2.16	0.54	$y = 0.0165x + 0.0042$	0.998
Glc_4	5	10–200	2.76	0.33	$y = 0.0144x - 0.0557$	0.999
Glc_5	10	20–150	2.79	3.50	$y = 0.0111x - 0.06$	0.998
Glc_6	10	50–200	5.68	1.36	$y = 0.007x + 0.0075$	0.989
20% (v/v) [DMIM]DMP						
Glc	<5	5–100	1.03	0.41	$y = 0.039x + 0.1677$	0.999
Glc_2	<5	5–100	1.89	0.94	$y = 0.0248x + 0.0928$	0.999
Glc_3	5	10–300	9.04	0.98	$y = 0.0159x + 0.0422$	0.998
Glc_4	5	20–200	4.74	3.60	$y = 0.0135x - 0.041$	0.999
Glc_5	5	20–200	4.98	2.50	$y = 0.0098x - 0.0047$	0.999
Glc_6	5	50–200	9.36	3.41	$y = 0.0068x + 0.0123$	0.996
40% (v/v) [DMIM]DMP						
Glc	<5	5–50	2.50	0.94	$y = 0.0369x + 0.1084$	0.997
Glc_2	<5	5–300	5.09	1.37	$y = 0.0247x + 0.0728$	0.996
Glc_3	5	10–300	10.1	1.46	$y = 0.0146x + 0.0107$	0.989
Glc_4	5	10–200	8.35	0.53	$y = 0.011x + 0.0267$	0.996
Glc_5	5	20–150	8.82	10.3	$y = 0.0087x + 0.0228$	0.995
Glc_6	5	50–150	2.50	9.02	$y = 0.0065x + 0.0446$	0.993
20% (v/v) [EMIM]AcO						
Glc	<5	5–100	1.25	0.47	$y = 0.0385x + 0.0513$	1
Glc_2	<5	5–300	7.10	1.08	$y = 0.0273x - 0.0659$	0.998
Glc_3	5	50–300	2.74	2.19	$y = 0.0175x - 0.0909$	0.997
Glc_4	5	50–200	3.59	4.05	$y = 0.0136x - 0.0449$	0.998
Glc_5	20	50–200	2.42	5.01	$y = 0.0095x + 0.0271$	0.999
Glc_6	20	50–200	2.50	1.56	$y = 0.0071x + 0.0182$	0.999

Data have been acquired by six independent injections of the standard solution. LOD = limit of detection, LR = linearity range, RSD(low) relative standard deviation for the response factor at lowest determined point on calibration line, RSD(high) relative standard deviation of the response factor at highest determined point on calibration line, R^2 = regression coefficient. The response factor is defined as the parameter ratio y/x.

[DMIM]DMP, where the enzyme was found to lose its activity completely at 15% [DMIM]DMP. In this study, we followed the change caused by ILs on β-glucosidase action by analysing the individual cellooligomers in the hydrolysates.

Our results (Table 2) show that when the commercial β-glucosidase preparation is used all cellooligomers in the range of cellobiose to cellohexaose are hydrolysed to glucose in sodium citrate buffer at pH 5.0. The β-glucosidase preparation contained xylanase

and endoglucanase side-activities, which may play a role in hydrolysing the larger cellooligomers to smaller fragments. The β-glucosidase preparation seemed to be quite sensitive to the rise of pH, as the hydrolysis was not completed in phosphate buffer at pH 6.0.

The β-glucosidase preparation action in matrices containing 20% and 40% of [DMIM]DMP and [EMIM]AcO was studied with cellobiose and cellopentaose as substrates. When adding ILs to the hydrolysis matrix, the action of the β-glucosidase was greatly affected. In 20% [EMIM]AcO, the action of the enzyme was extremely low (Table 2). In 40% [EMIM]AcO, no cellooligomer hydrolysis took place. In the presence of [DMIM]DMP considerable enzymatic action was observed in 20% [DMIM]DMP, where both cellobiose and cellopentaose were completely hydrolysed to glucose. In 40% [DMIM]DMP, the cellooligomeric substrates were hydrolysed only partly. In the cases where partial hydrolysis occurred on cellopentaose, the intermediary products were cellotetraose, cellotriose and glucose, whereas very little cellobiose was produced. The hydrolysis matrices contained either citrate buffer at pH 5.0 or phosphate buffer at pH 6.0 in addition to the IL. There was a clear difference in the hydrolysis results depending on the matrix buffer. In all cases, hydrolysis results were lower in the phosphate buffer, as could be predicted from the results from the pure buffer matrices, probably due to the buffers' higher pH.

The changes in pH caused by the presence of the ILs (Table 2) are not considered as the main reason for the decreased enzymatic action in the presence of the ILs. [EMIM]AcO is in general to some extent more basic than [DMIM]DMP.[20] With the low contents of IL used in this work the pH is, however, practically the same for [DMIM]DMP and [EMIM]AcO in 0.1 M phosphate buffer (original buffer pH 6.0). This can be exemplified by comparing the hydrolysis of cellopentaose (Glc_5) in 20% [EMIM]AcO/phosphate buffer (Table 2, entry 7), where the pH was measured to 6.6, comparing to the hydrolysis of Glc_5 in 20% [DMIM]DMP/phosphate buffer (Table 2, entry 14), where the pH was measured to the same 6.6. In the presence of [EMIM]AcO, there is almost no hydrolysis observed, whereas the hydrolysis conversion in the corresponding conditions with [DMIM]DMP present is fairly high. Similar but smaller difference was also observed in the enzymatic hydrolysis of Glc_5 in 40% [DMIM]DMP/phosphate buffer versus 40% [EMIM]AcO/phosphate buffer (Table 2, entries 16 and 8). It is thus concluded that [EMIM]AcO is more inactivating for the studied β-glucosidase than [DMIM]DMP and that the main reason for this difference between [EMIM]AcO and [DMIM]DMP is not in their different basicities. The enzyme preparation as such did not contain significant amounts of oligosaccharides, which could disturb the saccharide quantitation. The reference samples containing cellooligomers in the hydrolysis matrix without addition of enzyme showed no signs of IL or buffer induced hydrolysis of oligosaccharides during the 20 h incubation. Some of the saccharides initially added to the hydrolysis mixture were lost during the treatment in IL solutions (Table 2). The reason for this is currently not known, but the reaction between the saccharide reducing ends and possibly formed carbenes, as was discussed in Section 3.2.4, provides a possible explanation.

3.4. Hydrolysis of microcrystalline cellulose by *T. reesei* Cel5A in the presence of [DMIM]DMP and [EMIM]AcO

Endoglucanases catalyse the hydrolysis of amorphous parts of cellulose resulting in oligosaccharides.[36] In our work, we used the CE method with pre-column derivatization described in this paper to analyse cellooligomers released from microcrystalline cellulose during hydrolysis with the *T. reesei* endoglucanase Cel5A (formerly known as endoglucanase II) in media containing the ILs [DMIM]DMP and [EMIM]AcO. Cel5A produced glucose, cellobiose and cellotriose but no larger cellooligomeric products in all media, where it was active. The hydrolysis products could be well separated and quantified, also in the presence of [DMIM]DMP and [EMIM]AcO, with the CE method, as can be seen in Figure 4. A more thorough analysis of the formed different cellooligomers in these systems has been published recently.[20]

The hydrolysis yield of the Cel5A treatment of microcrystalline cellulose in the different IL containing media was calculated for four different hydrolysis times (2, 24, 48 and 72 h), by adding the CE analysed amounts of each formed cellooligomer (Fig. 5). It can be seen that the hydrolysis yield is by far the highest in the pure buffer at pH 5.0 as expected and the ILs are inactivating for the enzyme in all reported concentrations. For all time points, it is clear that [DMIM]DMP is much less inactivating for the enzyme than [EMIM]AcO. Interestingly, the hydrolysis degree seems to be increasing throughout the whole studied time interval, in pure buffer as well as in 20% [DMIM]DMP suggesting that the hydrolysis has not yet at 72 h achieved its maximum level and some of the enzyme is still active also after 72 h incubation in 20% [DMIM]DMP. In 20% [EMIM]AcO the yields are much lower than in 20% [DMIM]DMP. The enzymatic hydrolysis reaches its maximum rather early, between the 2 and 24 h time points (Fig. 5) indicating a complete inactivation of the cellulase in 20% [EMIM]AcO in the

Table 2
Oligosaccharide composition and pH value of β-glucosidase hydrolysates in buffer or in ionic liquid containing matrices (C = 0.050 M citrate buffer (pH 5.0) and P = 0.100 M phosphate buffer (pH 6.0))

Substrate	Matrix	pH	Products (mg/L)				
			Glc	Glc_2	Glc_3	Glc_4	Glc_5
Glc_2	C	5.0	146.9	Traces			
Glc_3	C	5.0	142.9				
Glc_4	C	5.0	165.6				
Glc_5	C	5.0	172.9				
Glc_6	C	5.0	167.6				
Glc_5	P	6.0	112.0				35.9
Glc_5	20 m-% [EMIM]AcO+P	6.6	Traces			Traces	69.6
Glc_5	40 m-% [EMIM]AcO+P	7.4					83.2
Glc_2	20 m-% [DMIM]DMP+C	5.4	120.4				
Glc_5	20 m-% [DMIM]DMP+C	5.4	135.2				
Glc_2	40 m-% [DMIM]DMP+C	6.3	45.8	46.2			
Glc_5	40 m-% [DMIM]DMP+C	6.3	43.4		Traces	17.9	45.6
Glc_2	20 m-% [DMIM]DMP+P	6.6	161.9	16.8			
Glc_5	20 m-% [DMIM]DMP+P	6.6	89.7	4.5	Traces	11.3	6.9
Glc_2	40 m-% [DMIM]DMP+P	7.2	25.4	60.2			
Glc_5	40 m-% [DMIM]DMP+P	7.2	17.0			Traces	57.4

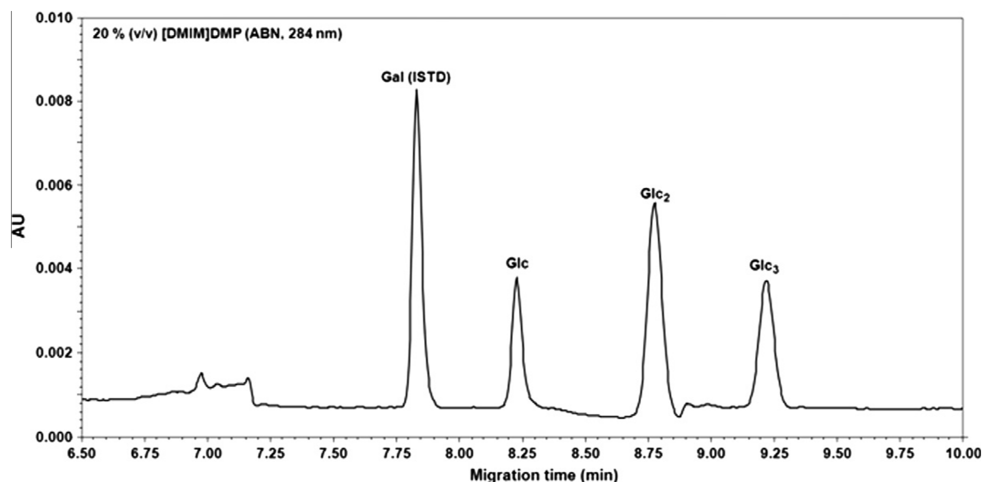

Figure 4. Analysis of cellooligomers produced in a 24 h hydrolysis in a medium containing 20% [DMIM]DMP. The derivatization reaction and CE conditions were as described in Section 2.3. The cellooligomers in this sample were quantified to Glc: 7.0 mg/L, Glc_2: 34.4 mg/L, Glc_3: 31.4 mg/L, peak labelling is as in Figure 1.

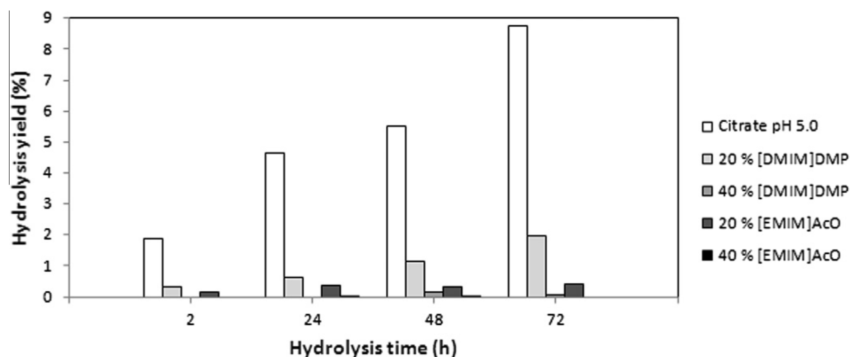

Figure 5. Yields from hydrolysis of microcrystalline cellulose hydrolysed by *T. reesei* Cel5A after 2, 24, 48 and 72 h hydrolysis in five matrices: 0.050 M citrate buffer (pH 5.0), 20% or 40% [DMIM]DMP or [EMIM]AcO in citrate buffer.

said time interval. In 40% of [DMIM]DMP only traces of saccharides were found and in 40% [EMIM]AcO none at all. No saccharides were found in any of the other high IL content matrices.

4. Conclusions

Imidazolium-based ionic liquids form difficult matrices for the analysis of carbohydrates with HPLC, colorimetric and CE methods. In this paper we have described a method for capillary electrophoresis with pre-column derivatization of saccharides with an UV active derivatization reagent. The method developed allows sensitive qualitative and quantitative analysis of carbohydrate mixtures comprising both mono- and oligosaccharides in the presence of significant quantities of the ionic liquids [DMIM]DMP and [EMIM]AcO.

The CE analysis method described in this paper is expected to be of good use in the sensitive analysis of carbohydrates in IL containing sample matrices related to biorefinery applications. The usefulness of the described CE method is demonstrated here by two independent examples: the enzymatic hydrolysis of cellobiose and cellopentaose with β-glucosidase in different media containing the two cellulose dissolving ionic liquids [DMIM]DMP and [EMIM]AcO and the hydrolysis of microcrystalline cellulose with a *T. reesei* endoglucanase in the same media during a time span of 2–72 h. The results show that even if the employed cellulases are very different in their function, their hydrolysis efficiency is decreased in the same way by the presence of the studied ionic liquids. [EMIM]AcO was in both the experiments much more inactivating than [DMIM]DMP.

The reaction of carbenes generated in imidazolium-based ILs with the reducing end of saccharides still needs further elaboration, as this reaction would directly influence any quantification results of reducing saccharides. Very little has been published about high sensitivity analysis of carbohydrates in the presence of ILs. This study shows that taking carbohydrate analysis from aqueous systems to IL containing matrices is not straightforward and many crucial questions still need to be answered in future studies.

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PUBLICATION III

Cellulose hydrolysis with thermo- and alkali-tolerant cellulases in cellulose-dissolving superbase ionic liquids

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PAPER

Cellulose hydrolysis with thermo- and alkali-tolerant cellulases in cellulose-dissolving superbase ionic liquids

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Ronny Wahlström,*[a] Alistair King,[b] Arno Parviainen,[b] Kristiina Kruus[a] and Anna Suurnäkki[a]

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Pretreatment with ionic liquids (ILs) is known to greatly increase the subsequent biomass hydrolysis with enzymes. However, the presence of even low amounts of ILs has negative effects on cellulase action. Most studies on cellulase inactivation by ILs have focused on imidazolium-based ILs, which until recently were one of the few IL classes known to dissolve cellulose. In this article we describe results of cellulase action in matrices containing ILs belonging to two IL classes recently reported as cellulose solvents. These ILs are based on the organic superbases 1,1,3,3-tetramethylguanidine (TMG) or 1,5-diazabicyclo-[4.3.0]non-5-ene (DBN). In this study commercial thermo- and alkaline stabile cellulase products were employed, as these were anticipated to also have a higher stability in ILs. For comparison, hydrolysis experiments were also carried out with a well-characterised endoglucanase (Cel5A) from *Trichoderma reesei* and in matrices containing 1-ethyl-3-methylimidazolium acetate, [EMIM]AcO. Two different substrates were used, microcrystalline cellulose (MCC) and eucalyptus pre-hydrolysis kraft dissolving grade pulp. The hydrolysis yields were on the same level for both of these substrates, but decreases in molecular weight of the cellulose was observed only for the dissolving grade pulp. By using commercial cellulases with good thermo- and alkali-stability some benefits were obtained in terms of IL compatibility. Enzyme thermostability correlated with higher hydrolysis yields in IL-containing matrices, whereas activity at high pH values did not offer benefits in terms of IL tolerance. The new classes of cellulose-dissolving superbase ILs did not differ in terms of cellulase compatibility from the well-studied imidazolium-based ILs. Of the novel superbase ILs tested, [TMGH]AcO was found to inhibit the enzymatic hydrolysis the least.

Introduction

Lignocellulosic biomass such as wood and straw is rich in cellulose and other polysaccharides (*e.g.* hemicelluloses). These form a potential source of monosaccharides, useful as starting materials for microbial fermentations in the production of fuels and value-added chemicals.[1] A great deal of research is currently going on in enzymatic hydrolysis of lignocellulosic biomass for ethanol production. Lignocellulosic biomass is, however, very recalcitrant towards enzymatic hydrolysis due to the complex and tight matrix structure of the main polymers cellulose, hemicelluloses and lignin,[2] cellulose crystallinity,[3] low surface area for enzyme binding,[2] low solubility in conventional solvents[4] and the presence of enzyme inhibitors.[2] Different chemical, physical, biological pretreatments, or combinations thereof, have been proposed to reduce the substrate recalcitrance towards hydrolysis.[2] Most pretreatment methods usually apply high pressures and temperatures or harsh chemical environments, and the formation of compounds inhibitory for downstream processing (fermentation) is a considerable problem.[5]

Ionic liquids (ILs), generally defined as salts with melting points <100 °C,[6] were introduced as cellulose solvents by Swatloski *et al.* in 2002.[7] It has been demonstrated that 1-ethyl-3-methylimidazolium acetate [EMIM]AcO is even capable of dissolving wood.[8] The efficient dissolution of lignocellulosic biomass in ILs opens up new routes for biomass modification and deconstruction.

