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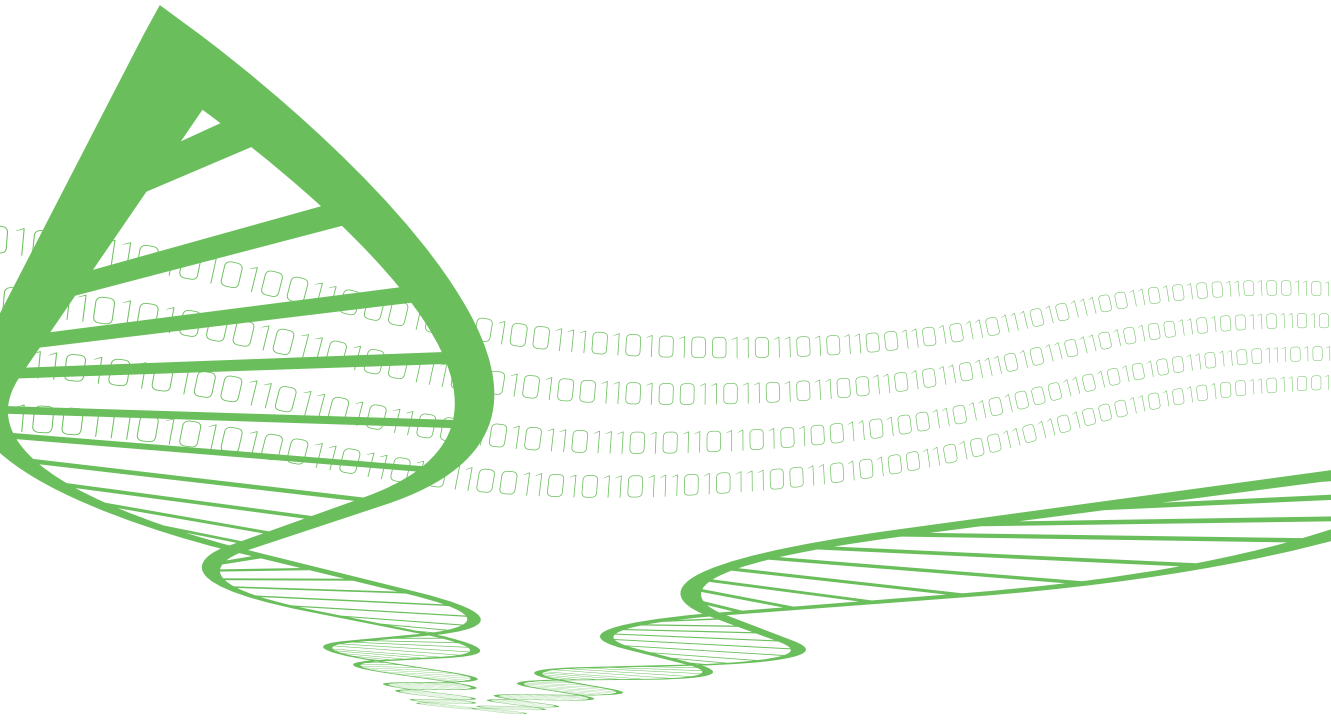
Impact of water content on enzymatic modification of wheat bran

Outi Santala



VTT SCIENCE 59

Impact of water content on enzymatic modification of wheat bran

Outi Santala

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Impact of water content on enzymatic modification of wheat bran

Vesipitoisuuden vaikutus vehnäleseen entsymaattisessa muokkauksessa. **Outi Santala.** Espoo 2014. VTT Science 59. 97 p. + app. 52 p.

Abstract

Enzymatic treatments of plant-based materials are generally conducted in excess water because reduction of water content usually decreases enzymatic conversion. Processing at high solids content would offer economical advantages, but in the area of enzymatic bioprocessing of plant materials for food applications, the role of water content has seldom been studied. Wheat bran is one of the most important by-products of the cereal processing industry and comprises the outer tissues of grain. Bran is a good source of dietary fibre (DF), protein and phytochemicals, but its use in food applications is limited because unprocessed bran is usually detrimental to product quality. The present work aimed to examine and develop techniques to utilize hydrolytic enzymes, especially xylanase, at reduced water content in order to increase the technological functionality of wheat bran in food applications.

The impact of water content on the action of xylanase was studied by treating wheat bran with a commercial xylanase enzyme preparation at water contents ranging from 20 to 92% using different processing methods including both continuous mixing and short pre-mixing combined with stationary incubation. The action of xylanase was measured by the solubilisation of bran arabinoxylan (AX), the main DF polysaccharide of wheat. The minimum required water content for the action of xylanase on wheat bran during continuous mixing was between 20 and 30%, corresponding to a water activity (a_w) of 0.83–0.89. Xylanase action was significantly enhanced at a water content of 40% (a_w 0.93), at which the granular material was transformed to a continuous paste. AX was solubilised at a similar level at 40 and 90% water contents when continuous mixing was used, but at water contents of 50–80% AX solubilisation was lower. Furthermore, it was shown that the use of an extruder for pre-mixing and forming a bran-water mixture increased the action of xylanase during stationary incubation at a water content of ≤54%, as compared to pre-mixing with a blade-mixer. The results indicated that the formation of a continuous paste is important for efficient enzyme action at low water content, and that it is possible to increase the enzyme action by changing the granular structure of the material to a continuous paste using an extruder, without increasing the water content. The extruder-aided pre-mixing process enabled efficient xylanase action at low water content without the requirement for continuous mixing.

Neither water content nor processing method affected the apparent average molecular weight (MW) of water extractable AX (WEAX) precipitated with 65% EtOH at water contents above 40%. When bran was treated with continuous mixing,

the A/X ratio of the bran water extract decreased similarly at both water contents of 40% and 90%, suggesting that AX was solubilised from the same bran tissues regardless of the processing conditions studied. The bran treated at a water content of 40% was characterized by higher solubilisation of DF polysaccharides, smaller average particle size, lower water holding capacity and more changes in bran proteins than the treatment at a water content of 90%. The more intensive changes in the properties of bran treated at low water content were related to the compact consistency and thus higher impacts of shear exerted on the bran-water mixture. Reduction of particle size, either prior to the treatment by grinding or during the treatment by intensive mixing and shear, was shown to enhance AX solubilisation and xylanase action, presumably due to improved substrate availability as a result of increased surface area. Small particle size also favoured the transformation of the bran-water mixture from granular mass to a continuous paste, which also enhanced enzyme action.

The technological functionality of modified bran was demonstrated in endosperm-flour based expanded extrudates supplemented with 20% of bran. Bran was treated with commercial xylanase and cellulase enzymes at a water content of 48% using the extrusion-aided low-water process, followed by oven or freeze drying. The modified bran ingredients increased the crispiness and reduced the hardness and bulk density of the bran-enriched expanded extrudates. The improvements in extrudate properties were attributed to the increased WEAX content and decreased water holding capacity of the modified brans.

The results of the work showed that enzymatic solubilisation of bran AX and improved technological functionality of bran can be achieved by enzymatic modification at a water content of 40–50%, which is well below the point of absence of free bulk water (70–80%). The consistency of the reaction mixture, mixing method and bran particle size were found to be important factors affecting the intensity of the modification process at low water content. The results can be utilized for improving the technological functionality of bran in food applications and for developing new processes for the enzymatic modification of plant raw materials at reduced water content.

Keywords wheat bran, arabinoxylan, xylanase, enzymatic modification, solubilisation, water content, high solids hydrolysis, bran particle size, high torque mixing, stationary incubation, extrusion, mechanical properties, extrudate structure

Vesipitoisuuden vaikutus vehnäleseen entsymaattisessa muokkauksessa

Impact of water content on enzymatic modification of wheat bran. **Outi Santala.**
Espoo 2014. VTT Science 59. 97 s. + liitt. 52 s.

Tiivistelmä

Kasviperäisten materiaalien entsymaattinen muokkaus tehdään yleensä suuressa vesimäärässä, koska vesipitoisuuden vähentäminen useimmiten heikentää entsyymien toimintaa. Teollisissa prosesseissa vesipitoisuuden vähentäminen toisi taloudellisia hyötyjä, mutta toistaiseksi vesipitoisuuden vaikutusta kasvimateriaalien entsymaattisessa muokkauksessa elintarvikesovelluksia varten on tutkittu hyvin vähän. Jyvän kuorikerroksista koostuva vehnälese on yksi viljateollisuuden tärkeimmistä sivutuotteista. Lese on hyvä ravintokuidun, proteiinin ja fytokemikaalien lähde, mutta sen käyttö elintarvikkeissa on hankalaa, koska käsittelemätön lese yleensä heikentää tuotteen laatua. Työn tarkoituksena oli tutkia ja kehittää menetelmiä hydrolyyttisten entsyymien, erityisesti ksylanaasien, käyttämiseen matalassa vesipitoisuudessa vehnäleseen teknologisen toimivuuden lisäämiseksi elintarvikesovelluksissa.

Vesipitoisuuden vaikutusta ksylanaasin toimintaan tutkittiin käsittelemällä vehnälesettä kaupallisella ksylanaasia sisältävällä entsyymiseoksella vesipitoisuuksissa 20–92 %. Työssä käytettiin erilaisia prosessointimenetelmiä, joissa lesettä sekoitettiin inkuboitaessa joko jatkuvatoimisesti tai vain lyhytaikaisesti käsittelyn alussa. Ksylanaasin toiminta mitattiin määrittämällä vehnän tärkeimmän ravintokuitukomponentin, arabinoksylaanin (AX) liukenemista. Vähimmäisvesipitoisuus, jossa ksylanaasi alkoi toimia jatkuvaa sekoitusta käytettäessä, oli 20:n ja 30 prosentin välillä, vastaten veden aktiivisuutta (a_w) 0.83–0.89. Ksylanaasin toiminta tehostui huomattavasti vesipitoisuudessa 40 % (a_w 0.93), jossa koostumukseltaan rakeinen materiaali muuttui yhtenäiseksi plastiseksi massaksi. AX:n liukeneminen jatkuvan sekoituksen prosessissa oli yhtä tehokasta 40:n ja 90 %:n vesipitoisuuksissa, mutta vesipitoisuuksissa 50–80 % AX:a liukeni vähemmän. Lisäksi osoitettiin, että verrattuna lapasekoittimeen lese-vesimassan esisekoitus ekstruuderilla ja sen aikaansaama plastisen massan muodostuminen tehosti ksylanaasin toimintaa ilman sekoitusta tapahtuvassa inkuboinnissa ≤54 %:n vesipitoisuudessa. Ekstruuderiavusteinen prosessi mahdollisti ksylanaasin tehokkaan toiminnan matalassa vesipitosuudessa ilman jatkuvaa sekoitusta. Tulokset osoittivat, että yhtenäisen, plastisen massan muodostuminen on tärkeää tehokkaalle entsyymin toiminnalle matalassa vesipitoisuudessa ja että entsyymin toimintaa on mahdollista tehostaa nostamatta vesipitoisuutta muuttamalla materiaali rakeisesta yhtenäiseksi massaksi ekstuuderin avulla.

Käsittelyn vesipitoisuus tai prosessointimenetelmä ei vaikuttanut 65 %:n etanolipitoisuudessa saostetun vesiliukoisen AX:n (WEAX) keskimääräiseen molekyyli-

painoon yli 40 %:n vesipitoisuudessa. Kun käytettiin jatkuvaa sekoitusta, leseen vesiuutteen arabinoosi-ksyloosisuhde laski samalla tavalla vesipitoisuuksissa 40 ja 90 %, viitaten siihen että liuennut AX oli lähtöisin samoista leseen soluseinän osista riippumatta käytetyistä prosessiolosuhteista. Verrattuna 90 %:ssa käsiteltyyn leseeseen 40 %:n käsittelyn jälkeen lese sisälsi enemmän liukoisia ravintokuitupolysakkarideja, leseen proteiineissa havaittiin enemmän muutoksia ja leseen partikkelikoko ja vedensidontakapasitetti oli pienempi. Suuremmat muutokset matalassa 40 %:n vesipitoisuudessa käsitellyssä leseessä johtuivat todennäköisesti seoksen kompaktista rakenteesta ja siitä johtuvasta suuremmasta leikkausvoimien vaikutuksesta leseeseen. Partikkelikoon pienentäminen joko ennen käsittelyä lesettä jauhamalla tai käsittelyn aikana tehokkaan sekoituksen ja leikkausvoimien vaikutuksesta ja siitä seurannut partikkelien pinta-alan kasvu lisäsi AX:n liukenemista ja ksylanaasin toimintaa, mikä todennäköisesti johtui substraatin saatavuuden parantumisesta. Pieni partikkelikoko myös edesauttoi seoksen rakenteen muuttumista rakeisesta yhtenäiseksi massaksi, mikä myös tehosti entsyymin toimintaa.

Muokatun leseen teknologinen toimivuus osoitettiin endospermijauhopohjaisissa puffatuissa ekstrudaateissa, joissa 20 % jauhoista oli korvattu leseellä. Lesettä käsiteltiin ensin kaupallisilla ksylanaasi- ja sellulaasientsyymeillä 48 %:n vesipitoisuudessa käyttämällä ekstruusioavusteista matalan vesipitoisuuden prosessia, jonka jälkeen lese kuivattiin joko uuni- tai kylmäkuivauksella. Muokatut lesetuotteet paransivat lesettä sisältävien ekstrudaattien rapeutta ja vähensivät niiden kovuutta ja tiheyttä. Ekstrudaattien ominaisuuksien parantumisen katsottiin johtuvan muokattujen leseiden kasvaneesta WEAX-pitoisuudesta ja pienentyneestä vedensidontakapasiteetista.