The potential of ILs in the pretreatment of cellulose for hydrolysis has been reported in many publications employing different cellulosic substrates, ILs and enzyme cellulase preparations.[3,9–18] Dadi *et al.* described a procedure where microcrystalline cellulose (MCC) is dissolved in IL and thereafter regenerated by precipitating it by the addition of an anti-solvent, such as water or alcohol, thus yielding a regenerated cellulose, which is more amorphous than the starting material.[3,11] The regenerated cellulose showed much better hydrolysis kinetics than untreated MCC. The regeneration pathway requires a thorough washing of the regenerated substrate, both to recover the IL and to avoid IL-induced cellulase inactivation. The

[a]VTT – Technical Research Centre of Finland, P.O. Box 1000, VTT, Tietotie 2, Espoo, FI-02044, Finland. E-mail: ronny.wahlstrom@vtt.fi; Fax: +358 20 722 7071; Tel: +358 40 02 54 073

[b]Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, PO Box 55, A. I. Virtasen Aukio 1, FIN-00014, Helsinki, Finland

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washing requires, however, large amounts of water and recycling of the much diluted IL from the washing liquid is energy intensive and costly.[19] Furthermore, some IL is usually left in the regenerated biomass, leading to enzyme inactivation in the hydrolysis step[20] and loss of IL. Finding processing conditions allowing enzymatic hydrolysis without washing off the IL after regeneration is thus desirable. Kamiya *et al.* introduced a concept where the cellulosic substrate is first dissolved in an IL and then buffer and enzyme are added for hydrolysis, without removing the IL between the pretreatment and hydrolysis steps.[21] In this study, 40% (v/v) of IL in the matrix was found to be the limit for significant enzymatic cellulose hydrolysis to take place.

The presence of ILs during cellulose hydrolysis has been reported to be very inactivating for cellulases.[22] As imidazolium-based, cellulose dissolving ILs have been found to be quite basic when mixed in different proportions with buffer as hydrolysis matrix,[23–25] it has been proposed that the basicity of the matrix could be one major reason for the observed enzyme inactivation.[23] However, another recent study came much to the opposite conclusion.[24] Thermostability has also been suggested to correlate with IL tolerance for cellulases.[26] Increased viscosity[25,27] and greatly increased ionic strengths[25] due to the presence of IL have both been shown to be detrimental for enzymatic activity in ILs. However, it has also been shown that these effects alone are not the only reason for the observed inactivation.[25]

The inactivating effect of both IL cations and anions has been shown to correlate with the Hofmeister series.[28] Hydrophilic anions such as AcO^-, NO_3^- and $CH_3SO_3^-$ in ILs have been found to be very inactivating, whereas fluorinated hydrophobic anions, such as PF_6^-, have, on the other hand, been shown to be more compatible with enzymes.[29] ILs with PF_6^- anions are not, however, reported to dissolve cellulose. Whether the enzymatic inactivation is reversible or irreversible depends very much on the IL anion.[29]

Enzymatic hydrolysis of cellulose in ILs has mainly been carried out with commercial cellulase cocktails used in total hydrolysis of lignocellulosics.[21,27] In addition, the effect of IL on monocomponent cellulases has been studied to elucidate enzymatic action in the presence of ILs in more detail.[10,24,30] Cellulase cocktails have also been specifically optimized for action in IL-containing hydrolysis matrices.[19,30]

Most of the studies regarding the use of ILs as cellulose solvents in pretreatments prior to hydrolysis and in chemical and physical cellulose modifications, have been carried out using imidazolium-based ILs. Recently new classes of cellulose-dissolving ILs with new interesting properties have been introduced. Cholinium-based carboxylates[31] and pyridinium-based ILs[32] have been shown to be efficient ILs for biomass pretreatment for saccharification. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU)-based ILs have been described as cellulose solvents in a patent by BASF.[33] An interesting and potentially bio-compatible new class of cellulose dissolving ILs with amino acid-anions was recently described by Ohira *et al.*[34] Ammonium-based ILs show good cellulase compatibility and have also been reported to dissolve biomass.[27] Aqueous tetrabutylphosphonium hydroxide (60% w/w) represents another newly reported cellulose solvent which very effectively dissolves cellulose under ambient conditions.[4] King *et al.* recently introduced a new class of ILs as cellulose solvents composed of acid–base conjugates of the organic superbase 1,1,3,3-tetramethylguanidine (TMG).[35] This class of ILs is particularly appealing for large scale biomass processing operations as these ILs are distillable under certain conditions, which greatly facilitates the recycling of IL in any process. In an extension of this work, Parviainen *et al.* described the cellulose dissolving ability of 1,5-diazabicyclo[4.3.0]non-5-ene (DBN)-based ILs.[36]

In this article, we report the action of different cellulases in the presence of the cellulose-dissolving TMG- and DBN-based ILs. Two different substrates, MCC and a hardwood dissolving pulp were used. MCC represents a highly crystalline and pure cellulose model substrate, whereas the hardwood dissolving pulp is a practical cellulosic fibre substrate. Two commercial endoglucanase rich cellulase preparations, IndiAge® ONE and Puradax® HA 1200E were selected for enzymatic hydrolysis based on their salt and alkaline tolerance. For comparison, the experimental monocomponent endoglucanase Cel5A from *Trichoderma reesei* and the commercially available thermophilic cellulase from *Thermotoga maritima* were also studied in the same systems. Cellulase action was also studied in matrices containing [EMIM]AcO to relate the results against previous studies.

Experimental

Materials

[EMIM]AcO (purity >98%) was purchased from Ionic Liquid Technologies GmbH (Iolitec, Heilbronn, Germany) and used without further purifications. The halide content of the [EMIM]AcO determined by ion chromatography was: chloride <100 ppm and bromide <50 ppm. TMG and DBN-based ILs were prepared as described by King *et al.*[35] and Parviainen *et al.*[36] MCC, (research grade, particle size 0.020 mm) was from Serva Electrophoresis GmbH (Heidelberg, Germany) and the pre-hydrolysis kraft dissolving grade pulp from *Eucalyptus urograndis* was acquired from Specialty Cellulose, Brazil. According to carbohydrate analysis[37] the pulp contained 94.9% cellulose and 2.5% xylan. The pulp was cut manually into small pieces (0.5 × 0.5 cm) and milled with a Fritsch Pulverisette 14 variable speed rotor mill to a final size of <1 mm. The dry weight of the MCC and milled pulp substrates was determined as the average mass loss for three parallel samples by keeping the cellulose at 105 °C for 14 h. The cellulase preparation of *Trichoderma reesei* Cel5A was produced, isolated and purified at VTT according to a method described by Suurnäkki *et al.*[38] Thermostable cellulase from *Thermotoga maritima* was purchased from Megazyme International (Bray, Ireland). The cellulase granules of IndiAge® ONE and Puradax® HA 1200E were a kind gift from DuPont Industrial Biosciences (Hanko, Finland).

Enzyme preparations and characterization

The IndiAge® ONE and Puradax® HA 1200E enzyme granules were dissolved as 1% (w/v) solution in 0.100 M phosphate buffer (pH 6.0) by stirring for one hour. The cloudy crude enzyme solutions were centrifuged in glass test tubes 3000 rpm for

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5 min. Econo-Pac® 10 DG desalting columns (Bio-Rad Laboratories Inc., USA) were used to remove stabilizing agents from the enzyme solution. Based on the dinitrosalicylic acid (DNS) assay[39] the enzyme preparations did not contain detectable amounts of reducing sugars. The commercial *T. maritima* cellulase and the *T. reesei* Cel5A were in solution and not further processed before usage. The purity of the *T. maritima* cellulase, IndiAge® ONE and Puradax® HA 1200E preparations were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1).[40] Samples were run in precast Tris–HCl gradient gels (4–20%, Bio-Rad, Hercules, CA, USA) and visualized with a Criterion stain-free imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA) in which protein visualization is based on a UV-light driven reaction of tryptophan residues in the presence of a trichlorocompound.[41] According to SDS-PAGE, IndiAge® ONE and the *T. maritima* cellulase were very pure monocomponent protein preparations, whereas the Puradax® HA 1200E showed one major band, probably corresponding to the endoglucanase, and also some minor bands of unknown origin (Fig. 1).

Protein content was measured with a commercial kit (DC Protein Assay from Bio-Rad, Hercules, CA, USA) based on the Lowry method[42] using bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) as standard. The protein content measurement was preceded by a precipitation step with acetone, followed by

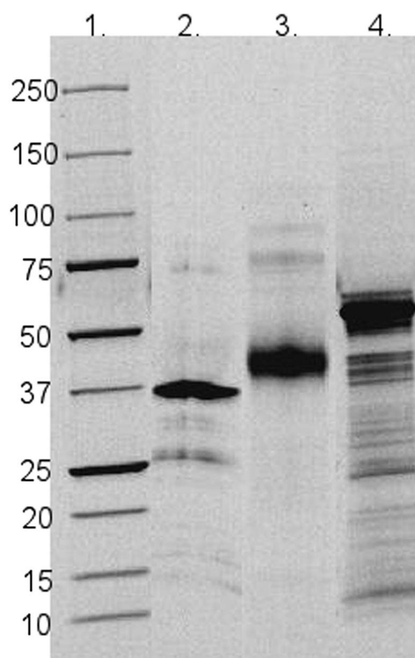

Fig. 1 Purified enzymes visualised using SDS-polyacrylamide gel electrophoresis. Lane 2 *Thermotoga maritima* cellulase; lane 3 IndiAge® ONE; lane 4 Puradax® HA 1200E; lane 1 is the molecular weight standards in which the size of each protein is shown in kDa. Extra lanes have been removed from the gel image.

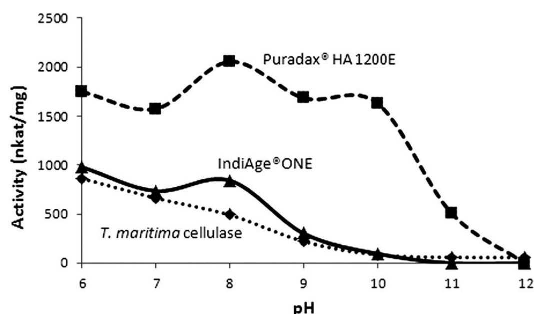

Fig. 2 pH dependence of Puradax® HA 1200E, IndiAge® ONE and *Thermotoga maritima* cellulase activities on CMC. Endoglucanase activities were measured on 1% CMC in Britton–Robinson buffer for 10 min at 45 °C, with the amount of catalysed reactions determined by DNS assay.

re-dissolution in alkaline Na_2CO_3 solution, to reduce the amount of oligopeptides in the enzyme sample.

The endoglucanase activities of the enzyme preparations were measured with 1% (w/v) carboxymethylcellulose (CMC) as substrate in a Britton–Robinson buffer, containing 0.04 M borate, 0.04 M phosphate and 0.04 M acetate, with pH adjusted by addition of NaOH. The activities were measured in the pH range of 6 to 12 to determine whether the studied cellulases had activities in neutral and alkaline hydrolysis matrices, as it was known that the cellulose-dissolving ILs render buffer solutions rather basic.[24] After a reaction time of 10 min at 45 °C the reaction was terminated by adding DNS reagent solution to the samples and boiling for 5 min. The endoglucanase activity was determined by measuring the absorption of the boiled samples at 540 nm and comparing the absorption to that of glucose standards treated in the same way as the enzyme samples. IndiAge® ONE and the *T. maritima* cellulase had a falling activity trend from neutral to high pH with little activity at pH 10, whereas Puradax® HA 1200E had high activity over the whole pH range of 6–10, but with quickly decreasing activity at pH values above 10 (Fig. 2). The pH dependence of *T. reesei* Cel5A activity (on β-glucan) is reported to be a bell-shaped curve with the optimum at pH 5 having only little activity at pH 8 or higher.[43]

Hydrolysis

MCC and pulp samples for hydrolysis were prepared with 0, 20, 40, 60, 80 and 90% (w/w) IL concentration in sodium phosphate buffer (0.100 M, pH 6.0). 30 mg (dry weight) of substrate was measured into a test tube, the defined amount of buffer was added and the mixture was stirred to homogeneity. The defined volume of IL was added to the mixture and the mixture was again stirred to homogeneity. TMG-based ILs were melted in an oil bath due to their high melting points[35] before weighing by pipetting into the hydrolysis tubes. The enzyme dosage was 1.0 mg of protein per gram of dry cellulose (Table 1). The final substrate consistency was 1% (w/w) and the total sample mass 3 g. The hydrolysis was carried out at 45 °C in a closed test tube with continuous magnetic stirring, with a hydrolysis time of 72 h. The temperature-dependence experiments were carried

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Table 1 Enzyme dosages in the hydrolysis experiments in terms of mg enzyme per g of substrate and activity (CMC) per g of substrate. Activities were measured in 0.100 M phosphate buffer (pH 6.0) except for *Trichoderma reesei* Cel5A which was measured in 0.050 M citrate (pH 5.0)

Enzyme	Dosage (mg per g substrate)	Spec. activity (nkat per mg prot.)	Dosage (nkat per g substrate)
T. reesei Cel5A	0.98	2034	2000
Puradax® HA 1200E	0.98	1750	1720
IndiAge® ONE	0.99	980	963
T. maritima cellulase	0.98	863	847

out in 0.100 M phosphate buffer, the hydrolysis temperature was 40, 50, 60 or 70 °C and the hydrolysis time 2, 4, 24 and 72 h. The hydrolysis was stopped by boiling the sample for 600 s. After cooling to room temperature, the sample was centrifuged at 3000 rpm for 10 min and the clear hydrolysate was separated from the solid cellulose residue. In the samples containing 80 or 90% (w/w) of IL, the dissolved cellulose was regenerated by adding 3 g of distilled H_2O and vigorously mixing before centrifugation. All experiments were carried out in triplicates. Reference samples were treated under the corresponding conditions, but without adding enzyme.

Analysis

The total amount of carbohydrates solubilized from the substrate was analysed by DNS assay according to Sumner *et al.*,[39] with absorption measurements at 540 nm carried out using a Hitachi U-2000 spectrophotometer. Hydrolysates were analysed qualitatively and quantitatively for cello- and xylooligomers by capillary electrophoresis (CE) using pre-column derivatization according to a previously published procedure.[44] The molecular mass distributions of residual cellulose samples were determined by gel-permeation chromatography (GPC), with the samples dissolved and diluted in LiCl/DMAc.[24]

Results and discussion

Hydrolysis in superbase ILs

In this work, four enzyme preparations consisting of endoglucanase activities were studied in cellulose hydrolysis in the presence of novel superbase ionic liquids. The enzymes include a purified monocomponent preparation of the mesophilic *T. reesei* Cel5A and three commercial enzymes, Puradax® HA 1200E, IndiAge® ONE and a thermophilic cellulase from *T. maritima*. Puradax® HA 1200E is a commercial enzyme manufactured for dish applications and IndiAge® ONE is used for denim finishing in the textiles industry. Based on the manufacturer's note, these two cellulase preparations have relatively broad pH and temperature applicability ranges. The thermophilic cellulase from *T. maritima* was considered a promising candidate for hydrolysis in ILs as thermostability has been shown to correlate with IL-tolerance in the literature[26] and previously *T. maritima* cellulases have been found to have some IL-tolerance.[20,26]

Seven different superbase ILs were initially tested for enzyme compatibility with *T. reesei* Cel5A, in matrices containing 20% (w/w) of IL and 1% (w/w) of MCC. The tested ILs were [TMGH]COO, [TMGH]AcO, [TMGH]EtCOO, [TMGH]*n*-PrCOO, [DBNMe]DMP, [DBNH]AcO and [DBNH]EtCOO (Fig. 3). The experiment in 20% (w/w) [EMIM]AcO was carried out as a reference. In all cases, yields of MCC hydrolysis, analysed by DNS colorimetry,[39] ranged from trace amounts to 2% of the dry substrate (Fig. 4). The *T. reesei* Cel5A compatibility with the TMG-based ILs was the following: [TMGH]AcO > [TMGH]COO > [TMGH]EtCOO > [TMGH]*n*-PrCOO. The highest MCC hydrolysis yield in this experiment was observed in 20% (w/w) [EMIM]AcO, followed by [TMGH]AcO. The hydrolysis yields with *T. reesei* Cel5A in DBN-based ILs were more strongly reduced than in the TMG-based ILs. In presence of 20% (w/w) of [DBNH]AcO or [DBNH]EtCOO the amount of reducing saccharides was below

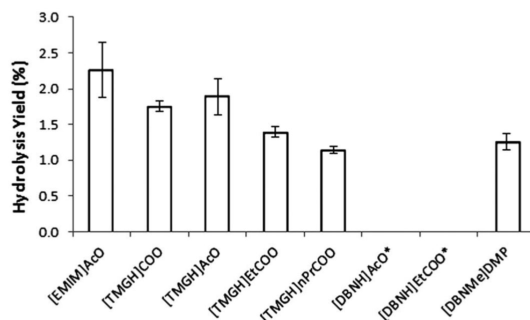

Fig. 4 Hydrolysis yields from 72 h hydrolysis of MCC (1% w/w) at 45 °C with *Trichoderma reesei* Cel5A in the presence of 20% (w/w) of superbase ILs. Error bars represent the standard deviation of the sample triplicates. * denotes that the hydrolysis yield was below the quantification limit of the DNS assay used.

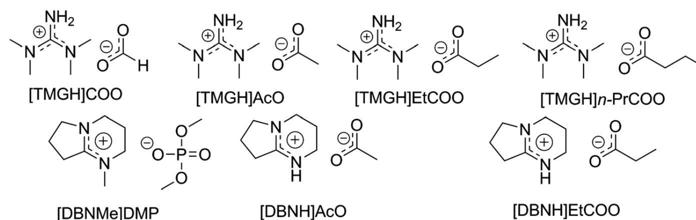

Fig. 3 Cellulose-dissolving superbase ionic liquids tested for compatibility with enzymes.

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the quantification limit of DNS colorimetry (corresponding to a 1% yield). For [DBNMe]DMP, the saccharide yield corresponded to the yields obtained for [TMGH]EtCOO and [TMGH]*n*-PrCOO (Fig. 4). There were clear differences between the three acetates which were studied here. As well, differences were noticed between the TMG-based ILs, depending on their carboxylate anion. This indicates that both the cation and the anion play a role in enzyme inhibition. [TMGH]AcO was chosen for further studies based on these results.