Tulokset osoittivat, että leseen AX:n entsymaattinen liuottaminen ja leseen teknologisten ominaisuuksien parantaminen on mahdollista tehdä entsymaattisella muokkauksella 40–50 %:n vesipitoisuudessa, joka on selvästi matalampi kuin rajapitoisuus (70–80 %) jossa kaikki seoksen vesi on sitoutuneena leseeseen. Reaktioseoksen fysikaalinen koostumus, sekoitusmenetelmä sekä leseen partikkelikoko todettiin tärkeiksi tekijöiksi, jotka vaikuttavat muokkausprosessin tehokkuuteen matalassa vesipitoisuudessa. Tutkimuksen tuloksia voidaan hyödyntää leseen teknologisen toimivuuden parantamiseen elintarvikesovelluksissa. Tulosten perusteella voidaan myös kehittää uusia entsymaattisia prosesseja kasvimateriaalien muokkaamiseksi matalassa vesipitoisuudessa.

Avainsanat wheat bran, arabinoxylan, xylanase, enzymatic modification, solubilisation, water content, high solids hydrolysis, bran particle size, high torque mixing, stationary incubation, extrusion, mechanical properties, extrudate structure

Preface

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Espoo, May 2014

Outi Santala

Academic dissertation

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Supervising professor	Professor Heikki Ojamo Department of Biotechnology and Chemical Technology Aalto University Espoo, Finland

List of publications

This thesis is based on the following original publications which are referred to in the text as I–IV.The publications are reproduced with kind permission from the publishers.

I Santala, O., Lehtinen, P., Nordlund, E., Suortti, T., & Poutanen, K. (2011). Impact of water content on the solubilisation of arabinoxylan during xylanase treatment of wheat bran. Journal of Cereal Science, 54, 187–194.

II Santala, O., Nordlund, E., & Poutanen, K. (2013). Treatments with xylanase at high (90%) and low (40%) water content have different impacts on physicochemical properties of wheat bran. Food and Bioprocess Technology, 6, 3102–3112.

III Santala, O., Nordlund, E., & Poutanen, K. (2013). Use of an extruder for pre-mixing enhances xylanase action on wheat bran at low water content. Bioresource Technology, 149, 191–199.

IV Santala, O., Kiran, A., Sozer N., Poutanen, K., & Nordlund, E. (2014). Enzymatic modification and particle size reduction of wheat bran improves the mechanical properties and structure of bran-supplemented expanded extrudates. Journal of Cereal Science, in press.

Author's contributions

I The author planned the work together with Prof. Kaisa Poutanen, Dr. Pekka Lehtinen and Dr. Emilia Nordlund, and carried out the experimental work. The author interpreted the results together with the other authors. Dr. Tapani Suortti had the main responsibility for the HP-SEC analysis of arabinoxylan. The author was responsible of writing the publication in cooperation with Prof. Kaisa Poutanen, Dr. Emilia Nordlund and Dr. Pekka Lehtinen.

II The author planned the work together with the other authors and carried out the bran treatments with the help of a trainee (Ninon Piacere). The author carried out the analyses, except for the gas chromatography analyses, and interpreted the results together with the other authors and MSc. Ulla Holopainen (microscopy). The author wrote the publication in cooperation with the other authors.

III The author was responsible for planning the work together with the other authors. The author conducted the bran treatments with the help of a trainee (Anish Kiran) and carried out the analyses except for dietary fibre analyses. The author intreperted the results and wrote the publication in cooperation with the other authors.

IV The author had the main responsibility for planning the work in collaboration with the other authors. The author carried out the bran treatments and the production of the expanded extrudates together with an MSc student Anish Kiran, who performed the analyses with the help of technicians. The author supervised the MSc work of Anish Kiran together with Dr. Emilia Nordlund. The author interpreted the results together with Dr. Emilia Nordlund and Dr. Nesli Sozer and wrote the paper in cooperation with the other authors.

Contents

List of abbreviations

a_w	water activity
AX	arabinoxylan
A/X	arabinose to xylose ratio
AGP	arabinogalactan peptide
C_i	crispiness index
DF	dietary fibre
DM	dry matter
DTT	dithiothreitol
Dv50	median of the volumetric distribution of particle sizes
ER	expansion rate
F_{cr}	crushing force
F_{max}	maximum point of the force-deformation curve
GC	gas chromatography
GH	glycosyl hydrolase
HPLC	high performance liquid chromatography
HP-SEC	high performance size exclusion chromatography
MW	molecular weight
SDS	sodium dodecyl sulphate
WEAX	water extractable arabinoxylan
WBC	water binding capacity
WHC	water holding capacity
WUAX	water unextractable arabinoxylan

1. Introduction

Waste and side streams of agriculture, forestry and the food industry are currently considered as valuable resources for conversion to value added products such as food, feed, fuel and chemicals. These residues include renewable materials such as brans, straws, cobs, grasses and woody biomass, and are mainly composed of plant cell wall constituents, i.e. cellulose, hemicellulose and lignin (Singh nee' Nigam et al. 2009; Modenbach and Nokes 2013). Bran is one of the most important by-products of the cereal industry. Bran comprises the outer layers of grain separated in the milling process during the production of refined flours. High nutritional quality, especially the high content of dietary fibre, protein and phytochemicals, makes bran an interesting raw material for food products, but it is currently under-utilized as a food ingredient due to its technological and sensory challenges. In baked and extruded products bran is known to have an adverse effect on the volume, texture, flavour and appearance of the product (Coda et al. 2014; Lebesi and Tzia 2011; Robin et al. 2011a; Brennan et al. 2008).

Enzymatic conversions have a fundamental role in several industrial food manufacturing processes and in the upgrading of agro-industrial residues with the aim of increasing the efficacy of the production processes and utilization rate of the raw material, and improving the sensory quality of the end product. Hydrolases are the most commonly used enzymes in industrial processes, and depending on the process the aim is either complete or partial degradation of the substrate. Enzymes are also commonly used in the cereal industry. Solubilisation and degradation of cell wall components of bran by bioprocessing with hydrolytic enzymes such as xylanases has shown potential as a means to improve the technological and nutritional properties of the bran in food applications (Coda et al. 2014; Lebesi and Tzia 2011; Mateo Anson et al. 2011; Nordlund et al. 2013).

In current industrial processes, enzymatic reactions are typically conducted in excess water due to technological considerations. In general, reduction of the water content leads to reduced enzymatic conversion. However, increasing interest in reduction of water content as a means of improving the overall economy of industrial processes has promoted research in the area of processing of lignocellulosic materials under high-solids conditions. In the area of enzymatic processing of plant materials for food applications, the role of water content has been studied to a much smaller extent.

1.1 Enzymatic processing of plant-based materials at low water content

1.1.1 Role of water in enzymatic processing

Water has a unique role in biological structure and function. Enzymes are generally stable and active catalysts in their natural, cellular microenvironments. In addition to the role of water in maintaining the structure of enzyme proteins, water molecules can mediate enzymatic catalysis either directly by taking part in the reaction or indirectly by providing a medium for the reactants and products (Simpson et al. 2012). In relation to its amount and availability, water affects several physicochemical properties of plant-based substrate materials, with effects on the rate of enzymatic reactions.

Biological function of water in retaining protein structure

Water plays a crucial role in maintaining the active conformation of enzymes through hydrophobic and other non-covalent interactions that provide thermodynamic stability to folded protein structures in aqueous solution. The degree of enzyme hydration required for function is not fully resolved, but despite the varying estimates, 0.2 g H_2O /g protein is generally accepted as a threshold value of water required for enzyme activity, which is actually less than the amount of water needed for the formation of a monomolecular layer of water molecules on the polar groups of protein (Lind et al. 2004; Beliz et al. 2009).

It is generally accepted that completely anhydrous solvents do not support enzymatic activity, but that enzymes may be active even in almost non-aqueous solvents containing only traces of water. The use of enzymes in non-aqueous solvents has been studied intensively since the 1980s as a means of producing chemicals by reactions that are not feasible in aqueous media. In non-aqueous solvents such as supercritical fluids, gases, ionic liquids and organic solvents, enzymes may exhibit altered selectivities, pH memory, increased activity and stability at elevated temperatures, and hydrolytic enzymes may carry out synthetic reactions, as reviewed by Hari Krishna (2002).

Water as a reactant

Hydrolases are the most commonly used enzymes in industry (Hari Krishna 2002), and in their reactions, water takes part as a reactant. In the hydrolysis reaction, chemical bonds are cleaved by the addition of water to the cleavage point. This means that a certain amount of bulk water is consumed during the reaction, thus increasing the mass of the solute. This phenomenon is referred to as the hydrolytic gain (Marchal and Tramper 1999). The role of the hydrolytic gain in the water usage of an industrial process was illustrated by the following example. Industrial processes producing glucose syrups from starch typically use an initial dry matter

content of about 35%. During hydrolysis the cleavage of each glycosidic linkage results in the net addition of one molecule of water. In a solution containing 35% starch, approximately 5% of the initial water concentration is used for the hydrolytic gain (Van der Veen et al. 2006).

Water as a solvent, reaction medium and plasticizer

Water is a good solvent due to its polarity, and it is a universal solvent in biological systems. Many biological catalyses involve soluble enzymes acting on insoluble substrates, and water is the medium for dissolution, dispersion, transition and reaction of the components.

Water has an essential role in the mass transfer of enzymatic processes, including the diffusion and convection of enzyme, substrate and product molecules in the reaction medium. Mass transfer impacts enzymatic hydrolysis rates mainly by affecting the rate at which (1) enzymes are distributed in the reaction medium and transferred to reaction sites and (2) solubilised hydrolysis products are transferred away from the reaction site (Roberts et al. 2011). Efficiency of mass transfer is affected by the viscosity and other rheological properties of the enzyme-substrate mixture, and it can be enhanced mechanically by mixing. Increase of water content generally decreases viscosity, which makes mixing easier and less energy demanding. However, viscosity also depends on the intrinsic characteristics of the solutes and solids, as well as on temperature (Guillon and Champ 2000; Modenbach and Nokes 2013).

The rate of biochemical reactions is also dependent on the physical state of the substrate, which can be affected by the amount of water in the system. Water can act as a plasticizer converting amorphous materials from a solid glassy state to a rubbery state (Ruan and Chen 1998). This conversion is known as glass transition, and it is accompanied by a considerable decrease in viscosity and increase in molecular mobility. Although many authors have suggested that reactant mobility and diffusion within a matrix is related to both solvent characteristics (i.e. water availability) and system mobility, described by glass transition temperature and viscosity, studies on the individual or synergistic effects of these parameters on enzyme activity are still scarce, as reviewed by Neri et al. (2010).

States of water in biomaterials and measurement of hydration

The properties of water are affected by its microenvironment. The amount and chemical nature of solutes and the sorption of water within the insoluble plant cell wall structures define the level of “free” or “available” water in these systems. The determination of the state and location of water in biomaterials is of importance when identifying their susceptibility to enzymatic action.

In food science, the state of water has classically been described by the concept of ‘‘water activity” (a_w), defined as the ratio of partial vapour pressure in equilibrium with the food to the saturation vapour pressure of pure water at the same temperature. Solutes decrease the vapour pressure of a solvent by imposing a

physical constraint on solvent molecules directly interacting with the solute molecules (Israelachvili 2006). The extent to which a solute reduces a_w is a function of the chemical nature of the solute. The relationship between water content and water activity in a specific material is indicated by the moisture sorption isotherm at equilibrium. Classically, water activity measurements have been used to determine the product quality and stability based on the "stability map" introduced by Labuza et al. (1970), which shows the general relationship between the occurrences of various reactions (chemical, physical, biochemical, microbial) as a function of a_w. According to the stability map, enzymatic activity is virtually non-existent below a_w 0.2 and starts to increase gradually at a_w 0.3, until a rapid increase occurs above a_w 0.8 (deMan 1999).

Three different conditions of water present in food are classically differentiated on the basis of the sigmoid shape of a typical sorption isotherm, i.e. 1) a monomolecular layer of water, 2) additional layers of water, 3) the water in capillaries and pores of the material (deMan 1999). However, this definition is not very precise, and as reviewed by Roberts et al. (2011), up to five different 'pools' i.e. states of water have been observed in cellulosic suspensions by using time-domain nuclear magnetic resonance, including: (1) primary bound water, also known as non-freezing bound water, which is constrained by interactions with the surface of cellulose; (2) secondary bound water, also known as freezing bound water, which is constrained by primary bound water; (3) water bound by capillary forces within cell wall lumens; (4) restricted bulk water or bulk water the movement of which is restricted by the presence of the other pools of water; and (5) free water. The division of water between the different states depends on a number of factors including the chemical structure of the components, the associations between molecules, the porosity and size of the particles, and temperature (Thebaudin et al. 1997).

The approximate areas of the different states of water in relation to the water content and physical state of the substrate-water mixture are presented in Table 1. According to Felby et al. (2008), below the saturation point of approximately 25–30% moisture, the major part of the water will be present as primary or secondary bound water within the cell wall. Similar values (28–30%) have also been reported for wheat flour, as reviewed by Lee (1970). Above the saturation point water fills the cell lumens until full saturation in the area of 60–70% moisture content (Felby et al. 2008). Even above a water content of 80% (the area of restricted bulk water in which the material typically is in the form of a slurry), the extent of the binding of water might be dependent on the water content of the system, as suggested by Roberts et al. (2011), who found that water was held more tightly in cellulose suspensions as the solids content increased from 5 to 20%. It has also been shown that it is possible to link the state and location of water within the cellulose fibre with structural changes occurring in enzymatic hydrolysis (Felby et al. 2008).