Release of saccharides from MCC and eucalyptus pre-hydrolysis kraft dissolving grade pulp in enzymatic hydrolysis in [TMGH]AcO and [EMIM]AcO matrices

The hydrolysis yields obtained with Puradax® HA 1200E, IndiAge® ONE and *T. reesei* Cel5A after 72 h hydrolysis of MCC and eucalyptus pre-hydrolysis kraft dissolving grade pulp at 45 °C are presented in Fig. 5A and B. The hydrolysates were analysed for cello- and xylooligomers, in the range of monosaccharide to hexasaccharide, with a recently reported CE method.[44]

IndiAge® ONE was the most efficient enzyme in MCC hydrolysis, in terms of hydrolysis yield, in all the studied matrices (Fig. 5A). In buffer the hydrolysis yield was 9.6% (w/w of dry MCC). The hydrolysis yields decreased to 3.8% and 1.4% in the 20 and 40% (w/w) [TMGH]AcO matrices, and 2.6% and 0.7% in the 20 and 40% (w/w) [EMIM]AcO matrices, respectively. Puradax® HA 1200E, having the highest activity on soluble substrates (CMC) for all pH values between 6 and 12 (Fig. 2), had comparably low hydrolysis yields on solid substrates compared to IndiAge® ONE (Fig. 5A and B). This difference in substrate specificity can possibly be attributed to the different end uses of these two cellulases: Puradax® HA 1200E is used as a washing liquid enzyme whereas IndiAge® ONE finds its use in textile processing and is thus optimized to

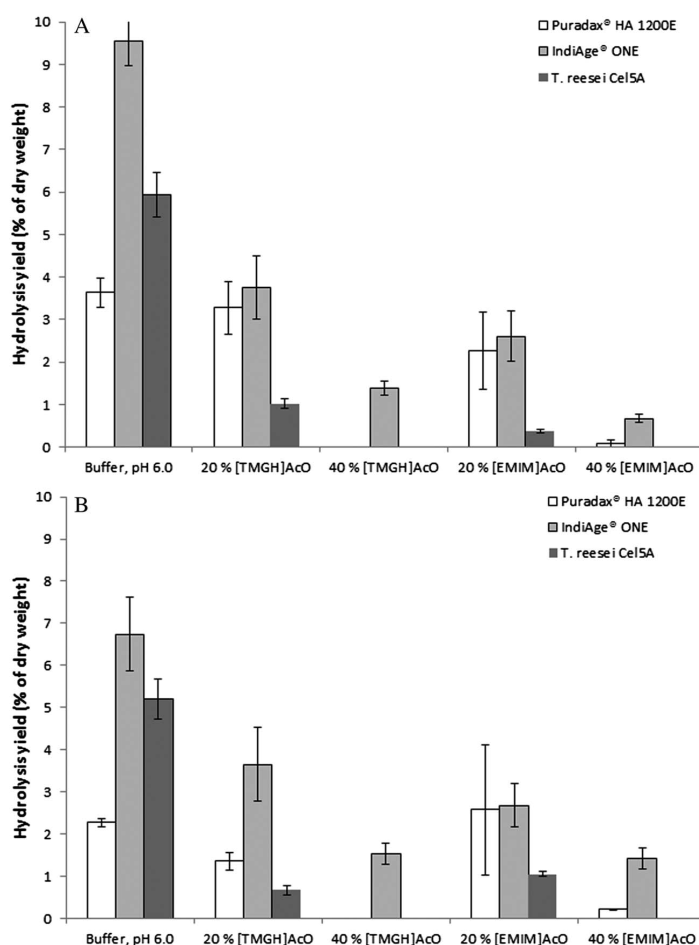

Fig. 5 Hydrolysis yields from 72 h hydrolysis of (A) MCC and (B) eucalyptus pre-hydrolysis kraft dissolving grade pulp at 45 °C by three different cellulase preparations in buffer and IL containing matrices. Error bars represent the standard deviation of the sample triplicates. Hydrolysis yields were quantified by CE with pre-column derivatization.[44]

work on solid substrates. It should also be noted that a lower amount of active enzyme in terms of moles, was present in the Puradax® HA 1200E hydrolysis than in the experiments with the other enzymes, due to the higher molecular weight and lower purity of this enzyme preparation (Fig. 1). The thermostable cellulase from *T. maritima* was not efficient under the employed conditions, with yields below 1% on MCC in all cases (results not shown). MCC hydrolysis with this cellulase was also carried out at 80 °C, the optimum temperature of the enzyme, but raising the temperature did not significantly increase the yields. *T. maritima* cellulases have previously been shown to display an increased IL tolerance, as compared to mesophilic enzymes.[20] IL tolerance has been linked to the enzyme's thermostability in several studies.[20,26] Based on our study, no conclusions of the IL tolerance of *T. maritima* could be made. No release of soluble saccharides could be detected in the enzymatic hydrolysis with the studied cellulases in matrices containing >40% (w/w) of IL (Fig. 5A and B).

The commercial IndiAge® ONE and Puradax® HA 1200E cellulases both showed better hydrolysis yields on MCC in IL matrices than the mesophilic Cel5A. The hydrolysis yields by IndiAge® ONE were reduced directly proportionally to increasing amounts of IL in both [EMIM]AcO and [TMGH]AcO matrices. *T. reesei* Cel5A responded in a similar manner to IL presence, even if Cel5A was more sensitive to IL. Cel5A did not in any case show activity in 40% (w/w) IL matrices, but some hydrolysis took place in 20% (w/w) IL matrices. For Puradax® HA 1200E, the response pattern to IL was different from the hydrolysis yield reductions observed for IndiAge® ONE and *T. reesei* Cel5A. The presence of 20% (w/w) of IL had fairly little effect on the hydrolytic action of this enzyme on MCC. However, when increasing the IL content to 40% (w/w) of the hydrolysis matrix, the hydrolysis yield rapidly collapsed, with only little hydrolysis taking place in 40% (w/w) of [EMIM]AcO and no hydrolysis in 40% (w/w) of [TMGH]AcO. This is somewhat contrary to the observation for the other cellulases that [TMGH]AcO is more enzyme compatible than [EMIM]AcO.

Hydrolysis yields with eucalyptus pre-hydrolysis kraft dissolving grade pulp as substrate were generally on the same level as with MCC in the presence of ILs (Fig. 5B). The reduction in enzymatic hydrolysis yield caused by the presence of ILs followed roughly the same patterns as described above for MCC hydrolysis. The relatively low hydrolysis yields of the cellulose pulp were expected to be due to mixing problems, as the mixing of the 1% (w/w) pulp suspension led to some clustering of the ground pulp particles. The mixing of MCC, on the other hand, was always close to ideal due to the small and even particle size. The effect of mixing on hydrolysis yield was studied by carrying out some hydrolyses with pulp in two times diluted systems, *i.e.*, in 0.5% (w/w) pulp consistency, and keeping the same level of catalytic enzyme activity per weight of substrate. The hydrolysis yields did, however, not increase but rather stayed at the same level as for the 1% (w/w) pulp consistency (results not shown). Apparently the mixing was not the limiting factor in the hydrolysis. The cellulose accessibility in the MCC and the pulp could, however, affect the enzymatic hydrolysis of these two substrates.

Table 2 Cellooligomer distribution in hydrolysates of IndiAge® ONE and *Trichoderma reesei* Cel5A on microcrystalline cellulose. Glc denotes glucose, Glc_2 cellobiose, Glc_3 cellotriose and Glc_4 cellotetraose. Oligosaccharides were quantified by CE with pre-column derivatization[44]

	Produced oligosaccharides (mg L^{-1})			
Treatment	Glc	Glc_2	Glc_3	Glc_4
IndiAge® ONE				
Buffer, pH 6	350	552	85	37
20% [TMGH]AcO	34	187	110	62
40% [TMGH]AcO	0	57	47	41
20% [EMIM]AcO	36	144	62	33
40% [EMIM]AcO	0	25	25	21
***T. reesei* Cel5A**				
Buffer, pH 6	119	298	212	0
20% [TMGH]AcO	5	48	53	0
40% [TMGH]AcO	0	0	0	0
20% [EMIM]AcO	0	15	18	Traces
40% [EMIM]AcO	0	0	0	0

The distribution of oligosaccharides produced in enzymatic hydrolysis of MCC and the impact of IL presence on the distribution, was studied. For IndiAge® ONE, the presence of ILs led to a distinct shift from shorter to longer cellooligomers (Table 2). In addition to glucose, cellobiose and cellotriose, cellotetraose was also produced. *T. reesei* Cel5A, producing glucose, cellobiose and cellotriose in buffer, has previously been reported to be influenced by the presence of ILs so that the oligomer distribution is shifted towards cellobiose and cellotriose, with less glucose produced.[24] In this study, Cel5A was found to produce also trace amounts of cellotetraose in the IL matrices, in addition to the general shift of the distribution towards the larger cellooligomers. The *T. maritima* cellulase produced glucose, cellobiose, cellotriose and cellotetraose, but in very low yields (results not shown). Puradax® HA 1200E produced glucose, cellobiose and cellotriose as major products in the hydrolysis of both MCC and pulp. In buffer, longer cellooligomers were also produced. The oligomer distribution was the same in terms of oligomers and their relative occurrence in all IL matrices. For any of the employed enzymes, no significant differences in the product distributions or the yields could be recorded for the two substrates tested, MCC and eucalyptus pre-hydrolysis kraft dissolving grade pulp. In many cases traces of xylose or xylobiose were also found in the hydrolysates.

All of the studied ILs (Fig. 3) were quite basic in aqueous/buffer solution (Table 3). [TMGH]AcO was the least basic of the four [TMGH] carboxylates. This may play a role in the higher

Table 3 pH values for IL in buffer (0.100 M phosphate, original pH 6.0) solutions, with ILs representing different classes of cellulose-dissolving ILs

	pH at IL content in matrix, % (w/w)		
IL	20	40	60
[EMIM]AcO	6.5	7.4	8.8
[TMGH]AcO	5.6	6.1	7.1
[DBNMe]DMP	6.8	8.1	10.5

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hydrolysis yield obtained in this IL solution, compared to the other [TMGH] carboxylates (Fig. 4). Based on previous studies,[23–25] the basic effect of imidazolium-based ILs is a well-established fact. There is, however, some controversy in interpreting whether the basicity is one of the main reasons for the observed strong inactivation of cellulases, when exposed to IL matrices. By comparing the pH of the IL containing matrices (Table 3), with the data on the pH dependency of IndiAge® ONE and Puradax® HA 1200E activity on CMC (Fig. 2), it appears that these enzymes should still have substantial activity at the neutral or alkaline pH values of the matrices with 20 or 40% (w/w) IL. Nevertheless, the enzymatic hydrolysis yields decreased strongly at these IL contents. Apparently, the IL basicity is not the only reason for the observed low yields in the studied systems.

The temperature influence on hydrolysis yields for IndiAge® ONE, Puradax® HA 1200E and *T. reesei* Cel5A was compared in the temperature range 40–70 °C, for different time points, in phosphate buffer at pH 6.0. IndiAge® ONE was clearly the most effective cellulase in terms of saccharide production, and also the most thermostable cellulase (Fig. 6A). In 60 °C only little reduction in hydrolysis yield was observed and even in 70 °C a fair hydrolysis yield could be obtained. In contrast, Puradax® HA 1200E was very temperature sensitive with no hydrolysis at temperatures higher than 50 °C (Fig. 6B). *T. reesei* Cel5A showed mediocre thermostability with the highest yields at 40 and 50 °C and low yields still at 60 °C (Fig. 6C). IndiAge® ONE, which had the best thermostability, also showed the best IL tolerance in this study, which indicates that thermostability could be at least one factor that predicts IL tolerance. On the other hand, Puradax® HA 1200E, which showed the lowest thermostability, was more effective in IL matrices, than *T. reesei* Cel5A. These conclusions suggest that thermostability alone is not enough for predicting the IL tolerance of a cellulose preparation.

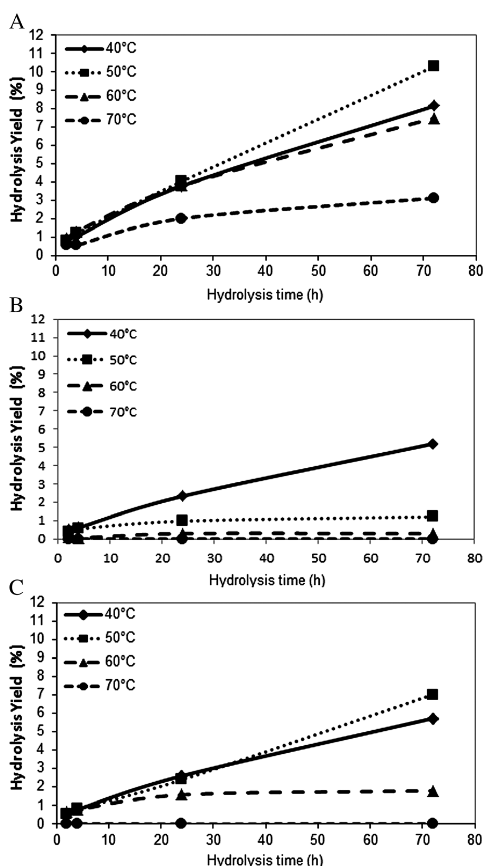

Fig. 6 Temperature dependence curves for (A) IndiAge® ONE, (B) Puradax® HA 1200E and (C) *T. reesei* Cel5A. Experimental conditions: 1% (w/v) MCC, 0.100 M phosphate buffer, pH 6.0, enzyme dosage 1.0 mg/g of the dry substrate.

Cellulose molecular weight after hydrolysis in ionic liquid matrices

In general, none of the employed enzymes were able to cause significant changes to the weight average molecular mass (M_w) of the MCC substrate in any matrices, even though up to almost 10% of the substrate was hydrolysed to soluble saccharides, under optimum conditions in buffer. In the 20 and 40% (w/w) IL matrices, in which some soluble saccharides were released during hydrolysis, no cellulose dissolution was yet taking place. In matrices with high IL content (80–90% (w/w) of IL), where partial or even complete cellulose dissolution already takes place, some decreases in MCC molecular weight have earlier been reported when employing *T. reesei* endoglucanases in imidazolium-based IL.[24]

The enzymatic hydrolysis of eucalyptus pre-hydrolysis kraft dissolving pulp resulted in considerable decreases in the M_w in several different IL matrices (Fig. 7). For *T. reesei* Cel5A the decrease in M_w correlated with the released saccharide yields in the hydrolysates; the largest decrease of 40–50% of the initial M_w value occurred in buffer where also the highest yield of soluble saccharides was obtained. In addition, a similar decrease in M_w was also measured for samples treated with *T. reesei* Cel5A in the 20% (w/w) [EMIM]AcO matrix. With Puradax® HA 1200E and IndiAge® ONE, the decrease in pulp M_w was only very marginal in buffer although 2.3% and 6.7% of the pulp was hydrolysed to soluble saccharides, respectively (Fig. 5B). In some cases, the pulp M_w was affected in IL matrices by the enzymatic treatments: Puradax® HA 1200E caused a decrease in the pulp M_w in 20% (w/w) [EMIM]AcO, whereas a decrease in M_w with IndiAge® ONE was observed only in 40% (w/w) [TMGH]AcO. For Puradax® HA 1200E and IndiAge® ONE no clear correlation between the pulp hydrolysis yields and changes in the M_w values was observed.

Conclusions

The recently described cellulose-dissolving superbase ILs, based on TMG or DBN, are easy to synthesize from cheap starting materials and potentially easy to recycle by

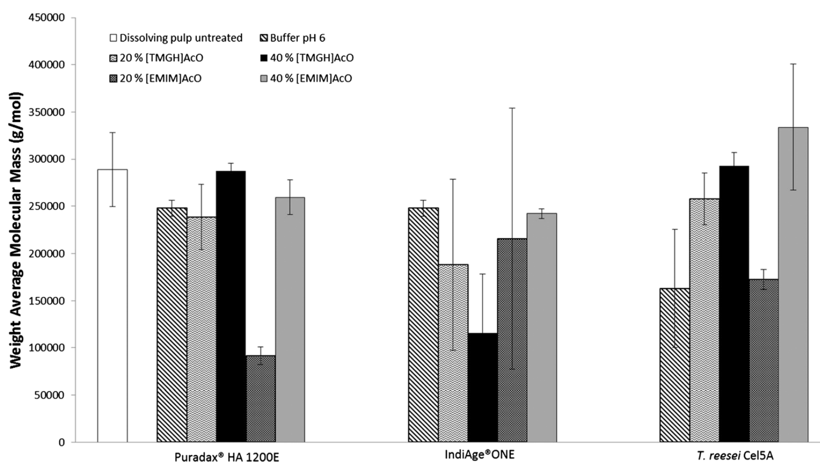

Fig. 7 Weight average molecular masses (M_w) for enzymatically treated eucalyptus pre-hydrolysis kraft dissolving grade pulp in IL matrices. Error bars represent the standard deviation of the sample triplicates. Cellulose molecular weights were measured by GPC with the cellulose dissolved and eluted using LiCl/DMAc.

distillation.[35,36] In this study their enzymatic compatibility was elucidated by the hydrolysis of microcrystalline cellulose, MCC, and a eucalyptus pre-hydrolysis kraft dissolving grade pulp, with commercial and experimental cellulases. It was found that the TMG and DBN based ILs offer little benefit in terms of enzyme compatibility over [EMIM]AcO. [TMGH]AcO turned out to affect the hydrolysis efficiency of the enzymes studied somewhat less than [EMIM]AcO. Employing the commercial cellulases Puradax® HA 1200E and IndiAge® ONE, with enhanced thermo- or alkali-tolerance, higher hydrolysis yields in the presence of IL was obtained, as compared with the experimental mesophilic *T. reesei* cellulase. In terms of enzymatic saccharide production, MCC and hardwood dissolving pulp were hydrolysed to a similar level in IL matrices, whereas the pulp was the only substrate to show molecular mass reductions during cellulase treatment. Enzyme-catalysed saccharide production and decreases in the substrate molecular mass could not be correlated in all treatments. For designing real enzyme-compatible and industrially feasible cellulose solution systems, much work is still needed in both developing IL tolerant enzymes, as well as in designing "enzyme-friendly" ILs, with good cellulose-dissolving capability, recyclability and technically and economically feasible production routes.