A practical approach to determine the water-substrate interactions is measurement of the hydration properties of the material. The water holding capacity of the substrate determines the point of disappearance of the free bulk liquid phase from the mixture, which has dramatic impacts on the rheological properties and physical form of the mixture (Table 1). When water content is reduced below this point, the

material changes from a pourable suspension into a thick, paste-like substance that can be moulded and formed into shapes (Coussot 2007; Stickel et al. 2009). When water content is further reduced, the material reaches a point at which the liquid is fully absorbed into the solids. At this point the mixture can no longer be called a slurry because it is unsaturated and acts more like a wet, granular substance (Stickel et al. 2009; Viamajala et al. 2009). The measurement of the kinetics of water absorption or the overall water holding capacity of the material can be made either without the application of external forces or by the application of external forces such as centrifugation or pressure (Guillon and Champ 2000; Robertson et al. 2000).

Table 1. Different states of water in relation to water content and physical form of the substrate-water mixture.

Water content	State of water	Physical form of the mixture
< 30%	Primary or secondary bound water, no liquid phase	Powder
30–60%	Water starts to fill the capillaries, no liquid phase	Powder / granular
60–80%	Full saturation of the capillaries, appearance of liquid phase	Granular
> 80%	Restricted bulk water / free bulk water	Paste / slurry

1.1.2 Effects of water content reduction on enzymatic processing

High-solids (i.e. low-water) enzymatic hydrolysis has been loosely defined to take place at solids contents at which significant amounts of free liquid are not initially present (Hodge et al. 2009). In the case of many lignosellulosic substrates, this corresponds to a solids content of 15–20%.The benefits of working at high solids concentrations include reduced downstream processing costs due to higher product concentrations, as well as reduced disposal and treatment costs due to the lower water usage. In addition, higher solids concentrations mean lower requirements for reactor volume and thus reduced capital costs, as well as reduced energy demands for heating and cooling. However, the reduction of water content is generally accompanied by reduced enzyme activity. The effect appears to be rather linear (Jørgensen et al. 2007) and to hold for a variety of substrate materials, as reviewed by Kristensen et al. (2009). Several studies have attempted to elucidate the mechanisms behind this phenomenon, as recently reviewed by Modenbach and Nokes (2013), but the exact cause has not been determined. Many of the encountered problems are related to mass transfer limitations in a direct or indirect way, although it appears to be difficult to quantify and assign the challenges of operating at high solids content to a specific factor because many of them are strongly interrelated. The benefits and challenges related to reduction of the water content of an enzymatic process are summarized in Table 2 and further discussed below.

Table 2. Benefits and challenges related to enzymatic processing at high-solids / reduced water content.

Benefits of low-water processing		**Challenges and related phenomena**	
Lower water usage	Enzyme performance	Properties of the substrate-water mixture	Enzyme performance
• lower requirements for reactor volume • reduced energy demands for heating and cooling • higher product concentrations • reduced downstream processing costs (e.g. drying, concentration) • reduced wastewater treatment and disposal costs • simpler process layout	• Increased enzyme stability	• increased viscosity and changes in the state of water → mass transfer limitations • mixing difficulties; increased shear and power requirements for mixing • increased concentrations of end-product inhibitors	• reduced rate of enzyme action → lower yield, long hydrolysis times • higher degree of polymerization of hydrolysis products • formation of side products • reduced enzyme adsorption on substrate • non-productive binding of enzymes • overcrowding of available substrate sites

Handling of the substrate mixture and mass transfer limitations

Inadequate mixing is considered to be one causative factor for reduced enzyme action at low water content. Mixing is important as a means to enhance the mass transfer of enzyme reactions. When water content is reduced, mixing becomes difficult due to changes in the rheological properties of the material. At high water content, molecules or particles are well separated from each other and are free to move independently. Reduction of water content increases the viscosity of slurry when the particles and/or solutes start to touch and entangle with each other causing increased friction, and consequently mixing and handling of the material become more difficult (Viamajala et al. 2009). Increased viscosity increases the required shear stress necessary to produce a given shear rate, necessitating higher power input for mixing (Hodge et al. 2009; Kristensen et al. 2009). However, increasing the shear rate is not always a viable option for enzyme stability; for example, cellulase enzymes have been shown to be sensitive to both shear and temperature (Reese and Ryu 1980; Gunjikar et al. 2001).

Roberts et al. (2011) found that water was more tightly bound to lignocellulose as solids loadings increased, and they hypothesized that the negative effects of reduced water content on the hydrolysis rate are caused by this ‘water constraint’. Their data suggested that the primary mechanism by which the increased constraint (caused either by increased solids content or increased monosaccharide content) results in decreased saccharification rates is probably the increase in

mass transfer resistances. Increased viscosity of the fluid in the suspensions resulted in decreased diffusivities of solutes such as protein and monosaccharides. They also pointed out that uniform distribution of enzymes in high solids saccharification reactions is difficult to achieve because mass transfer resistances in the reaction increase, and this might limit the synergistic action of enzymes.

Changes in enzyme performance

End-product inhibition by hydrolysis products can play an important role in enzymatic hydrolysis. For example, cellobiose and glucose have been demonstrated to significantly inhibit endoglucanases, cellobiohydrolases and β-glucosidase (Bezerra and Dias 2005; Xiao et al. 2004). At low water contents, the inhibition may become more apparent due to the increased concentration of the inhibiting sugars, especially since it is often coupled with reduced mass transfer rates, which hinder diffusion of the inhibitors away from the reaction site (Hodge et al. 2009). Another mechanism related to the increased concentration of hydrolysis products was suggested by Kristensen et al. (2009). They showed that increasing concentrations of glucose and cellobiose inhibited the adsorption of enzymes onto cellulose, and suggested this to be the main factor behind the “solids effect” limiting enzyme action (Kristensen et al. 2009). On the other hand, some authors have explained decreased enzyme action at higher solids contents by the non-productive adsorption of cellulases to insoluble solids, especially to lignin and cellulose (Rosgaard et al. 2007), and the same mechanism has also been suggested for xylanases binding to xylan substrates (Sørensen et al. 2006).

Long hydrolysis time is one of the challenges specific to high solids enzymatic hydrolysis (Modenbach and Nokes 2013). As pointed out by Kristensen et al. (2009), the inhibition effect of high solids content primarily affects the hydrolysis rate and not the maximum conversion or yield, given sufficient time. In principle the hydrolysis rates could be increased by an increase of enzyme dose, but as reviewed by Modenbach and Nokes (2013), recent studies have suggested that increased enzyme loading may not improve the hydrolysis rate as expected (Bommarius et al. 2008; Olsen et al. 2011; Xu and Ding 2007). It has been suggested that enzymes can completely saturate the substrate by binding to all the accessible sites, which would prevent achievement of the full hydrolytic potential of the given enzyme loading.

In addition to reduced enzymatic activity, the reduction of water content may cause changes in product composition. Van der Veen et al. (2005) investigated the hydrolysis of maltodextrins at dry matter contents of 30–70% w/w, and reported that increased dry matter content resulted in increased formation of condensation products so that both the quantity and the length of the product polymers increased. They concluded that the product composition was kinetically controlled, and that the yield of glucose showed an optimum in time so that the slow side reactions could virtually be excluded by short reaction times (van der Veen et al. 2005). Hardt et al. (2013) showed that wheat gluten can be hydrolysed at solids concentrations as high as 60%. However, they noted that high solids concentra-

tions resulted in a higher weight fraction of peptides with a high molecular mass of > 25 kDa than low solids concentrations. This was attributed to high viscosity and mass transfer limitations (Hardt et al. 2013).

The possible increase in enzyme temperature stability is one beneficial impact of increased substrate concentration. This phenomenon has been noted in non-aqueous media but also in systems with water as the reaction medium (Baks et al. 2008; de Cordt et al. 1994; Sola-Penna and Meyer-Fernandes 1998; Warmerdam et al. 2013). It has been suggested that the higher enzyme stability at higher substrate concentration might be caused by the mechanism of molecular crowding or by complexation with the substrate (Warmerdam et al. 2013). It has also been proposed that the protection of enzymes by soluble sugars is caused by the exclusion of water from the hydration layer of the enzymes, which would cause a decrease in enzyme flexibility, finally resulting in increased enzyme stability but reduced activity (Sola-Penna and Meyer-Fernandes 1998).

1.1.3 Process solutions for hydrolytic enzyme treatments at low water content

A number of processing strategies have been developed and studied as means of modifying different plant-based raw materials with hydrolytic enzymes at reduced water content for various purposes (Table 3). The features of the different processes are discussed below.

Table 3. Processes applying hydrolytic enzymes at low-water conditions for the modification of plant-based raw materials.

Process type	Aim	Raw materials	Mixing type / process solution	Solids content	Enzymes	References
High-solids hydrolysis of lignocellulosic biomass	Hydrolys to monosaccharides	Various lignocellulosic biomasses	Stirred tank reactor	up to 30%	cellulolytic enzymes	e.g. Zhang et al. 2010)
			Rotating drum	up to 40%		Jørgensen et al. (2007)
			Peg mixer	20%		Zhang et al. (2009)
			Roller bottles	15–30%		Roche et al. (2009)
			Fed batch feeding	up to 30%		e.g. Hodge et al. (2009): Yang et al. (2011)
Enzymatic hydrolysis of cereal materials using extruders	Production of glucose syrups	Native/pre-gelatinized starches	Extrusion or extrusion+batch incubation	30–80%	α-amylase, glucoamylase	e.g. Baks et al. (2008); Linko (1989)
	Modification of baking properties	Brewery spent grains		65%	xylanase and cellulase enzyme mixtures	Steinmacher et al. (2012)
	Partial depolymerisation of β-glucan	β-glucan-enriched oat bran fraction		50%	hydrolytic enzyme mixture	Sibakov et al. (2013)
Solid-state enzymatic bioconversions of agricultural food raw materials	Release of antioxidants	Wheat bran	Static incubation	57–70 %	β-glucanase, carboxylic esterase, polygalacturonase, aminopeptidase, cellulase	Moore et al. (2006)
	Hydrolysis of starch to monosaccharides	Chestnut		0.165–0.495 g/l	α-amylase, glucoamylase	López et al. (2005)
	Reduction of water absorbing capacity	Psyllium		*not specified*	xylanase enzyme mixtures	Yu and Perret (2003)
Baking and other low-water cereal food processes applying enzymes	Improved dough properties and/or end-product quality	Various cereal fractions from wheat, rye, oat etc.	Static incubation / intermittent mixing	20–50%	hydrolytic, oxidative and transferase enzymes	e.g. Martinez-Anaya and Jimenez (1997); Lebesi and Tzia (2012); Coda et al. (2014)
	Improved gluten functional properties	Wheat gluten	Continuous mixing	10–60%	protease mixture	Hardt et al. (2013)

High-solids hydrolysis of lignocellulosic biomass

Hydrolysis of lignocellulosic biomass is an area in which much research has been performed to target the challenges related to working at low water content. At high solids content, the method of mixing can have a substantial impact on the conversion of lignocellulosic substrates. Traditional mixing designs resembling a standard, vertical stirred tank reactor require excessive power for adequate mixing and generally perform poorly for high yield stress fluids (Ehrhardt et al. 2010; Lavenson et al. 2012; Modenbach and Nokes 2013; Roche et al. 2009; Saeed et al. 2008). Approximately 12–15% of insoluble solids is generally considered to be the upper limit at which biomass slurries can be mixed and hydrolysed effectively in conventional stirred-tank reactors (Hodge et al. 2009; Kristensen et al. 2009). However, the geometry of the impeller can play a significant role in the efficiency of the mixing, as shown by Zhang et al. (2010), who demonstrated the superior performance of a helical impeller as compared to the traditional Rushton impeller at high solids contents up to 30%. Several studies have shown that horizontal orientation of the reactor and free-fall mixing provide numerous advantages over typical stirred tank reactors, including minimized particle settling, easy scale-up and lower power requirements, as reviewed by Modenbach and Nokes (2013). Examples of this type of mixing systems are the peg mixer, roller bottles and the rotating drum, which have been used up to solids contents of 40% (Table 3).

A special approach to overcome the challenges related to working at high solids content is the use of fed-batch feeding strategies, i.e. sequential loading of substrate or substrate plus enzymes during enzymatic hydrolysis (Hodge et al. 2009; Yang et al. 2011). One benefit of fed-batch feeding is lower initial viscosity as compared to simple batch processing. The viscosity of lignocellulosic substrates is known to decrease as a result of cellulolytic activity, and in fed-batch processing the initial viscosities can be low since this allows time for the slurry to liquefy before adding additional solids. However, when a fed-batch approach is selected, it must be considered how and when to add substrate, as well as enzymes, in order to maintain high rates of conversion (Modenbach and Nokes 2013). The possible problems include inability of the enzyme to desorb from partially hydrolysed substrate and find accessible cellulose sites in the fresh substrate (Chandra et al. 2011), but sequential addition of enzymes with each addition of fresh substrate has given positive results (Hodge et al. 2009; Yang et al. 2011). Although many studies support the use of fed-batch processing for high-solids hydrolysis, the results concerning its applicability are currently still inconclusive (Modenbach and Nokes 2013).

Specific viscosity-modifying additives, such as surfactants and water soluble polymers, have also been investigated as a means to decrease the viscosity effects in high-solid slurries, but the economics of their use in industrial scale are still to be validated (Knutsen and Liberatore 2010). Another studied approach is the reduction of particle size of the substrate (Dasari and Berson 2007). Viamajala et al. (2009) and Dasari and Berson (2007) reported that smaller particle sizes re-

sulted in lower apparent viscosities under equivalent conditions, as well as enhanced enzymatic hydrolysis. Size-reduction could have reduced the amount or size of macro-pores in the biomass particles, so that less liquid is entrained in these particles and thus more free water remains in the suspension, resulting in decreased interactions between particles and lower apparent slurry viscosities (Viamajala et al. 2009).