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PUBLICATION IV

Cellulose hydrolysis and binding with *Trichoderma reesei* Cel5A and Cel7A and their core domains in ionic liquid solutions

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Cellulose Hydrolysis and Binding With *Trichoderma reesei* Cel5A and Cel7A and Their Core Domains in Ionic Liquid Solutions

Ronny Wahlström, Jenni Rahikainen, Kristiina Kruus, Anna Suurnäkki

VTT - Technical Research Centre of Finland, P.O. Box 1000 FI-02044 VTT, Espoo, Uusimaa, Finland; telephone: +358-40-02-54-073; fax: +358-20-72-27-071; e-mail: ronny.wahlstrom@vtt.fi

ABSTRACT: Ionic liquids (ILs) dissolve lignocellulosic biomass and have a high potential as pretreatment prior to total enzymatic hydrolysis. ILs are, however, known to inactivate cellulases. In this article, enzymatic hydrolysis of microcrystalline cellulose (MCC) and enzyme binding onto the cellulosic substrate were studied in the presence of cellulose-dissolving ILs. Two different ILs, 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP) and 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO), and two mono-component cellulases, *Trichoderma reesei* cellobiohydrolase Cel7A and endoglucanase Cel5A, were used in the study. The role and IL sensitivity of the carbohydrate-binding module (CBM) were studied by performing hydrolysis and binding experiments with both the intact cellulases, and their respective core domains (CDs). Based on hydrolysis yields and substrate binding experiments for the intact enzymes and their CDs in the presence of ILs, the function of the CBM appeared to be very IL sensitive. Binding data suggested that the CBM was more important for the substrate binding of endoglucanase Cel5A than for the binding of cellobiohydrolase Cel7A. The CD of Cel7A was able to bind well to cellulose even without a CBM, whereas Cel5A CD had very low binding affinity. Hydrolysis also occurred with Cel5A CD even if this protein had very low binding affinity in all the studied matrices. Binding and hydrolysis were less affected by the studied ILs for Cel7A than for Cel5A. To our knowledge, this is the first systematic study of IL effects on cellulase substrate binding.

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KEYWORDS: cellulase; carbohydrate-binding module; hydrolysis; substrate binding; ionic liquid; protein-carbohydrate interaction

Correspondence to: R. Wahlström
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Introduction

Total hydrolysis of lignocellulosic biomass is currently a very active research area, aiming at the sustainable production of biofuels and chemicals. Enzymatic total hydrolysis of the main polysaccharides in plant biomass produces monosaccharides which can be further converted to products such as ethanol, butanol, lactic acid, and fatty acid ethyl esters by microbial fermentations (Bokinsky et al., 2011; Hofvendahl and Hahn–Hägerdal, 2000). The main bottleneck of industrial-scale processing is the recalcitrance of lignocellulosics towards enzymatic hydrolysis. Thus, efficient and economical pretreatment methods are needed. Many different physical, chemical, and biological pretreatment methods have been proposed, though all of them suffer from their own drawbacks (Chandra et al., 2007; Shill et al., 2011). Some ionic liquids (ILs), defined as salts with melting points below 100°C (Sun et al., 2011), are able to dissolve cellulose and have during the last decade gained significant interest in biomass pretreatment prior to hydrolysis. Swatloski et al. (2002) were the first to demonstrate that cellulose can be dissolved in imidazolium-based ILs, whereafter the dissolution of native lignocellulosic biomass, even wood, has been reported (Kilpeläinen et al., 2007). Dissolution of microcrystalline cellulose (MCC) in IL followed by regeneration by the addition of an anti-solvent, has been shown to significantly increase the kinetics of enzymatic hydrolysis (Dadi et al., 2006, 2007). To omit the regeneration step, Kamiya et al. (2008) proposed a procedure where enzymatic hydrolysis of cellulose is carried out in the same vessel as its regeneration from IL, without removing the IL between the steps. This one-pot procedure suffers, however, from the fact that enzymes hydrolyzing cellulose are inactivated in the presence of high concentrations of IL (Turner et al., 2003).

For efficient total hydrolysis of cellulose, several cellulase activities are needed. According to the classical hydrolysis theory (Bhat and Bhat, 1997), endoglucanases (EC 3.2.1.4) catalyze random hydrolysis along the amorphous regions of the cellulose chains, whereas cellobiohydrolases (exoglucanases, EC 3.2.1.91 and 3.2.1.176) hydrolyze the crystalline cellulose regions either from the reducing or non-reducing

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end, liberating cellobiose as their main product. β-Glucosidases (EC 3.2.1.21) finally hydrolyze the released soluble cellooligomers to glucose. Cellulases are different in their structure and their mode of action but very few studies comparing the IL tolerance of the different cellulase main categories have been published. In a recent study, the effect of IL on the hydrolytic action of monocomponent *Trichoderma reesei* endoglucanases, cellobiohydrolases and *Aspergillus niger* β-glucosidase was evaluated (Engel et al., 2012). Based on these studies, β-glucosidases appear to be particularly sensitive towards ILs. As cellulases are inactivated by ILs to different degrees, cellulase cocktails have been optimized specifically for their use in IL containing matrices (Engel et al., 2012; Park et al., 2012).

ILs have been proposed to have harmful effects on cellulase action through their properties including: high ionic strength and viscosity of the hydrolysis medium (Engel et al., 2010) and high basicity in aqueous solutions (Engel et al., 2010; Li et al., 2012; Wahlström et al., 2012). In addition to these factors cellulase inactivation probably proceeds through specific molecular effects or IL-induced unfolding of proteins (Engel et al., 2010). ILs with hydrophilic anions are more inactivating for enzymes than those with hydrophobic anions (Kaar et al., 2003) which, however, are not generally reported to dissolve cellulose. IL induced enzyme inactivation has been shown to be either reversible or irreversible (Kaar et al., 2003).

T. reesei cellulases, also used in commercial cellulase preparations, are the most extensively studied cellulolytic enzymes. Of the *T. reesei* cellobiohydrolases, Cel7A hydrolyses the cellulose from the reducing chain end and Cel6A from the non-reducing chain end and both hydrolyses cellulose in a processive manner (Teeri, 1997). Endoglucanases catalyze cellulose chain hydrolysis randomly through cycles of adsorption and desorption (Linder and Teeri, 1996). *T. reesei* endoglucanases have their catalytically active site in a cleft on the protein surface (Teeri, 1997), whereas the active site of *T. reesei* cellobiohydrolases is tunnel-shaped (Divne et al., 1994; Rouvinen et al., 1990). Many fungal endoglucanases and cellobiohydrolases have a two-domain structure consisting of a core domain (CD) and a carbohydrate-binding module (CBM), which are linked through a heavily *O*-glycosylated peptide linker (van Tilbeurgh et al., 1986). The major endoglucanases (Cel5A, Cel7B) and both cellobiohydrolases (Cel6A, Cel7A) of *T. reesei* all have a two-domain structure with a CD linked to a family 1 CBM (Linder et al., 1995b). The CBM has been proposed to have multiple roles in the hydrolysis of cellulose: to increase the concentration of cellulase close to the substrate (van Tilbeurgh et al., 1986), to target the CD to specific sites on the substrate (Carrard et al., 2000; Fox et al., 2013) and to disrupt the crystalline structure of the substrate (Arantes and Saddler, 2010; Din et al., 1994). The presence of CBMs has a great impact on the hydrolysis of solid substrates, but does not affect the hydrolysis rates of soluble substrates (van Tilbeurgh et al., 1986). Recent results also show that the presence of CBM does not affect the actual turnover number of Cel7A on solid cellulose and both the CD and the intact enzyme proceed along a cellulose chain with a similar speed (Igarashi et al., 2009; Jalak and Väljamäe, 2010). Recently it was shown, that the CD of *T. reesei* Cel7A is equally efficient in high solid content hydrolysis as the intact cellulase, suggesting that the CBM only improves hydrolysis at low solid contents (Várnai et al., 2013). Both the CBM and the CD bind to the substrate, but the binding affinity of the CBM is much higher (Palonen et al., 1999; Ståhlberg et al., 1991).

Family 1 CBMs are small wedge-shaped domains with a rough and a flat face (Kraulis et al., 1989). CBM interaction with crystalline cellulose surfaces takes place through three aromatic amino acid residues aligned on the flat face of the CBM (Linder et al., 1995b). In the CBM of *T. reesei* Cel7A all three aromatic residues are tyrosines, whereas in the CBM of Cel5A one of the tyrosines is replaced by a tryptophan. Furthermore, there are one asparagine and one glutamine residue on the flat face, which are conserved in the studied family 1 CBMs, and suggested to form hydrogen bonds with cellulose. The CBM of Cel7A binds completely reversibly to cellulose (Linder and Teeri, 1996), whereas the binding of the CBM of the other *T. reesei* cellobiohydrolase, Cel6A, does not show complete reversibility (Carrard and Linder, 1999), even though they both belong to family 1 CBMs (Linder et al., 1995b). Replacement of a single tyrosine with a tryptophan on the flat binding face of Cel7A CBM was shown to significantly increase CBM affinity to cellulose (Linder et al., 1995a). Thus, large differences in cellulose binding behavior can be expected even when the CBMs are closely related.

Up to date, little data has been published regarding how ILs affect the function of CBMs and cellulase binding to cellulose. Pottkämper et al. (2009) carried out mutations on the CBMs of several bacterial cellulases and based on activity measurements concluded that the CBM may play a key role for cellulase performance in the presence of ILs. We have also previously observed that the presence of low concentrations 20% (v/v) of the IL 1,3-dimethylimidazolium dimethylphosphate [DMIM]DMP did not affect the efficiency of *T. reesei* Cel5A CD in MCC hydrolysis, whereas the intact Cel5A carrying a CBM suffered a drastic decrease in hydrolysis yield as compared to hydrolysis in buffer (Wahlström et al., 2012). In this article, we report how the presence of two cellulose-dissolving imidazolium-based ILs, [DMIM]DMP, and 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO), affects the hydrolysis of MCC by *T. reesei* Cel5A and Cel7A (formerly known as endoglucanase II and cellobiohydrolase I, respectively) and their CDs. Furthermore, the effect of ILs on the binding on MCC of these intact *T. reesei* cellulases and their CDs is elucidated for the first time.

Materials and Methods

Chemicals and Enzymes

[DMIM]DMP (>98%) and [EMIM]AcO (>98%) were purchased from Ionic Liquid Technologies (IoLiTec,

Heilbronn, Germany). For the preparation of 3,5-dinitrosalicylic acid (DNS) reagent solution according to Sumner (1924), DNS and potassium sodium tartrate tetrahydrate were acquired from Merck (Darmstadt, Germany). [^{3}H] $NaBH_4$ (100 mCi, 10.9 Ci/mmol) and Ultima Gold^{TM} XR liquid scintillation cocktail were purchased from PerkinElmer (Boston, MA) and 37% formaldehyde solution with 10–15% methanol added as preservative was from Sigma–Aldrich (Steinheim, Germany). Microcrystalline cellulose (MCC, research grade, particle size 0.020 mm) was purchased from Serva Electrophoresis Gmbh (Heidelberg, Germany). The dry weight of MCC was determined as the mass loss for triplicate samples when kept at 105°C overnight.

T. reesei Cel5A, Cel5A CD, and Cel7A CD were produced, isolated, and purified as described in Suurnäkki et al. (2000) and *T. reesei* Cel7A according to Rahikainen et al. (2013). Endoglucanase activity was determined on carboxymethyl cellulose (CMC) as described in Wahlström et al. (2012). Cellobiohydrolase activity was determined on 4-methylumbelliferyl-β-D-lactoside with an assay based on the method in van Tilbeurgh et al. (1988). The purity of the enzyme preparations was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Samples were run in precast Tris–HCl gradient gels (4–20%, Bio-Rad, Hercules, CA) and visualized with a Criterion stain-free imaging system (Bio-Rad Laboratories, Inc.) in which protein visualization is based on a UV-light driven reaction of tryptophan residues in the presence of trichlorocompounds (Kazmin et al., 2002). The molar concentrations of the monocomponent cellulase preparations were determined based on their absorbance at 280 nm using the following molar extinction coefficients (ε): Cel5A 91,000 $M^{-1}cm^{-1}$; Cel5A CD 77,000 $M^{-1}cm^{-1}$; Cel7A 83,000 $M^{-1}cm^{-1}$, and Cel7A CD 80,000 $M^{-1}cm^{-1}$ (Palonen et al., 1999).

Enzymatic Hydrolysis of MCC in IL Matrices

Enzymatic hydrolysis of MCC (1% w/w suspension) was carried out by *T. reesei* Cel5A, Cel5A CD, Cel7A, and Cel7A CD in matrices containing [DMIM]DMP or [EMIM]AcO (0–50% w/w) in 0.050 M citrate buffer. MCC (30 mg by dry weight) was weighed in a test tube, and buffer was added followed by the addition of IL under mixing. The total amount of liquid was 3 g. The hydrolysis was started by the addition of enzyme (dosage 400 nM, corresponding to 1.6–2.2 mg protein per g of MCC). The hydrolysis temperature was 45°C and hydrolysis time 2 or 72 h. The hydrolysis was ended by boiling the samples for 600 s, whereafter the samples were centrifuged 2,500 rpm for 10 min, and the supernatants were collected for analysis. The amount of reducing sugars in the supernatant was determined by DNS assay according to the IUPAC protocol (Ghose, 1987) with the DNS reagent solution prepared as described by Sumner (1924).

Enzyme Binding to MCC in IL Matrices

Radiolabeling of Enzymes Through Reductive Methylation

T. reesei Cel5A, Cel5A CD, Cel7A, and Cel7A CD were labeled with tritium through reductive amination with formaldehyde and tritium-enriched [^{3}H]$NaBH_4$ based on a method by Means and Feeney (1968) with the following modifications: the reaction buffer had a pH of 8.5, [^{3}H]$NaBH_4$ was added as solution in 0.01 M NaOH and the reaction time was 60 min. After the reaction, the reaction mixture was eluted twice in 1 mL fractions through an Econopac® 10 DG gel column (Bio-Rad Laboratories, Inc.). The protein containing fractions were pooled, concentrated, and the buffer was changed to 0.050 M citrate buffer (pH 5.0). All buffer exchanges and enzyme concentrations were carried out using 6 mL Vivaspin centrifugal concentrators with polyethersulfone (PES) membranes with a molecular weight cut-off of 5,000 Da (Sartorius Stedim Biotech GmbH, Göttingen, Germany). The final concentration procedure also served as purification of the ^{3}H-labeled enzyme preparation, as some residual radioactive material moved to the permeate in the first concentration with very little radioactivity detected in the second permeate.

The specific radioactivity of the ^{3}H-labeled enzyme preparation was calculated by determining the radioactivity of the preparation through liquid scintillation counting (LSC, see Binding experiments to MCC with ^{3}H-labeled cellulases and liquid scintillation counting) and dividing the value with the molar protein concentration. The specific radioactivities were 1.7, 2.0, 2.2, and 2.4 Ci/mmol for *T. reesei* Cel5A, Cel5A CD, Cel7A, and Cel7A CD, respectively. The specific hydrolytic activity of the labeled cellulases was unaffected by the labeling reaction. The labeled cellulase preparations were also analysed by SDS-PAGE, which indicated that no degradation of the cellulase preparations had taken place (Supplementary Fig. 1 and 2).

Binding Experiments to MCC with ^{3}H-Labeled Cellulases and Liquid Scintillation Counting

Binding experiments were carried out in 1% (w/w) MCC suspension in 0.050 M citrate buffer (pH 5.0) dispersions with added [DMIM]DMP or [EMIM]AcO (0, 20, and 40% w/w) and initial cellulase concentrations of 0.1–10 μM. Prior to the experiment, the ^{3}H-labeled cellulase preparations were mixed in a 1:50 dilution ratio with unlabeled cellulase, which allowed detection of at least 0.1 μM enzyme concentration in solution. In the binding experiments, the MCC (dosed in 2% w/w suspension), buffer and IL in a total weight of 0.250 g were mixed in 2 mL polypropene microtubes and cooled to 4°C followed by addition of the cellulase preparation. An equilibration time of 4 h, which was checked to be enough for equilibration in all the studied matrices at 4°C, was used. 4°C was chosen as binding temperature to suppress any enzymatic hydrolysis of the MCC, which would lead to changes in the cellulose morphology and amount and

seriously complicate comparison between binding results in the different IL solutions. The experiment was stopped by centrifuging the tubes 8,000 rpm for 4 min, followed by separating the supernatant from the MCC. All procedures were carried out in environments thermostated to 4°C. The cellulase binding was calculated based on the amount of unbound cellulase in the supernatant as quantified by LSC, by comparing samples with MCC to reference samples without MCC. For LSC, aliquots of supernatant was mixed with Ultima Gold™ XR liquid scintillation cocktail and the counts per minute were determined with a liquid scintillation counter (Tri-Carb 2810 TR, PerkinElmer) with a 15 min counting time. All binding experiments were carried out in triplicates. Isotherms were plotted based on the calculated bound enzyme per gram of MCC against the concentration of free enzyme at equilibrium. The maximal binding level (B_{max}, expressed as μmol/g MCC) and the equilibrium dissociation constant (K_d, expressed as concentration units) were estimated by fitting the data to a hyperbolic, one site binding model (Equation 1) using the GraphPad Prism® 4 software.

$$\text{Amount of bound enzyme} = \frac{B_{max} \times C_{free}}{C_{free} + K_d} \qquad (1)$$

where c_{free} denotes the concentration of unbound enzyme in the supernatant.

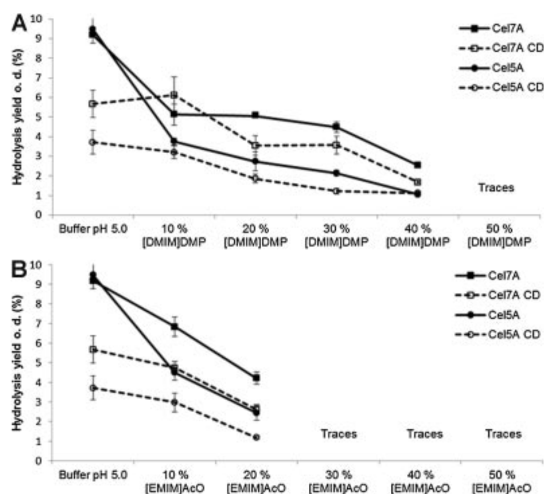

Figure 1. Yields of 72 h hydrolysis of MCC at 45°C with *Trichoderma reesei* Cel5A, Cel5A CD, Cel7A and Cel7A CD, in citrate buffer matrices containing **A**: 0–50% [DMIM] DMP or **B**: 0–50% [EMIM]AcO. Hydrolysis yields were measured by DNS assay, with a limit of quantification of 1% yield. Detectable hydrolysis yields below 1% have been designated as Traces. o. d. denotes of dry weight (of the substrate).

Results and Discussion

Hydrolysis of MCC with *T. reesei* Endoglucanase Cel5A and Cellobiohydrolase Cel7A and their CDs in IL Matrices

The enzymatic hydrolysis of MCC with *T. reesei* Cel5A and Cel7A and their CDs was carried out for 2 or 72 h. The hydrolysis yield was quantified by analyzing the reducing sugars liberated in hydrolysis by the DNS assay, having a limit of quantification (LOQ) of 1% hydrolysis yield in the used setup. In the 2 h hydrolysis Cel5A was the only enzyme producing over 1% yields, with yields of 1.5%, 1.3%, and 1.1% in buffer, 10% (w/w) [DMIM]DMP, and 10% (w/w) [EMIM]AcO, respectively (results not shown). The studied ILs do not dissolve MCC at IL concentrations below 80% under the experimental conditions applied here, as has been shown in Wahlström et al. (2012) by light transmission experiments.

Results of the 72 h hydrolyses of MCC in [DMIM]DMP and [EMIM]AcO containing matrices are presented in Fig. 1A and B. As expected from previous studies (Datta et al., 2010; Kamiya et al., 2008; Wahlström et al., 2012), an increasing concentration of IL led to decreasing hydrolysis yields. [EMIM]AcO (Fig. 1B) had a stronger inhibiting effect than [DMIM]DMP (Fig. 1A) on the studied cellulases in matrices containing over 10% (w/w) of IL, which is in accordance with our previous studies (Wahlström et al., 2012). All the studied cellulases had hydrolysis yields above 1% in up to 40% (w/w) [DMIM]DMP. Cel5A and Cel5A CD had clearly lower yields than their Cel7A counterparts and Cel5A was the cellulase most affected by the presence of both ILs. In [EMIM]AcO all four cellulases produced over 1% yields in up to 20% (w/w) IL, but the yields were under the LOQ for matrices containing 30% (w/w) [EMIM]AcO or more. By comparing the 2 and 72 h hydrolysis results it can be concluded that Cel5A was more efficient than the other cellulases in the beginning of the hydrolysis, whereas Cel7A (and Cel7A CD in most matrices) produced higher yields in the longer 72 h hydrolysis.

The effect of increasing the [DMIM]DMP concentration on the yield in MCC hydrolysis was different for the intact cellulases and their CDs (Fig. 1A). The intact cellulases Cel5A and Cel7A both had higher yields in buffer than their corresponding CDs. This was expected as over 50% yield losses have been reported in MCC hydrolysis with low substrate concentrations when the CBM has been removed from *T. reesei* cellobiohydrolases (Tomme et al., 1988). When changing the matrix from buffer to 10% (w/w) [DMIM] DMP, a drastic decrease in hydrolysis yield was observed in the case of the intact cellulases, being 43% for Cel7A, and 60% for Cel5A. Thereafter, when further increasing the IL concentration only moderate decreases in the hydrolysis yields were observed. The hydrolysis yields of the Cel5A CD and Cel7A CD were, however, evenly decreased by each

increment of [DMIM]DMP in the matrix, being on the same level as for intact cellulases in the [DMIM]DMP matrices. These results suggest that the CBM does not promote the hydrolysis in the presence of [DMIM]DMP. A similar effect of [DMIM]DMP addition on the yield of MCC hydrolysis has previously been reported with *T. reesei* Cel5A and its CD (Wahlström et al., 2012).

Surprisingly, MCC hydrolysis yields with Cel7A were differently affected in [EMIM]AcO than in [DMIM]DMP. Cel7A did not suffer a drastic decrease in yield when adding 10% (w/w) of [EMIM]AcO and the yield decreased quite evenly for each increment of [EMIM]AcO (Fig. 1B). The higher hydrolysis yield of 6.8% with Cel7A in 10% (w/w) [EMIM]AcO as compared to 5.1% in 10% (w/w) [DMIM] DMP was unexpected, as [EMIM]AcO in higher concentrations was more harmful to cellulase performance than [DMIM]DMP. In the case of Cel5A, a 53% decrease in the hydrolysis yield was observed when going from buffer to 10% (w/w) [EMIM]AcO (Fig. 1B), as was observed with [DMIM] DMP. The addition of [EMIM]AcO decreased the hydrolysis yields of both Cel7A CD and Cel5A CD similarly as those of the intact Cel7A. Unlike in [DMIM]DMP, the hydrolysis yields of intact enzymes were higher than those of the CDs in the presence of [EMIM]AcO (Fig. 1B). It appears, that the influence of the CBM on the hydrolysis yield is IL dependent.

Binding of Intact and Core Domain Cellulases to MCC in IL Solutions

Cellulase Binding to MCC in Buffer

The substrate binding of *T. reesei* Cel7A, Cel7A CD, Cel5A, and Cel5A CD was followed by determining binding isotherms under standardized conditions. The radiolabeled enzymes were incubated with MCC at 4°C for 4 h after which the solid cellulose was removed and the concentration of unbound enzyme was measured from the liquid phase by liquid scintillation counting. The four studied cellulases clearly showed different binding behavior to MCC in buffer solution (0.050 M citrate, pH 5.0) (Fig. 2). The two intact cellulases, Cel5A and Cel7A, both exhibited similar binding to MCC. Cel7A CD, lacking CBM, also bound rather well to MCC even though the binding was not as high as for the two intact cellulases. Cel5A CD, on the other hand, had only minor binding to MCC in buffer. Thus, the CBM appeared to be very important for the binding of Cel5A to cellulose, whereas Cel7A was able to bind significantly to cellulose even without CBM. The reason for this difference between the binding of the CDs could be in the different structures of their catalytically active sites, which in cellobiohydrolases (Cel7A) is a tunnel and in endoglucanases (Cel5A) an open-site cleft (Teeri, 1997). With the cellulose bound in the active site tunnel of Cel7A the need for a CBM for efficient binding appears not to be so pronounced as with Cel5A. The tunnel-shaped active site of Cel7A has previously been suggested to cause the tight binding of *T. reesei* Cel7A

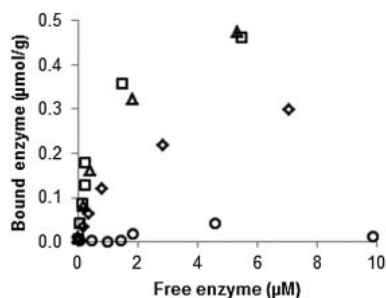

Figure 2. Binding isotherms for *Trichoderma reesei* Cel5A(Δ), Cel5A CD(○), Cel7A(□), and Cel7A CD(◇) on MCC in 0.050 M citrate buffer (pH 5.0) at 4°C.

to cellulose in buffer (Linder and Teeri, 1996). Intact Cel7A have been shown to have the highest binding affinity to cellulose, while the binding affinity is lower for isolated CBMs and even lower for isolated CDs (Palonen et al., 1999). The binding of Cel7A CD through the active site tunnel has also previously been proposed by Kotiranta et al. (1999) who studied *T. reesei* cellulase binding to steam-pretreated willow. Cellulase binding appears, however, to be much dependent on the substrate and applied conditions based on previous studies (Kotiranta et al., 1999; Palonen et al., 2004).

Cellulase Binding to MCC in Aqueous Ionic Liquid Solutions

The binding isotherms of *T. reesei* Cel7A, Cel7A CD, and Cel5A clearly showed that the enzyme binding to MCC was reduced by the presence of ILs (Fig. 3A–C). The binding of Cel5A CD to MCC was very low in all matrices and the influence of IL could not be assessed (results not shown). For all the studied cellulases, [EMIM]AcO affected the binding more than [DMIM]DMP and increasing the IL concentration of the binding matrix solution led to lower cellulase binding to MCC, similarly to how the hydrolysis yields were affected by ILs. The binding of Cel5A was clearly more sensitive to the presence of IL than Cel7A and Cel7A CD. In 40% (w/w) [DMIM]DMP, some binding of Cel5A still occurred, whereas 40% (w/w) of [EMIM]AcO completely prevented Cel5A from binding to MCC (Fig. 3A). Cel7A and Cel7A CD were able to bind to MCC even in the highest IL concentrations in the studied matrices (Fig 3B and C). The binding of Cel7A CD was relatively much less affected by the presence of ILs than the binding of the intact cellulases in terms of isotherm shape and B_{max} value (Fig. 3C and Table I). The binding isotherm of Cel7A CD was even alike in buffer and in the 20% (w/w) [DMIM]DMP matrix. Based on the results of the binding studies in IL matrices, the following conclusions could be drawn: The binding ability of *T. reesei* cellulase CBMs

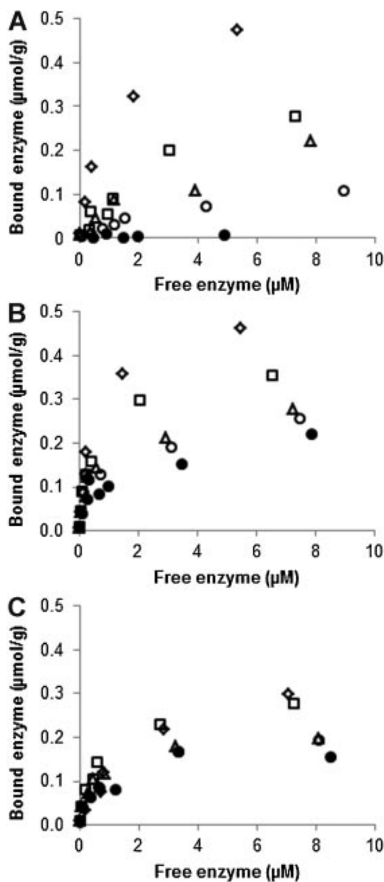

Figure 3. Binding isotherms for **A**: *Trichoderma reesei* Cel5A, **B**: *T. reesei* Cel7A and **C**: *T. reesei* Cel7A CD in 0.050 M citrate buffer (pH 5.0) containing 0% IL (w/w) (◇), 20% (w/w) [DMIM]DMP (□) or [EMIM]AcO (Δ), or 40% (w/w) [DMIM]DMP (◯) or [EMIM]AcO (●) at 4°C.

is very sensitive to the studied ILs. Cel5A relies to a high extent on its CBM for substrate binding, whereas Cel7A substrate binding takes place efficiently via both the CD and the CBM. Interestingly, the binding of Cel7A CD is not very IL sensitive.

The binding of the CBM to cellulose happens mostly through hydrophobic interactions between the three hydrophobic amino acid residues on the flat face of the CBM (Linder et al., 1995b). It has been shown that the addition of organic solvents to the binding matrix decreases the binding affinity of cellobiohydrolase CBMs (Carrard and Linder, 1999), probably due to interference of the solvent with the hydrophobic interactions. The interference of the hydrophobic interactions by ILs may be one reason for the reduced cellulase binding to MCC observed in this study. In Carrard and Linder (1999), the effect of matrix pH on CBM (from *T. reesei* Cel6A and Cel7A) binding was found to be less than 10% between pH 2.5 and 11. Both [DMIM]DMP and [EMIM]AcO are basic in buffer solution, but the pH of 40% (w/w) [DMIM]DMP or [EMIM]AcO in citrate buffer does not exceed 6 or 7.5, respectively, reported in Wahlström et al. (2012). Thus, it is assumed that the basicity of the ILs as such would not cause large changes to the CBM binding to cellulose. Due to different temperatures used in binding and hydrolysis experiments, binding and hydrolysis results cannot directly be correlated, but it could be expected that the substrate binding of the cellulases is even more interfered by the ILs at higher temperatures than at 4°C. Finally, ILs have been proposed to induce conformational changes to enzymes leading to inactivation (Sheldon et al., 2002), and a similar effect of the ILs used in this work on the CBM is possible, which would also lead to lower binding affinity. The observed decrease in the cellulases' cellulose binding and hydrolysis performance caused by the ILs might be due to both the interference of the ILs with the hydrophobic interactions mediating substrate binding and conformational changes. The conformational changes and protein unfolding caused by ILs should clearly be studied in the future. However, method development may be necessary to carry out such studies in IL environments, as ILs have been reported to cause significant baseline problems in many common methods used for studying enzyme conformation in solution, such as circular dichroism (CD) (Sandoval et al., 2012).

Effect of ILs on B_{max} and K_d of Cellulase Binding to MCC

The maximal binding level ($B_{\max}$) and equilibrium dissociation constant (K_d) for the binding isotherms were evaluated by fitting the equilibrium points to a hyperbolic function for one site binding using nonlinear regression (Table I). $B_{\max}$ describes the maximal amount of bound enzyme or alternatively the available binding sites on the sorbent, that is, the isotherms plateau value, and K_d is the concentration of free enzyme where the binding corresponds to half the $B_{\max}$. K_d is thus a measure of binding affinity (a small K_d indicates high affinity). For *T.reesei* Cel5A, Cel7A, and Cel7A CD, the $B_{\max}$ values decreased with increasing IL concentrations. For Cel7A CD, the IL effect on $B_{\max}$ was less pronounced than on the intact enzymes. For Cel5A, the K_d values increased with increasing IL concentration, indicating less binding affinity. Interestingly, the calculated K_d value decreased with increasing IL concentration compared to buffer for Cel7A and Cel7A CD, which was partly in conflict with the general inspection of the binding isotherms. As K_d is directly dependent on $B_{\max}$, K_d values from different experiments should, however, be compared with care.

Conclusions

The effect of IL on cellulose hydrolysis and substrate binding with *T. reesei* monocomponent cellulases and their CDs was studied systematically for the first time. Our results show that

Table I. Estimated values for the maximal binding level (B_{max}) and the equilibrium dissociation constant (K_d) for *Trichoderma reesei* Cel5A, Cel7A, and Cel7A CD in 0.050 M citrate buffer (pH 5.0) containing 0, 20, or 40% (w/w) of IL.

Matrix	Cellulase	B_{max}	St. error	K_d	St. error	R^2
Buffer	Cel5A	0.58	0.02	1.26	0.11	0.99
	Cel7A	0.51	0.02	0.64	0.06	0.99
	Cel7A CD	0.39	0.02	2.25	0.26	0.98
20 % [DMIM]DMP	Cel5A	0.45	0.05	4.41	0.93	0.95
	Cel7A	0.37	0.01	0.47	0.06	0.97
	Cel7A CD	0.23	0.01	0.67	0.08	0.97
40 % [DMIM]DMP	Cel5A	0.16	0.04	4.43	2.04	0.78
	Cel7A	0.24	0.01	0.39	0.06	0.94
	Cel7A CD	0.21	0.01	0.85	0.11	0.98
20 % [EMIM]AcO	Cel5A	0.37	0.09	6.19	2.74	0.90
	Cel7A	0.28	0.01	0.49	0.06	0.96
	Cel7A CD	0.21	0.01	0.49	0.06	0.96
40 % [EMIM]AcO	Cel5A	Does not converge (very low binding affinity)				
	Cel7A	0.22	0.02	0.99	0.201	0.91
	Cel7A CD	0.17	0.01	0.65	0.18	0.85

St. Error denotes the estimated standard error for the B_{max} and K_d values.

both the hydrolysis of MCC with *T. reesei* Cel5A and Cel7A and their binding to MCC are severely interfered by the presence of ILs ([DMIM]DMP and [EMIM]AcO), but to different degrees depending on the structure of the cellulase. Both hydrolysis and binding were found to be more severely interfered by ILs with intact cellulases than with their CDs, suggesting that ILs highly affect the function of the CBM. The CBM was clearly more important for Cel5A substrate binding than for the substrate binding of Cel7A and accordingly Cel5A substrate binding was also much more sensitive to the presence of IL. [EMIM]AcO had a stronger detrimental effect on both hydrolysis and substrate binding for all the cellulases as compared to [DMIM]DMP. The decreasing hydrolysis yields in IL matrices cannot be attributed only to lower binding affinities. Substrate binding took place even in matrices with high IL concentrations in which very low hydrolysis was observed. As was observed for Cel5A CD, high binding affinity is not always necessary for hydrolysis. It is suggested that the different binding affinities and the IL sensitivity of binding of Cel5A and Cel7A is dependent also on the structure of the catalytically active site and its substrate binding zone, which in addition to the CBM is the second site on the enzyme where substrate binding occurs. As ILs appear to affect especially the action of modular cellulases and their CBMs, it may be beneficial to concentrate on cellulases consisting of only a CD for the future screening of IL compatible cellulases.

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PUBLICATION V

The effect of 1-ethyl-3-methylimidazolium acetate on the enzymatic degradation of cellulose

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The effect of 1-ethyl-3-methylimidazolium acetate on the enzymatic degradation of cellulose

Gerald Ebner[a,1], Philipp Vejdovszky[a,1], Ronny Wahlström[b], Anna Suurnäkki[b], Michael Schrems[a], Paul Kosma[a], Thomas Rosenau[a], Antje Potthast[a,*]

[a] University of Natural Resources and Life Sciences, Vienna, Department of Chemistry, Muthgasse 18, A-1190 Wien, Austria
[b] VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Espoo, Finland

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ABSTRACT

The effect of cellulose pre-treatment with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc) on the enzymatic hydrolysis with a cellulase from *Trichoderma reesei* was studied. Enzymatic assays were performed with three different pulps – cotton linters, sulfite dissolving pulp, and eucalyptus Kraft pulp. The reaction kinetics were determined by two different methods: (i) the classical test based on the measurement of released reducing sugars from a water soluble cellulosic substrate (carboxy methyl cellulose (CMC)), and (ii) a novel approach where the enzymatic activity is determined as function of the molecular weight decrease of underivatized cellulose. Furthermore the impact of the pure ionic liquid on the stability of the enzyme was investigated. We found that enzymatic degradation of cellulose I followed a completely different degradation pattern in the molecular weight distribution and sugar solubilzation compared to the corresponding regenerated cellulose II.