Hydrolysis of cereal materials using extruders as bioreactors

The enzymatic hydrolysis of starch to glucose syrups is an important industrial process that consists usually of two steps: 1) gelatinization and liquefaction, and 2) saccharification. The industrial gelatinisation and liquefaction with α-amylase are usually carried out in excess water (at 30–35% dry solids content) in order to facilitate gelatinisation and ensure sufficient mixing during the reactions (Van der Veen et al. 2006), but these processes can also be efficiently performed at dry matter contents from 50% up to 80% using extruders (Govindasamy et al. 1997a, 1997b; Linko 1989; Van Zuilichem et al. 1990). Extruders are able to continuously create high shear, which is required when the gelatinization process is performed at reduced water content (Barron et al. 2001; Van der Veen et al. 2006). An extruder comprises a horizontal barrel with one or two conveying screws and a die exit. The raw material becomes efficiently mixed and subjected to high pressure and shear as it is conveyed across the barrel to the die with a restricted opening. In a typical extrusion process temperatures are between 100 and 180°C and residence times range from seconds to a few minutes (Guy 2001). Extrusion cooking usually involves low moisture conditions between 10 and 40%, but since the 1980s, “wet extrusion” with a feed moisture content above 40% has been possible due to developments with twin screw extruders including sophisticated barrel designs, screws and dies (Akdogan 1999), which have enabled the use of extruders as bioreactors for enzymatic treatments.

As reviewed by Baks et al. (2008), extruders have been used for combined gelatinisation and enzymatic hydrolysis of native starches (Govindasamy et al.1997a, 1997b; Lee and Kim 1990; Vasanthan et al. 2001) and for hydrolysis of pregelatinised starch (Komolprasert and Ofoli 1991), as well as in combination with a batch reactor to increase the hydrolysis time (Chouvel et al. 1983; Linko 1989; Reinikainen et al. 1986; van Zuilichem et al. 1990). In many cases the enzymes have been added at the beginning of the extruder together with the starch-water mixture, but in order to avoid the shear-induced enzyme deactivation it is preferable to add the enzyme at the end of the gelatinisation (Baks et al. 2008; Grafelman and Meagher 1995; Van der Veen et al. 2006). The impact of water content has also been studied, and in most cases the conversion has been highest at the highest water content studied, typically at 55–70% water content, as reviewed by Linko (1989) and Akdogan (1999), although (Tomás et al. 1997) reported maximum starch hydrolysis at an intermediate water content of 60% when studying in the range of 55–65%.

Studies concerning the use of extruders for enzymatic modification of other cereal materials than starch or for other targets are rare. However, two recent papers reported the use of cell wall degrading enzymes for enzymatic hydrolysis of oat bran β-glucan at a water content of 50% (Sibakov et al. 2013) and for modification of brewer's spent grain at a water content of 65% (Steinmacher et al. 2012).

Solid-state enzymatic bioconversions

Solid-state (or substrate) fermentation (SSF) is generally defined as the growth of microorganisms on (moist) solid material in the absence or near-absence of free water (Pandey et al. 2008). In SSF processes, the growth and metabolic reactions of microorganisms are utilized for a variety of applications, including the production of industrial enzymes, organic acids and secondary metabolites such as antibiotics and ethanol, as well as for bioremediation and biotransformation of agro-industrial raw materials and residues (Krishna 2005). Enzymatic reactions initiated by the metabolic activity of microorganisms have a key role in many SSF processes, and the same approach, i.e. incubation of substrates in solid state, has been applied for enzymatic conversions of some agro-industrial materials for food applications (Table 2). These processes have been conducted without agitation, and the reaction times last up to several days. The benefits of this type of processing are that they do not require expensive equipment or post-reaction processing for product recovery (Moore et al. 2006). Solid-state enzymatic procedures have been used to improve the physiochemical and functional properties of psyllium (Yu and Perret 2003), to hydrolyse chestnut starch (López et al. 2005) and to release insoluble bound phenolic acids from wheat bran (Moore et al. 2006). These studies have reported that solid-state enzyme treatments have promoted beneficial changes in the raw materials, and also investigated the effect of water content (e.g. Moore et al. 2006). However, the long process times and the lack of comparison of the low-water process to optional process variations (high-water content with mixing) complicate evaluation of the overall efficiency and industrial applicability of the solid-state enzyme treatments in the field of food and feed processing.

Baking and other low-water cereal food processes

Enzymes are routinely used in the baking industry as processing aids and for improving product quality. Baking can be considered as a low-water process since the reactions take place in the dough, i.e. in the absence of free liquid. The water content of bread dough is typically around 50%. Wheat is by far the most important cereal grain in bread making, and the essential wheat flour constituents include starch, gluten proteins, arabinoxylans and lipids (Goesaert et al. 2005). In the baking process, enzymes are mixed together with the other ingredients into a visco-elastic dough, which is fermented and baked. The enzymes used and studied in baking include hydrolytic enzymes such as amylases, lipases, proteases, cellulases, β-glucanases and xylanases, as well as oxidative enzymes such as

lipoxygenase, glucose oxidase and laccase, and also the transferase enzyme transglutaminase (Caballero et al. 2007; Goesaert et al. 2005; Martínez-Anaya and Jiménez 1997). The use of hydrolytic enzymes has also been shown to improve the quality of high-fibre breads produced using whole-grain flours or brans (Katina et al. 2006; Laurikainen et al. 1998; Shah et al. 2006).

In addition to direct addition in the bread dough, enzymes have been used as a pre-treatment for modification of the baking raw materials. The wheat flour milling process includes a tempering step, during which the moisture content of wheat grains is increased to 15.5%. Haros et al. (2002) examined the impact of addition of cellulase, xylanase, and β-glucanase to the tempering treatment (20 °C, 16 h), and reported that the quality of fresh bread improved when using flours obtained from the carbohydrase-treated wheat. Several studies have shown that bioprocesssing of bran with cell wall degrading enzymes can modify the physicochemical properties and structure of bran and improve its technological functionality in baking processes (Coda et al. 2014; Lebesi and Tzia 2012; Nordlund et al. 2013). These bran fermentations have been performed at water contents of 65–80%. The recent paper of Hardt et al. (2013) is a rare example of a study in the field of food processing in which the impact of a range of water contents on an enzymatic treatment has been studied. They examined the enzymatic hydrolysis of wheat gluten at solids concentrations varying from 10% to 60%, and concluded that wheat gluten can be hydrolysed at solid concentrations as high as 60%, but that increased reaction times at very high solid concentrations result in optimum productivity at 40% solids content (Hardt et al. 2013).

1.2 Xylanolytic enzymes and degradation of plant cell walls

Xylanolytic enzymes are a widespread group of enzymes catalysing the hydrolysis of xylan, which is a major structural polysaccharide in plant cell walls. Xylanases together with other xylanolytic enzymes have an important role in the hydrolysis of lignocellulosics as well as in the processing of agro-industrial plant materials for food and feed applications.

Xylan is a constituent of the hemicellulose fraction of plant materials and accounts for one third of all renewable organic carbon available on earth (Prade 1996). Most xylans occur as heteropolysaccharides, containing different substituent side chains in the backbone chain composed of 1,4-linked β-D-xylopyranosyl units. The substituents found on the backbone are glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and p-coumaroyl side-chain groups (Kulkarni et al. 1999; Liab et al. 2000). In cereals and other grasses, xylan occurs mainly as arabinoxylan (AX), and it is the predominant form of non-cellulosic polysaccharides of both primary and secondary cell walls (Izydorczyk 2009).