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1. Introduction

Fossil resources as the common raw materials for energy production and for chemical industries, are limited. Their energetic usage, i.e. burning, is the prime cause of carbon dioxide enrichment in the atmosphere. These negative aspects of the fossil starting materials, along with their prices steadily rising, make it understandable why renewable materials gain increasing interest. Due to its dominance with regard to both mass and available processing technologies, cellulose, the most abundant natural polymer [1], appears to be the logical alternative to successively replace fossil resources as raw material. Lignocellulosic biomass, which is not utilized for food production, is mainly composed of cellulose (35–50%), accompanied by hemicellulose (20–35%) and lignin (10–25%) [2]. These three biopolymers are assembled in the natural composite material "wood", which has been thoroughly optimized by nature toward strength, endurance and permanence. Separation of the components is consequently rather difficult and laborious. On one side, cellulose is the basis of established industrial branches, such as the pulp, paper and fiber industries as well as more recent cellulose material applications. On the other side, many biorefinery approaches rely on cellulose to be degraded to glucose and small cellooligosaccharides ("saccharification"), which can be fermented to a variety of products, such as ethanol, propanol, acetic acid, biogas, or bioenergy, or be further converted to platform chemicals, e.g. furfural and furan. Cellulose can be degraded in the presence of hemicellulose and lignin with comparably low yields. Alternatively, the three wood constituents are at least partly separated from each other prior to cellulose degradation, which normally gives better outcomes. The saccharification of cellulose is said to be the key step [3,4] in most biorefinery scenarios. Currently, two procedures are technically applied to hydrolyze cellulose. The first is the chemical cleavage of the 1,4-glycosidic bonds between the anhydroglucose units with the aid of mineral acids, usually sulfuric acid or hydrochloric acid, at elevated temperature and pressure, i.e. an acid-catalyzed hydrolytic cleavage. These processes suffer from relatively low yields and from the formation of significant amounts of byproducts that may interfere with further fermentation [5,6]. Furthermore they are quite cost-intensive since they require input of considerable amounts of energy and the use of corrosion-resistant production lines. The second approach is the more environmentally friendly, less energy-demanding hydrolysis of cellulose with cellulolytic enzymes produced from fungi or bacteria. The enzymatic hydrolysis is usually carried out in aqueous media at low temperature and generates less undesirable byproducts

* Corresponding author. Tel.: +43 147654 6071; fax: +43 147654 6059.
E-mail addresses: gerald.ebner@boku.ac.at (G. Ebner), philipp.vejdovszky@boku.ac.at (P. Vejdovszky), Ronny.wahlstrom@vtt.fi (R. Wahlström), anna.suurnakki@vtt.fi (A. Suurnäkki), paul.kosma@boku.ac.at (P. Kosma), thomas.rosenau@boku.ac.at (T. Rosenau), antje.potthast@boku.ac.at (A. Potthast).

[1] These authors contributed equally to this paper.

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[2]. However, also this strategy leads to low yields relative to the theoretical amount of glucose obtainable. The reason for this is the low accessibility of cellulose in many lignocellulosic materials. To tackle this problem, several methods of pretreatment have been developed. The aim of these pre-steps is to remove lignin and hemicellulose, reduce cellulose crystallinity and increase porosity and specific surface of the lignocellulosic material, while avoiding the formation of byproducts [7], which results in an overall improved accessibility of cellulose and thus higher glucose yields. The methods of pretreatment can be sub-classified into biological, chemical, physico-chemical and physical processes.

In recent years several research groups have successfully addressed the refining of lignocellulosic biomass prior to an enzymatic degradation of cellulose by means of a pretreatment with ionic liquids (ILs) [4,5,8–10]. Ionic liquids are molten salts, consisting of an organic cation together with a usually inorganic anion, comprising unique characteristics, such as negligible vapor pressure, chemical and thermal stability, non-flammability and high dissolving power [11]. A number of ILs are reported to be suitable as cellulose solvents [12–14], some are even able to completely dissolve wood [9,10,15]. This makes ILs a promising tool for pretreating woody biomass. The IL 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc), for example, was used to extract lignin from wood flour and decrease cellulose crystallinity – hence improving cellulose degradability to more than 90%[16]. The same IL was applied to dissolve wood and rye straw flour for subsequent precipitation of cellulose – which was this way separated from lignin – by addition of an antisolvent, such as methanol or water [9,15]. In this context, dissolution of celluloses in ionic liquids was shown to causes a significant decrease in the molecular weight of the polymer. This degradation is caused by several factors, such as high temperature during dissolution, side reactions triggered by degradation products of ILs, and the direct reaction with imidazolium-type ILs with reducing ends and other carbonyls in celluloses [17]. However, a loss in the degree of polymerization of cellulose is rather advantageous for further conversion to mono- and oligosaccharides as long as it does not chemically alter the cellulose. But ILs also exhibit disadvantages, such as their enzyme-inactivating effect [3,18–25] the difficulty of purifying them, and their rather high price.

ILs have been studied with regard to their qualification as reaction media to host biotransformations [26,27], especially in terms of hydrolase-catalyzed reactions (lipases and cellulases). In some cases ILs have been applied neat, in others they have been used in a mixture with aqueous buffer solutions. They have been shown to be an expedient alternative to conventional solvents, and to cause an enhancement in reaction rate, compared to aqueous buffer systems. The combination of ionic liquids and cellulase enzymes appeared to be an obvious and attractive option for cellulose processing in biorefineries. It would allow conducting the pretreatment of the cellulose containing biomass and its subsequent hydrolysis with enzymes in a one batch process and thus simplify saccharification of cellulose. Several accounts report about cellulose hydrolysis in a mixture of IL and aqueous buffer [3,21,28,29]. Hydrophobic ionic liquids were better tolerated by cellulases than hydrophilic ones; however, incubation of cellulases as well as lipases in pure ILs resulted in a complete inactivation of the enzymes after a certain time [20]. While 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) turned out to cause irreversible inactivation and unfolding of cellulase from *Trichoderma reesei* [18], [EMIM]OAc, a powerful cellulose and wood solvent [30], was reported to cause slow inactivation of the enzyme. The enhancing effect of increased cellulose availability caused by IL pretreatment overcompensated the reduced enzyme activity in subsequent hydrolysis [3,4,20,21,28]. The same cellulase was immobilized on a resin and thus showed increased stability in pure 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([BMIM]NTf_2) compared to an aqueous buffer. Thereby it became possible to perform enzymatic saccharification of cellulose in ionic liquid solution [23]. Another successful attempt to degrade cellulose in 80% [EMIM]OAc at 90 °C was achieved by employing heterologously expressed cellulase from a hyperthermophilic origin [31]. With tris(2-hydroxyethyl)methylammonium methylsulfate (HEMA) as the solvent (99%), cellulase from *Aspergillus niger* was used to saccharify cellulose with 70% of enzymatic activity measured on aqueous solution [32].

In the present study we were looking at enzymatic degradation of cellulose in ILs, but from the viewpoint of the cellulose – which had not been considered so far – and not from the viewpoint of enzymes as in previous studies. We investigated the depolymerization of cellulose by cellulase after cellulose regeneration from [EMIM]OAc solution. This system was compared with the enzymatic reaction on cellulose I in aqueous suspension. For the first time the enzymatic activity was determined as a function of chain cleavage events, which can be calculated from the molar mass of the residual cellulose, instead of quantifying the soluble low-molecular mass carbohydrate fragments. We used this method to characterize the enzymatic activity, or in other words to measure the deactivating power of the deployed ionic liquid on the cellulase. It was also tested whether cellulase is able to function in highly concentrated [EMIM]OAc with catalytic amounts of water, and eventually the influence of cellulose purity and morphology on enzymatic degradation with different cellulose pulps. We hope that this study – with its focus on cellulose and its integrity under different degradation conditions – will be seen as a useful complementation of previous work that mainly focused on such cellulose degradation systems from the enzyme point of view.

2. Materials and methods

2.1. Cellulosic substrates

In this study, cellulose from three different sources was used. The characteristics of these cellulose samples are given in Table 1.

The molecular weight distribution (MWD) of these three pulps as measured by GPC in the solvent system *N*,*N*-dimethylacetamide (DMAc)/LiCl is given in Fig. 1.

2.2. Solvents and reagents

All solvents and reagents, purchased from Sigma–Aldrich in the highest purity available, were used without further purification.

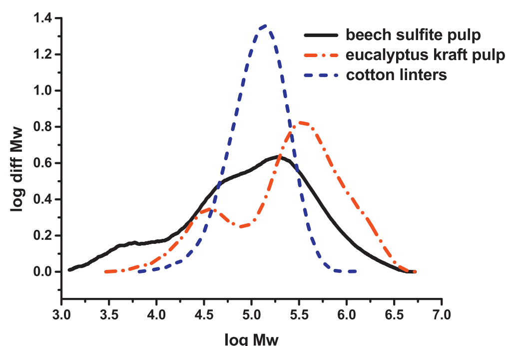

Fig. 1. Molecular weight distribution of the three types of cellulose used in the study: beech sulfite dissolving pulp, eucalyptus Kraft pulp, and cotton linters.

Table 1
Cellulose characteristics.

Origin	Type of pulp	Mn/Mw/Mz (kg mol^{-1})	*Kappa*-number	Crystallinity (%)	Content of hemicellulose (%)
Beech	Sulfite dissolving	26.7/250.2/862.3	~0.8	49	xyl 3.20/man 0.20
Eucalyptus	Kraft paper-grade	85.6/471.7/112.8	6.1	49	xyl 4.22/man 0.52
Cotton	Cotton linters	90.0/145.2/212.8	0.1	67	<1

The ionic liquid [EMIM]OAc was kindly provided by BASF Ludwigshafen, Germany (Basionic TM BC 01). The water content of the [EMIM]OAc was determined by Karl Fischer titration and was 7000 ppm. The enzyme used was a cellulase from *T. reesei* (ATCC 26921, C8546-10KU, lyophilized) obtained from Sigma–Aldrich, Schnelldorf, Germany. Although the supplier states that the product is a pure β-1,4-endoglucanase it is in fact a typical cellulase cocktail secreted by *T. reesei*, hence we call it cellulase.

2.3. Dissolution of cellulose and incubation of the enzyme in [EMIM]OAc

For dissolving cellulose the desired pulp was torn into pieces of about 1 mm in lateral length and then transferred into a 50 ml round-bottom flask. Then 0.05 ml [EMIM]OAc per mg of air-dried pulp (~2%, w/w) was added. In kinetic experiments usually 200 mg of air-dried pulp was mixed with 10 ml of ionic liquid. The flask was immediately sealed in order to avoid accumulation of moisture from the surrounding atmosphere. The cellulose was dissolved by heating the suspension to 110 °C with the aid of an oil bath and was mechanically stirred for 17 h. After dissolution, the clear cellulose solution was cooled to 40 °C in a water bath. Once the temperature was reached, for every mg of dry pulp 0.085 mg of cellulase was added and the solution was well mixed with a stirrer. After defined time intervals, samples of 1 ml were taken with a syringe and immediately transferred into water or sodium citrate buffer (10–20 ml). The presence of excess water causes sudden precipitation of cellulose from solution. In some experiments it was necessary to inactivate the enzyme, which was done by adjusting the pH of the mixture to 12 and boiling for five minutes. Simple boiling for 5 min was not sufficient to deactivate the enzyme.

2.4. Measurement of enzymatic activity

The activity of the cellulase after incubation in [EMIM]AcO was monitored in two different ways. The conventional way of determining the enzymatic activity is to use a soluble cellulose derivative (instead of cellulose) which is degraded by a cellulase to photometrically detectable reducing end groups which can be monomers or oligomers. Aliquots of the incubation mixture were taken and diluted by 0.05 M citrate buffer (pH 5.0) to give appropriate absorbances. The activity of the enzyme was measured by a modified version of the hydroxyethyl cellulose (CMC) assay [33]: a 1% (w/v) carboxy methyl cellulose (CMC) substrate solution in 0.05 M sodium citrate buffer at 50 °C was used. The measurement time was 10 min and the reaction was terminated by adding 1,5-dinitrosalicylic acid (DNS) reagent solution (prepared according to Sumner and Noback [34]) to the samples and boiling for 5 min. Afterwards the samples were cooled and their absorbances at 540 nm were measured, which covers all the reducing sugars formed. The [EMIM]OAc was found to give some background absorbance, which was corrected by background subtraction (blank samples containing the same dilution of completely inactivated incubated enzyme solution).

In a second approach, we determined the enzymatic activity by monitoring the molecular weight decrease of the cellulose substrate subjected to cellulose degradation. For that purpose the cellulose–[EMIM]OAc–enzyme mixture was transferred into an excess amount of water under vigorous stirring. The resulting suspension was kept at 40 °C while the precipitated cellulose was allowed to be degraded by the residual active enzyme for a certain time span. Then samples of the mixture were taken and vacuum-filtrated, to remove the supernatant. The residual cellulose was washed with 1 M Na_2CO_3 buffer (pH 12.2) and then prepared for GPC measurement to analyze its molecular weight distribution and the respective statistical molecular weight moments.

2.5. Enzymatic hydrolysis in buffer solution

200 mg of beech dissolving pulp was dispersed in 10 ml of a 0.05 M citrate buffer at pH 5.0 in a 25 ml flat-bottomed glass bottle. The dispersion was kept under constant magnetic stirring in a water bath at 40 °C. 17 mg of cellulase was added and aliquots (1 ml) of hydrolyzed pulp dispersion were taken after 0, 5, 10, 15, 30, 60, 90, 120, 150 min and 1 and 2 days of reaction time. The cellulose was separated from the supernatant by centrifugation and the cellulase was inactivated by washing the samples with 1 M Na_2CO_3 (pH 12.2) solution. The hydrolysis was stopped by heating the samples in a block heater at 98 °C for 10 min. After centrifugation, the sugar content of the supernatant was determined by the DNS assay [33] and a qualitative carbohydrate analysis was carried out by capillary electrophoresis with pre-column derivatization as described earlier [35]. The remaining solid cellulose residues were subjected to GPC analysis.

2.6. Determination of the molecular weight distribution

Excess of water from cellulose samples was removed by vacuum-filtration and the remaining cellulose was thoroughly washed, first with water, then with ethanol (96%) and finally by DMAc, in order to remove all residual water. In the next step, the cellulose pellets were transferred into vials, 4 ml of DMAc was added and the resulting suspensions were shaken at room temperature for 24 h. Excess DMAc was removed by filtration and 2 ml of DMAc/LiCl (9%, w/v) was added. After 24 h of shaking at room temperature, the samples were completely dissolved. The resulting solutions were diluted with pure DMAc (1:1, v/v) and filtered through a 0.2 μm PTFE syringe filter. These solutions were directly used for GPC analysis.

The GPC measurement was performed with the following equipment: four serial PL-gel mixed A LS columns, 20 μm, 7.5 mm × 300 mm; column oven: Gynkotek STH 585; eluent: *N*,*N*-dimethylacetamide containing 0.9% LiCl, filtered through a 0.02 μm PTFE-filter; degasser: Dionex DG-2410; pump: Kontron pump 420; autosampler: HP1100; light scattering detector: multi-angle laser light scattering (MALLS) detector Wyatt Dawn DSP with argon ion laser (λ = 488 nm); RI-detector: Shodex RI-71; operating conditions: flow rate: 1.00 ml/min, injection volume: 100 μl, room temperature, 45 min run time.

2.7. Activity measurement by cellulose degradation

For measuring the enzyme activity by GPC, the pulp was dissolved in [EMIM]OAc and the resulting solution was cooled to 40 °C

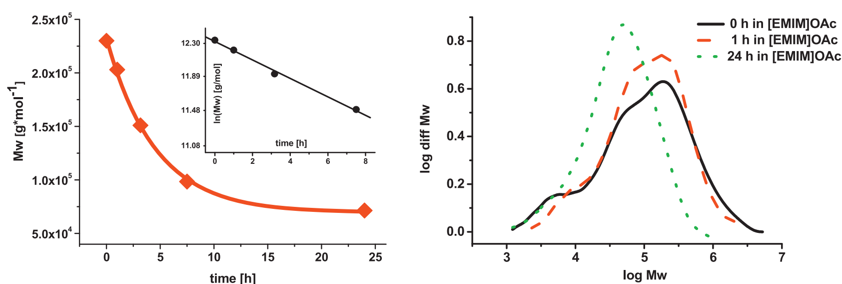

Fig. 2. Left: degradation of a sulfite dissolving pulp at 110 °C in pure [EMIM]OAc IL (without enzyme). Insert: first order rate law. Right: the corresponding molecular weight distribution of the starting material, after 1 h and after 24 h.

and kept at this temperature (different amounts of water were added to the cellulose solution in the neat, water-free IL in order to see the effect of water, water concentrations in the samples were 0.74% (7400 ppm) and 1.21% (12,100 ppm), respectively). Then, cellulase was added to the solution and the mixture was kept at 40 °C and homogenized. After defined time intervals, aliquots were taken and transferred into an excess amount of water. The obtained aqueous suspension was kept at room temperature under mechanical agitation for 4 h, after which a possible enzymatic activity was eliminated by increasing the pH to a value >12. The Mw-values of the samples after these 4 h of reaction time could then be related to the different incubation times, i.e. the time the enzyme was exposed to the concentrated ionic liquid. A relationship between the water concentration in the ionic liquid and the rate of enzyme inactivation could be established in this way.

Calculation of kinetic constants: The enzymatic degradation of cellulose by a cellulase obeys an exponential function, i.e., it follows a first-order reaction law. In order to compare the enzymatic activity of cellulase with different substrates it is advantageous to determine the actual number of chain scissions over a certain period of time. This can be done by calculating the ratio of cleaved glycosidic bonds relative to the total glucose units of a cellulose chain, the so called scission-faction of cellulose units or SFCU (Eq. (1)) [36]:

$$SFCU = \frac{1}{DP} - \frac{1}{DP_0} \quad (1)$$

DP_0 represents the initial degree of polymerization and DP the degree of polymerization after a given reaction time, with DP evidently being a smaller number. The DP value is calculated directly from the weight average molecular weight (Mw) by dividing this value in g mol^{-1} by 162.14 g mol^{-1}, which is the molecular weight of an anhydroglucose unit (AGU). A very common method is based on a slight adaption of the expression of the SFCU in order to obtain a linear relationship with reaction time (Eq. (2)), assuming first-order reaction kinetics [37]:

$$\ln\left(1 - \frac{1}{DP_0}\right) - \ln\left(1 - \frac{1}{DP}\right) = kt \quad (2)$$

3. Results and discussion

3.1. Degradation of cellulose upon dissolution in the IL

In order to distinguish between a loss in Mw catalyzed by the enzyme and a potential loss originating from the dissolution procedure, we initially looked at the effect of the relatively harsh conditions during dissolution of the pulp on the integrity of the cellulose. The dissolution was accomplished by adding pulp to the pure IL and heating the mixture to 110 °C for 17 h. After defined time intervals aliquots of the solution were taken, and the molecular weight of the dissolved cellulose was determined. This allowed for tracing possible changes in the molecular weight distribution of the pulp over time. Since no enzyme was present, these changes could be unambiguously assigned to the dissolution procedure and the medium. The results of these experiments are given in Fig. 2 for the beech sulfite pulp, as an example. It was evident, that the dissolution procedure had a significant degrading effect on the pulp, which is in agreement with an earlier study by Schrems et al. [15]. The Mw of the pulp decreased within 24 h from about 230.000 g mol^{-1} to less than 80.000 g mol^{-1}, just by the action of the ionic liquid at elevated temperatures. The corresponding changes of the molecular weight distribution are shown in Fig. 2 left.

Similarly the effect of the pure ionic liquid on the molecular weight of the cellulose was investigated at 40 °C, since this was the temperature used in experiments where the enzyme was incubated in the [EMIM]OAc–cellulose solution. Therefore, a solution of sulfite dissolving pulp in [EMIM]OAc was prepared under the conditions described above (110 °C, 17 h) and was then kept at 40 °C without addition of cellulase. Samples were taken after defined time intervals and the molecular weight distribution of the dissolved cellulose was measured. The respective results, illustrated in Fig. A 1, provided solid proof that under these conditions the dissolved cellulose is not further degraded by [EMIM]OAc. Even after a period of 48 h the molecular weight did not significantly deviate from the initial value of the sample taken directly after the dissolution period. Thus, while cellulose is strongly degraded during dissolution at 110 °C, it remains stable and its integrity is fully maintained when kept at 40 °C in pure [EMIM]OAc solution. This allowed for the assignment of any further changes in the Mw to the action of the enzyme, since other degrading effects under these conditions were ruled out.

3.2. Impact of the pure ionic liquid on the stability of the cellulase

In the next step, we studied the influence of pure [EMIM]OAc on the stability of the cellulase of *T. reesei*. Therefore, again a cellulose solution (sulfite dissolving pulp) in pure [EMIM]OAc was prepared. After cooling to 40 °C, lyophilized enzyme was directly added and the mixture was further incubated at 40 °C. Aliquots were taken after defined time intervals, and put into an excess amount of water at RT in order to regenerate the cellulose and to start the enzymatic degradation (provided any enzymatic activity was left). After 4 h of reaction in aqueous medium, the enzyme was inactivated by addition of 2.5 M Na_2CO_3 solution and the enzymatic activity was calculated as a function of the decrease of the molecular weight during these 4 h. The results were then related to the incubation time during which the enzyme had been exposed to

Table 2
Specific cellulase activity toward cellulose.

Incubation time in [EMIM]OAc (h)	Decrease of the molecular weight (%/h)	Specific activity of the cellulase (%/(h mg enzyme))	Relative specific activity (%)
0	21.6	1.6	100.0
1	16.4	1.2	76.0
3	14.8	1.1	68.3
6	8.7	0.6	40.0
24	0.0	0.0	0.0

pure [EMIM]OAc and had partly lost its activity. This approach was based on the assumption that no enzymatic catalysis can take place in the pure [EMIM]OAc–cellulose solution, which is indeed the case as demonstrated in other experiments (see below).

In contrast to the commonly used activity measurement with HEC or CMC [33], this approach allows to measure the activity of the cellulase toward its natural substrate (cellulose) and addresses possible effects of the IL toward the enzyme reaction. The Mw data were converted into %-values, defining 100% as the initial value before the reaction started. Similarly, the enzymatic activity was also converted into %-values, arbitrary setting 100% as the activity of the enzyme not exposed to the concentrated ionic liquid, and, trivially, 0% as no activity. Fig. A2 and Table 2 summarize the respective results.

The data obtained are in good agreement with other reports [3,21,22,24], demonstrating a deactivating effect of the pure IL on the enzyme. The actual time after which the enzyme was completely inactivated can be estimated under the assumption of a linear activity decrease, as shown in Fig. A2. The results demonstrate that the cellulase was not inactivated immediately by the pure IL, but rather lost its activity progressively within 10–11 h after which catalytic activity had completely disappeared.

3.3. The role of water in the system cellulose – enzyme – [EMIM]OAc

The role of water in different IL on cellulase activity has already been covered in the literature [3,18,20–23,31,32] From the IL tested in that respect, EMIM[OAc] has a rather strong deactivating effect on the enzyme, leaving roughly half of the catalytic activity, compared to e.g. when [EMIM]DEP is used at the same concentrations. Zhao et al. verified this strong deactivating effect of [EMIM]OAc at 17 and 34% and reported the complete inactivation at 51% IL content, based on measurements of the amount of reducing sugars after 48 h of enzymatic reaction on cellulose [22].

Our above-described stability tests of cellulase in [EMIM]OAc demonstrated that the inactivation of the enzyme in a highly concentrated IL solution proceeded relatively slow (within about 10 h). This is in line with successful attempts from other groups that used the same enzyme in different ILs at high concentration. This posed the question whether the cellulase from *T. reesei* can even act in pure [EMIM]OAc environment, provided that there is a certain minor amount of water available which is required for the glycosidic bond cleavage, and how the stability/activity of the enzyme is affected by varying water concentrations.

We first analyzed the enzyme stability with the aid of the modified CMC assay (see Section 2). Cellulase was incubated in 100% and 90% aqueous [EMIM]OAc at 40 °C. The enzymatic activity in pure aqueous buffer was set as reference level (100%). The residual activities of the incubated cellulase were compared to this initial level of 357 nkat/mg enzyme. The incubation of cellulase in IL was fairly inactivating as the enzyme was found to lose its activity practically completely after 4 h. The activity level of the enzyme decreased to about 40% after 1 h, to about 16% after 2 h and to <1% after 4 h of incubation, as shown in Fig. 3 left. However, the differences observed between 90% and 100% IL where only minor.

Inactivation in EMIM[OAc] proceeds over time without reaching any equilibrium activity. For the cellulose-dissolving IL 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP), such an activity profile has been shown previously, with incubated cellulase initially losing about 50% of its activity but then remaining stable at constant activity level for several days [24]. However, at this point it has to be mentioned that in this study a different cellulase had been investigated. In our study, the presence of 10% water did not have any significant influence on the enzyme activity and the activity decrease (see Fig. 3). A practically complete inactivation of the used cellulase according to the CMC assay was observed after 4 h of incubation both in neat [EMIM]OAc and in [EMIM]OAc containing 10% of aqueous buffer. After dilution of the samples with buffer (1:1–1:30, RT, 2 days, no agitation) the enzymes did not regain any activity under these conditions which suggests that the inactivation of the enzyme caused by [EMIM]OAc is permanent and irreversible.

3.4. Measurement of cellulase activity directly by degradation of cellulose

The commonly applied activity test for cellulases, i.e. formation of low Mw carbohydrates from water soluble cellulose derivatives like HEC or CMC is not directly addressing the action of the enzyme on cellulose. The number of reducing ends formed may even differ depending on the completeness of degradation to monomers. However, it will still correspond mainly to the endoglucanase activity (cellobiohydrolases are not considered to be active on CMC [43]). Hence we measured the degradation of the substrate directly by GPC and applied the number of chain scissions (SFCU) as a sensitive means to visualize the enzymatic activity (for details see Section 2).

In contrast to the activity determination via CMC, the inactivation kinetics showed a clear dependence on the water amount, indicating that an increased amount of water has a decelerating effect on the enzyme inactivation, as illustrated in Fig. 3. Again this illustrates the superiority of the activity determination via molecular weight, offering the possibility to detect enzymatic catalysis, which is overlooked by the CMC-test. However, after 10 h of incubation time the enzymatic activity practically disappeared in every case, regardless of the water concentration used. For comparative efforts an additional experiment was performed in which the enzyme was incubated at the same conditions, but then immediately deactivated (pH > 12) after regeneration of the substrate. Since the molecular weight of these samples was constant, and thus independent from the incubation time, a catalytic activity in the highly concentrated [EMIM]OAc could be ruled out.

Besides measuring the direct effect on cellulose, differences in structure and purity of the cellulosic substrates are accessible through this approach, which would otherwise not be detected.

3.5. Comparison of enzymatic hydrolysis of native cellulose to regenerated cellulose

A number of publications emphasized the higher hydrolytic susceptibility of cellulose in a form other than cellulose I, e.g. after dissolving and subsequent regeneration (e.g. cellulose II) [3–5,9,10,16,21,22,24,25,28,29]. In order to demonstrate the effect

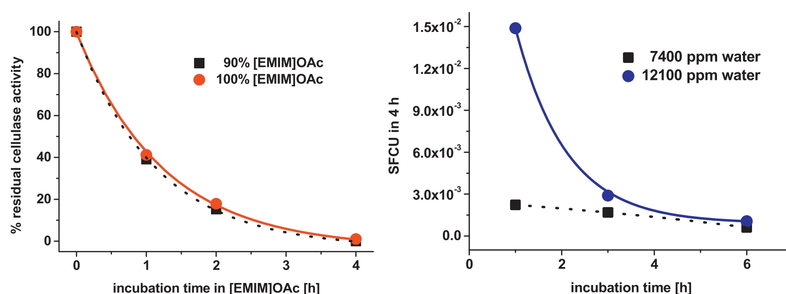

Fig. 3. Left: inactivation curves for *T. reesei* cellulase after incubation in 90% or 100% [EMIM]OAc (340 U/g pulp). Right: number of chain scissions of cellulose (SFCU) on sulfite pulp after incubation in [EMIM]OAc with different amounts of water, 7400 ppm and 12,100 ppm water respectively (340 U/g pulp).

of the pre-treatment of cellulose with [EMIM]OAc, the action of the cellulase on cellulose in aqueous buffer solution was compared to an enzymatic degradation of cellulose directly after regeneration from [EMIM]OAc solution. In the case of the saccharification in a buffer solution, the enzyme caused a fast hydrolysis of the cellulose as was determined by DNS colorimetric assay [33]. The hydrolysis settled at a final level of about 75% of solubilized sugars, as can be seen from Fig. 4. The residual pulp is inaccessible to the enzymes as the hydrolysis reaches its plateau level. In this enzymatic hydrolysis of pulp, glucose and cellotriose were the main products and cellobiose was a minor product. The product distribution, however, was not static. In the beginning of the hydrolysis (after 5 min) cellotriose was the dominating product whereas after 2 days of hydrolysis, when the hydrolysis had reached its plateau state, glucose was by far the most abundant product. This does to some extent influence also the outcome the DNS assay.

A prominent feature is the corresponding evolution of the molecular weight and the molecular weight distribution of the remaining polymers (Fig. 5). For a cellulase one would obviously expect to see mainly endo-cleavages and hence a progressing reduction of the molar mass of the cellulose. This, however, was not the case in the buffer solution. The statistical parameter Mw did almost not change, the distribution showed a slight change in the high molecular weight range accompanied by a mass loss in that region. Even after 75% of the cellulose was hydrolytically degraded to monomers and small oligomers as demonstrated by carbohydrate analysis, the remaining polymeric cellulose fraction was still very similar in its distribution to the corresponding starting material indicative of a layer-by layer mechanism. Du et al. [38] reported a decrease of Mw followed by a slight increase which was not observed in our case. This has certain significance for cellulose processing in enzymatically driven biorefinery setups. Evidently, a cellulose molecule, once attached to the enzyme's active site by the enzyme, is further "chewed up" until degraded to low-molecular weight fragments. The enzyme apparently did not switch between substrate molecules, but finished degradation of those molecules that were already attached before turning to "new" molecules. Similar macroscopic observation have been reported by Penttilä et al. [39] and Zhang et al. [40].

Fig. 6 contrasts the evolution of the molecular weight of native cellulose I and cellulose II, regenerated from [EMIM]OAc upon enzymatic degradation by the cellulase. For the second case two slightly different experiments were carried out. In the first one the enzyme was pre-incubated in the [EMIM]OAc–cellulose solution for 4 h. In the second one the enzyme was added directly after regeneration, hence has never been in contact with the highly concentrated ionic liquid. The significantly lower initial Mw of this cellulose compared to the cellulose used in the buffer experiment above (cellulose I) is a result of the dissolution conditions (20 h at 110 °C in pure [EMIM]OAc), which cause degradation of cellulose, as discussed above.

After addition of the enzyme, cellulose was degraded in a way that produced low-molecular weight fragments (similar to hydrolysis in buffer), but at the same time gradually decreased the molecular weight of the remaining polymer. The molecular weight distribution now showed exactly the expected image of cellulase (endoglucanase) action (see Fig. 6 right): long cellulose chains shortened by random cleavage, leading to a distinct shift

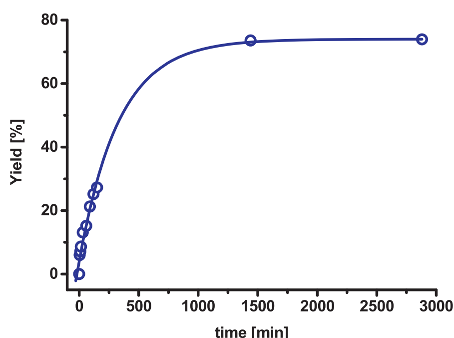

Fig. 4. Glucose release from cellulose I (beech sulfite pulp) in 0.05 M citrate buffer pH 5.0 (340 U/g pulp).

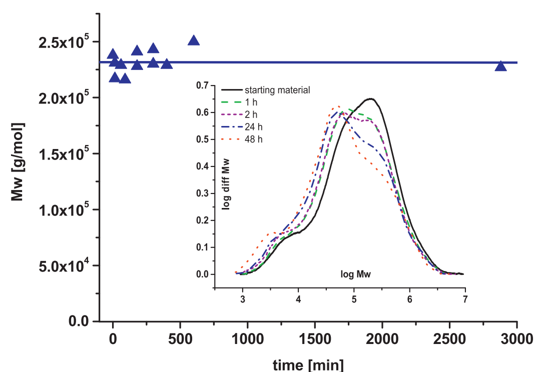

Fig. 5. Evolution of the average molecular weight (Mw) and the molecular weight profile (insert) of the remaining cellulose in buffer pH 5.0 after cellulolytic degradation (340 U/g pulp).

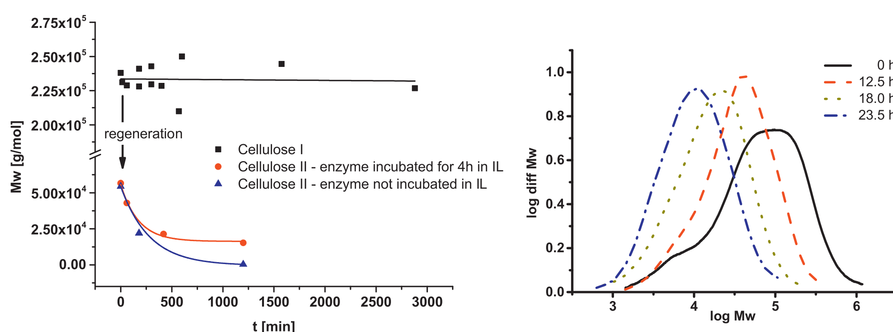

Fig. 6. Left: comparison of the degradation of native cellulose I (upper curve, squares) and cellulose regenerated from IL (cellulose II, circles, triangles) in buffer solution pH 4.8. Right: molecular weight distribution of regenerated cellulose after different times of enzymatic action (340 U/g pulp).

to overall lower molar masses. The results clearly demonstrate that the pre-treatment with [EMIM]OAc significantly enhances the enzymatic degradation kinetics determined as a function of the Mw-decrease of the substrate. When regenerated cellulose is subjected to enzymatic digestion with a fully active enzyme mixture, it is completely hydrolyzed to water soluble carbohydrates overnight. The conversion of native cellulose to soluble sugars at otherwise constant average Mw (see Fig. 6 left) of the residual cellulose seems to be a consequence of the fact that the enzyme clips to the cellulose chain and degrades it one at a time while the residual chains stay untouched. This corresponds to a cellulosic structure with a high order parameter Ω, used to define the preference of endoglucases to absorb on either crystalline or amorphous areas [42]. But according to Fox et al. [41] the difference between crystalline and amorphous regions is not solely responsible for differences in cellulose binding and subsequent degradation. In contrast, regenerated cellulose shows a constant loss in its average Mw (Fig. 6 left), which suggests that the enzyme does not stick to one chain but switches between them during the degradation process.

It was evident from our results, that dissolution of cellulose in IL and re-precipitation short before enzymatic hydrolysis clearly accelerated both the saccharification rate and the saccharification yield. This finding is in agreement with previous studies that focused on the impact of cellulose treatment with ionic liquids on the performance of cellulose degrading enzymes. However, it was shown for the first time that the degradation mechanism is different for cellulose degradation in aqueous buffer systems with and without IL pretreatment. When cellulose was enzymatically hydrolyzed in the aqueous buffer, remaining polymer had a very similar Mw and MWD as the starting material, i.e. a part of the cellulose molecules were completely degraded while others were completely unaffected, and the reaction stopped at about 75% conversion (degree of crystallinity for this pulp: 49%). When cellulose was enzymatically degraded after re-precipitation from IL solution, the Mw of the remaining polymer was progressively decreased (Fig. 6), and the conversion was complete. It might be concluded that there is a different mode of action for the enzyme in degrading native or regenerated cellulose, but surely this effect requires further in-depth studies which are currently underway.

3.6. Role of cellulose purity on cellulase activity

In order to investigate the influence of the cellulose purity on the enzymatic degradation, three pulps, namely beech sulfite dissolving pulp, eucalyptus Kraft pulp and cotton linters, have been subjected to enzymatic hydrolysis and the degradation rates were compared. The different pulps were first pre-treated (dissolved and reprecipitated) with pure [EMIM]OAc and then treated with the same cellulase used in the previous experiments. In order to allow a comparison the mathematical approach described in Section 2 had to be used, since the original Mw of the cellulose varies strongly among these three pulps, and thus the most different initial Mw-drops would have falsified the evaluation.

As illustrated in Fig. 7 there is a clear dependence of the reaction rate on the type of pulp employed. The enzyme displayed the highest activity on cotton linters ($k = 1.36\,\text{min}^{-1}$), followed by the sulfite dissolving pulp ($k = 1.20\,\text{min}^{-1}$) and the eucalyptus Kraft pulp ($k = 1.04\,\text{min}^{-1}$), which had the lowest reactivity of the three tested cellulose sources. It is reasonable to assume that these differences in the degradation rates are a result of the different purity degrees of the pulps used. Among these cotton linters is the most pure one, while the sulfite dissolving pulp and even more the eucalyptus Kraft contain residual amounts of hemicelluloses (~3.5% and ~12–15%, respectively). These impurities can also be seen in the molecular weight distributions, where the hemicelluloses are represented as a shoulder on the low-molecular-weight-end of the curves. The MWD-curve of cotton linters on the other hand displays a completely symmetric shape, indicating the absence of hemicellulose impurities (Fig. 1). These differences in the content of hemicelluloses have an impact on the reaction rate of the enzymatic degradation of the material by lowering the accessibility of the cellulose. This result is important to be kept in mind when working with “real-world” celluloses or with material from biorefineries,

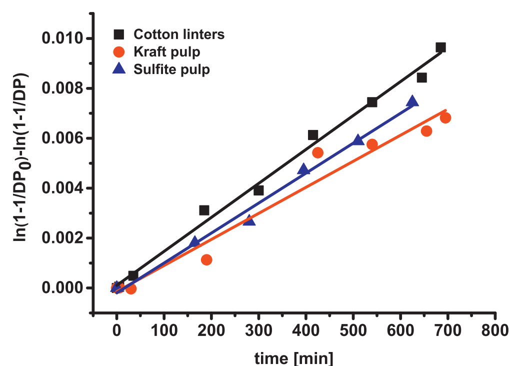

Fig. 7. Comparison of three different pulps – pseudo first order reaction kinetics of the enzymatic degradation of cotton (squares), sulfite pulp (triangles) and Kraft pulp (spheres), with 340 U/g pulp.

since many biorefinery-related studies have been performed with microcrystalline cellulose (Avicell) or cotton linters and thus pure cellulose substrates. These "idealized" substrates do not necessarily reflect the reactivity and reaction rates of cellulose and other cellulosic starting materials coming from conventional pulping or biorefinery processes. Therefore, when developing a process relying on the enzymatic degradation of cellulosic raw materials, one has to consider the potential amount of impurities, in particular hemicelluloses. Hence a method addressing the whole entity of cellulosic materials in enzymatic reactions will provide a more detailed inside on their reactivity.

4. Conclusions

The impact of cellulose pre-treatment with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc) on the reaction kinetics of the enzymatic hydrolysis of different celluloses was studied. The pre-treatment was performed by a complete dissolution of the cellulosic material in the pure ionic liquid, followed by reprecipitation in water. The interpretation of several assays conducted with a sulfite dissolving pulp, an eucalyptus Kraft pulp, and cotton linters, which were subjected to enzymatic degradation with a cellulase from *T. reesei*, led to four main conclusions, which are presented in the following paragraphs:

i. It could be shown that the pure [EMIM]OAc has a deactivating effect on the cellulase, which is in agreement with several earlier reports on this topic [3,21,22,24]. The apparent inactivation kinetics were dependent on the way the enzymatic activity was determined. In this context our approach to calculate the reaction rate on the basis of the molecular weight decrease of underivatized cellulose substrate based on a pseudo-first order rate law, rather than measuring the amount of released reducing sugars from carboxy methyl cellulose (CMC), turned out to be superior. This is supported by the fact that the former method was able to detect residual enzymatic activities after incubation in pure [EMIM]OAc for several hours, where the classical "CMC-test" failed to do so. According to our method, the cellulase from *T. reesei* is progressively and irreversibly deactivated in the pure ionic liquid within a time span of around 10–11 h.
ii. With regard to the incubation conditions of the enzyme in [EMIM]OAc, the concentration of water has a significant impact on the rate of enzyme inactivation, which could be shown by our method of activity determination via molecular weight decrease. Even a very slight increase of the water concentration from 0.74% to 1.21% during incubation in the pure ionic liquid resulted in a significantly higher enzymatic activity on sulfite dissolving pulp at room temperature.
iii. One of the major questions of this study was whether the pre-treatment of cellulosic substrates with [EMIM]OAc has a significant impact on the subsequent enzymatic hydrolysis. We could undoubtedly demonstrate, that cellulose regenerated from the ionic liquid was completely hydrolyzed to water-soluble carbohydrates overnight, while untreated cellulose I displayed a different behavior. While the major amount of the substrate is hydrolyzed to soluble sugars, there is a remaining polymeric fraction of which the mean molecular weight as well as the molecular weight distribution is practically unaltered.
iv. The origin of the employed cellulose, in particular the way it is produced, which results in different amounts of impurities, does have an influence on the enzymatic degradation after ionic liquid pre-treatment. Impurities, especially in the form of residual hemicelluloses originating from the lignocellulosic raw material, decelerate the rate of molecular weight decrease. The three tested pulps could be ordered according to their purity (cotton linters > sulfite dissolving pulp > eucalyptus Kraft pulp). Comparison of the rate constants of enzymatic degradation resulted in the same order, leading to the conclusion, that there is an inverse relationship between hemicellulose amount and enzymatic degradation rate.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.11.001.

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Series title and number

VTT Science 52

Title	**Enzymatic hydrolysis of cellulose in aqueous ionic liquids**
Author(s)	Ronny Wahlström
Abstract	Total enzymatic hydrolysis of the polysaccharides in lignocellulosic biomass to monosaccharides is currently a focus research area. The monosaccharides obtained from lignocellulose hydrolysis can be used for the production of platform chemicals and biofuels, most notably ethanol. One major challenge in the commercialization of lignocellulosic ethanol production is the recalcitrance of lignocellulosics towards enzymatic hydrolysis, necessitating efficient pretreatment of the lignocellulosic feedstock. Certain ionic liquids (ILs, salts with melting points below 100 °C) dissolve cellulose and even lignocellulosic biomass and are as such interesting candidates for pretreatment technology. However, cellulose-dissolving ILs have been found to severely inactivate the hydrolytic enzymes (cellulases) employed in cellulose hydrolysis. This work focuses on elucidating how certain ILs affect the action of cellulases in cellulose hydrolysis. The main emphasis was on the action of purified monocomponent *Trichoderma reesei* cellulases, but some commercial cellulase preparations were also studied in IL matrices. Hydrolysis experiments were made in solutions containing up to 90% of the two cellulose-dissolving ILs 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO) and 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP). The presence of increasing amounts of IL led to decreasing yields of solubilised saccharides in enzymatic hydrolysis. [EMIM]AcO was generally more harmful for cellulase action than [DMIM]DMP. Pure [EMIM]AcO completely inactivated *T. reesei* endoglucanase in 4 h in residual activity measurements, whereas pure [DMIM]DMP supported considerable cellulase activity for at least three days. The cellulase compatibility of several novel classes of cellulose-dissolving ILs were studied in hydrolysis, but these ILs were found to be at least as harmful for cellulase action as the studied imidazolium-based ILs. *T. reesei* endoglucanases were unable to reduce the molecular weight of microcrystalline cellulose (MCC) in buffer or in any aqueous matrix containing IL, except in 90% (v/v) [DMIM]DMP in which the MCC was partially dissolved. The studied ILs were found to have very detrimental effects on saccharide analytics. A capillary electrophoresis (CE) method was developed for the analysis of mono- and oligosaccharides in matrices containing ILs. The cellulase binding to MCC in solutions with [DMIM]DMP and [EMIM]AcO was studied with radiolabeled *T. reesei* Cel5A (endoglucanase II)and Cel7A (cellobiohydrolase I) and their respective core domains. Cel7A was able to bind to MCC with its core domain, whereas it was shown that Cel5A was very dependent on its CBM for efficient substrate binding. [EMIM]AcO interfered more with cellulase substrate binding than [DMIM]DMP. The binding ability of the *T. reesei* carbohydrate-binding modules (CBMs) was very IL sensitive.
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Seriens namn och nummer

VTT Science 52

Namn	**Enzymatisk cellulosahydrolys i vattenhaltiga jonvätskor**
Författare	Ronny Wahlström
Referat	Enzymatisk totalhydrolys av lignocellulosans polysackarider till monosackarider är för tillfället ett mycket aktivt forskningsområde. De sålunda producerade monosackariderna kan användas som råvara vid tillverkningen av plattformkemikalier och biobränslen, av vilka särskilt kan nämnas etanol. En av de största utmaningarna i kommersialiseringen av etanoltillverkning från lignocellulosa är lignocellulosans motståndskraft mot enzymatisk hydrolys, varför effektiva förbehandlingsmetoder är nödvändiga. Vissa jonvätskor (definierade som salt med smältpunkt under 100 °C) löser cellulosa och till och med fullständig lignocellulosa. Jonvätskorna utgör sålunda ett intressant alternativ som förbehandlingsteknologi för lignocellulosa. Jonvätskorna har emellertid i hög grad konstaterats inaktivera de hydrolytiska enzymer, cellulaser, som används i cellulosahydrolys. Detta arbete har haft som målsättning att klargöra hur cellulosalösande jonvätskor påverkar cellulasernas funktion i cellulosahydrolys. I första hand undersöktes hur funktionen hos cellulaser renade till enkomponentpreparat från *Trichoderma reesei*, men också hos kommersiella cellulaspreparat, påverkades i vissa jonvätskelösningar. Hydrolysexperimenten utfördes i lösningar med upp till 90 % jonvätska (1-etyl-3-metylimidazolium acetat ([EMIM]AcO) eller 1,3-dimetylimidazolium dimetylfosfat ([DMIM]DMP)). En ökande mängd jonvätska ledde till avtagande hydrolysutbyten i form av lösliga sackarider i enzymatisk hydrolys. [EMIM]AcO var i allmänhet mer skadlig än [DMIM]DMP för cellulasernas funktion. Ren [EMIM]AcO inaktiverade *T. reesei* endoglukanas fullständigt på mindre än 4 h, medan betydande restaktiviteter mättes efter inkubation i [DMIM]DMP under åtminstone tre dygn. Ett antal nya sellulosalösande jonvätskor, baserade på organiska superbaser studerades i hydrolysexperiment, men dessa jonvätskor konstaterades vara åtminstone lika skadliga för cellulasernas funktion som de imidazoliumbaserade jonvätskorna. *T. reesei*s endoglukanaser kunde inte reducera mikrokristallin cellulosas (MCC) molmassa i buffertlösning eller i någon jonvätskelösning, förutom i 90 % (v/v) [DMIM]DMP, vari MCC partiellt löste sig. De studerade jonvätskorna konstaterades vara mycket skadliga för många av de vanliga metoderna som används i kolhydratanalytik. En kapillärelektroforesmetod utvecklades för att analysera mono- och oligosackarider i jonvätskelösningar. *T. reesei* Cel5A (endoglukanas II), Cel7A (cellobiohydrolas I) och deras respektive katalytiska domäner märktes med radioaktivt tritium och dessa cellulasers förmåga att binda till MCC studerades i lösningar innehållande [DMIM]DMP och [EMIM]AcO. Cel7A kunde binda sig till MCC direkt via sin katalytiska domän, medan det kunde påvisas att Cel5A var ytterst beroende av sin kolhydratbindande modul för att binda till cellulosa. [EMIM]AcO konstaterades påverka cellulasernas bindingsgrad till MCC mer än [DMIM]DMP. Bindningsförmågan hos *T. reesei*s kolhydratbindande moduler konstaterades vara synnerligen känslig för de studerade jonvätskorna.
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Nimeke	**Selluloosan entsymaattinen hydrolyysi vesipitoisissa ioninesteissä**
Tekijä(t)	Ronny Wahlström
Tiivistelmä	Lignoselluloosan entsymaattista totaalihydrolyysiä tutkitaan nykyisin hyvin aktiivisesti. Lignoselluloosassa olevien polysakkaridien hydrolyysistä syntyviä monosakkarideja voidaan käyttää raaka-aineina kemikaalien, polymeerien ja biopolttoaineiden, erityisesti etanolin, tuotannossa. Iso haaste lignoselluloosapohjaisen etanolituotannon kaupallistamisessa on lignoselluloosan monimutkainen rakenne, joka vaikeuttaa entsymaattista hydrolyysiä. Tehokkaiden, lignoselluloosaa avaavien esikäsittelymenetelmien kehittäminen on siis tärkeää. Tietyt ioninesteet, jotka määritellään suoloiksi, joiden sulamispiste on alle 100 °C, liuottavat selluloosaa ja jopa lignoselluloosaa ja ovat näin ollen hyvin mielenkiintoisia käytettäviksi lignoselluloosan esikäsittelyssä. Selluloosaa liuottavien ioninesteiden on kuitenkin todettu inaktivoivan hydrolyyttisiä entsyymejä, sellulaaseja, joita käytetään selluloosan totaalihydrolyysissä. Tässä työssä tutkittiin pääasiassa *Trichoderma reesei* -homeen tuottamien ja puhdistettujen sellulaasien sekä myös joidenkin kaupallisesti saatavien sellulaasituotteiden toimintaa ja inaktivoitumista vesipitoisissa ioninesteliuoksissa. Hydrolyysikokeita tehtiin selluloosalla vesiliuoksissa, joiden ioninestepitoisuus vaihteli; suurimmillaan se oli 90 % (joko 1-etyyli-3-metyylimidatsoliumi asetaatti ([EMIM]AcO) tai 1,3-dimetyylimidatsoliumi dimetyylifosfaatti ([DMIM]DMP)). Kasvavat ioninestepitoisuudet aiheuttivat hydrolyysisaannon pienenemisen selluloosan entsyymaattisessa hydrolyysissä. [EMIM]AcO haittasi enemmän sellulaasien toimintaa kuin [DMIM]DMP. Puhtaassa [EMIM]AcO:ssa *T. reesei*n endoglukanaasi inaktivoitui täysin neljän tunnin käsittelyssä, kun taas aktiivisuus aleni hyvin vähän [DMIM]DMP:ssa kolmen vuorokauden aikana. Useamman uuden superemäspohjaisen, selluloosaa liuottavan ioninesteryhmän sellulaasiyhteensopivuus tukittiin hydrolyysikokeissa, mutta nämä ioninesteet eivät kuitenkaan olleet paremmin yhteensopivia sellulaasien kanssa kuin perinteiset imidatsoliumi-pohjaiset ioninesteet. *T. reesei*n endoglukanaasit eivät pystyneet vähentämään mikrokiteisen selluloosan (MCC) molekyylipainoa puskurissa eivätkä missään muussa ioninestettä sisältävässä liuoksessa, paitsi 90-prosenttisessa (v/v) [DMIM]DMP:ssa, johon MCC oli osittain liuennut. Tutkittujen ioninesteiden havaittiin olevan hyvin haitallisia hiilihydraattianalytiikkamenetelmille. Työssä kehitettiin kapillarielektroforeesimenetelmä mono- ja oligosakkaridien analyysiin ioninestepitoisissa matriiseissa. Sellulaasien sitoutumista MCC:aan tutkittiin radioleimatuilla *T. reesei* Cel5A:lla (endoglukanaasi II), Cel7A:lla (cellobiohydrolaasi I) ja niiden katalyyttisillä domeeneillä puskuriliuoksissa [DMIM]DMP:n ja [EMIM]AcO:n läsnä ollessa. Cel7A pystyi sitoutumaan MCC:aan pelkällä katalyyttisellä domeenillaan, kun taas Cel5A oli hyvin riippuvainen hiilihydraatteja sitovasta modulistaan sitoutuakseen tehokkaasti selluloosaan. [EMIM]AcO vaikutti [DMIM]DMP:a voimakkaammin sellulaasien selluloosaan sitoutumiseen. Yleisesti tutkittujen ioninesteiden todettiin vaikuttavan herkästi *T. reesei*n hiilihydraatteja sitovien moduulien toimintaan.
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Enzymatic hydrolysis of cellulose in aqueous ionic liquids

The rapidly growing global demand for energy and chemical resources, economic and political feedstock security issues and the concern for global warming direct us from the utilization of fossil to renewable feedstocks. Lignocellulosic biomass from different origins is a widely available and currently much underutilized feedstock for the production of e.g. liquid biofuels. Hydrolysis of lignocellulosic polysaccharides results in monosaccharides which can be further transformed into fuels and other chemical products by biotechnological means. The hydrolysis of lignocellulosics is preferably carried out using hydrolytic enzymes, cellulases and hemicellulases, as catalysts. The native lignocellulosic feedstock is, however, highly recalcitrant towards enzymatic hydrolysis and therefore costly pretreatments are needed prior to hydrolysis.

Ionic liquids are salts with low melting points (<100 °C). Some classes of ionic liquids have been noticed to dissolve cellulose, and more recently, to possess the quite unique property to dissolve even native lignocellulosic biomass. Thus, ionic liquids hold a great potential as pretreatment technology prior to enzymatic hydrolysis. It is, however, known that ionic liquids also inactivate cellulases used for cellulose hydrolysis. In this work, the performance of different cellulases in both commercial and non-commercial cellulose-dissolving ionic liquids was studied. The aim of this work was to elucidate the inactivation of cellulases in ionic liquids, study the effect of ionic liquids on the hydrolytic performance of cellulases during long hydrolysis experiments, and elucidate different ionic liquid- and enzyme-related factors affecting the hydrolytic ability of cellulases in the presence of ionic liquids.

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