1.2.1 Xylanolytic enzymes

Xylanolytic enzymes are produced mainly by microorganisms but are also found in plants, marine algae, protozoans, crustaceans, insects and snails (Sunna and Antranikian 1997; Dekker and Richards 1976). They participate in the breakdown of plant cell walls, and also digest xylan during the germination of seeds (Polizeli et al. 2005; Dekker and Richards 1976). Since the 1980s, industrially produced xylanolytic enzymes have found use in numerous commercial applications such as bleaching of cellulose pulp, waste water treatment, hydrolysis of biomass for biofuels production, animal feeds, baking and other food, ingredient and drink manufacturing processes, as well as in the pharmaceutical, chemical and textile industries (Beg et al. 2001; Polizeli et al. 2005). In the cereal industry, xylanases are frequently used to adjust processing, yield, and/or end product quality (Dornez et al. 2009), as well as for the production of special ingredients such as prebiotic oligosaccharides (Van Craeyveld et al. 2010).

The most important xylanolytic enzymes are the endo-(1,4)-β-D-xylanases (EC 3.2.1.8), also called endoxylanases or simply xylanases. They catalyse the hydrolysis of 1,4-β-D-xylosidic linkages in xylan backbone. Based on amino acid sequence and structural similarities, endoxylanases have been classified in glycoside hydrolase (GH) families 5, 7, 8, 10, 11 and 43 (Collins et al. 2005). The majority of microbial xylanases belong to GH families 10 and 11. As reviewed by Courtin and Delcour (2002), the differences in size and complexity of the protein structure of GH family 10 and 11 xylanases apparently reflect their substrate specificity and selectivity. In general, GH10 xylanases have greater catalytic versatility and lower substrate specificity, and tend to produce oligosaccharides with a low degree of polymerisation, whereas GH11 xylanases are more specific for xylan and produce larger oligosaccharides (Biely et al. 1997). Xylanases may also have selectivity towards either water extractable or water unextractable xylan (Courtin and Delcour 2001; Maes et al. 2004). It is generally accepted that GH11 xylanases preferentially cleave in unsubstituted regions of the xylan backbone, whereas GH10 enzymes cleave in the decorated regions (Berrin and Juge 2008; Biely et al. 1997). Fungal endoxylanases are typically stable over a wide pH range (3.0–10.0), whereas their pH optimum is generally 3.5–5.5. Bacterial endoxylanases usually have somewhat higher pH optima (6.0–7.0), and narrower pH stability (5.0–7.3) (Courtin and Delcour 2002). The optimum temperature for xylanase action ranges between 35 and 60°C (Beg et al. 2001), but bacterial and fungal xylanases typically have temperature optima between 40 and 50 °C, above which their stability is limited (Dekker and Richards 1976; Reilly, 1981).

Due to the heterogeneity and complex chemical nature of plant xylan, its complete breakdown requires the action of an array of several hydrolytic enzymes with diverse specificities and modes of action (Beg et al. 2001). The presence of such a multifunctional xylanolytic enzyme system is widespread among fungi, actinomycetes and bacteria, as reviewed by Collins et al. (2005). The cleavage of xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose is

catalysed by β-D-xylosidases (EC 3.2.1.37). Enzymes catalysing the removal of the side groups include α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73) and *p*-coumaric acid esterases (EC 3.1.1.). The sites of enzyme attack in xylan are presented in Figure 1 (Collins et al. 2005).

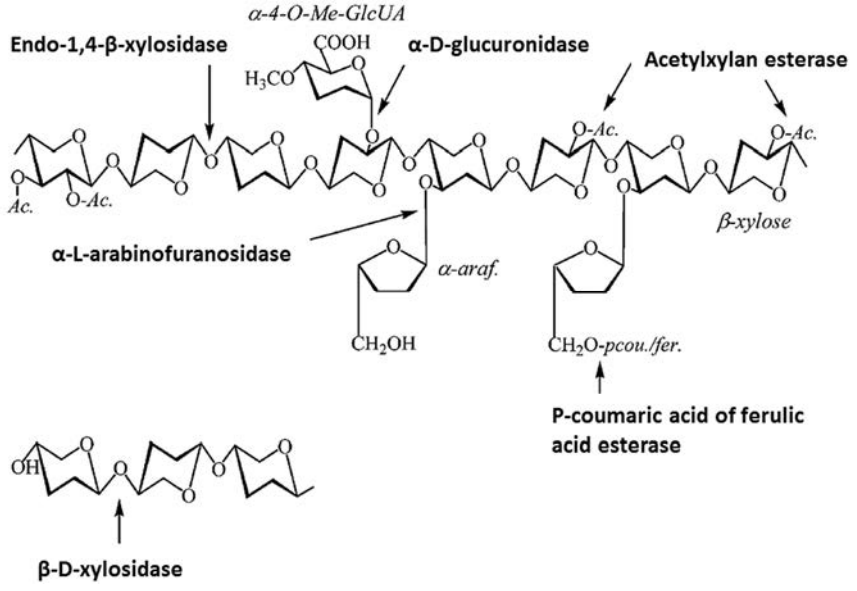

Figure 1. Structure of xylan and the sites of attack by xylanolytic enzymes. Adapted from Collins et al. (2005).

1.2.2 Enzymatic degradation of plant cell walls

Cell walls constitute the key structural components of plants and account for the bulk of lignocellulosic biomass. From a plant physiological perspective, the cell walls provide support and determine the cell shape and also take part in numerous activities in plant development, including cell growth and division, intercellular signalling and transport, protection against other organisms and environmental stresses and storage of food reserves (Waldron et al. 2003). The plant cell walls also have a major impact on the processing of plant materials for food and feed purposes (milling, malting, baking, juice processing etc.), (Cui and Wang 2009), and thus degradation of the cell walls with hydrolytic enzymes is often an essential step in the upgrading of plant-based materials for these applications.

Together with xylan, the major polymeric constituents of plant cell walls are cellulose (1,4-β-glucan) and lignin (a complex polyphenolic compound), and in cereal cell walls also β-glucan (1,3–1,4-β-glucan). The structure of a plant cell wall is generally described as a network of cellulosic microfibrils embedded in a matrix of

non-cellulosic polysaccharides, in which phenolic components, structural proteins and glycoproteins may also be present (Carpita 1996; Waldron et al. 2003). Within the cell wall matrix, different constituents are associated with each other through covalent and non-covalent linkages leading to the formation of a non-uniform, three-dimensional and compact structure (Andersson et al. 2003; Iiyama et al. 1994). The complex interaction between different components makes cell walls very resistant towards enzymatic action.

In the hydrolysis of biomass the use of a combination of different cell wall degrading enzymes is often feasible since enzymes may cooperate in a synergistic manner to degrade the substrate, meaning that the activity of enzymes working together is higher than the sum of their individual activities (Van Dyk and Pletschke 2012). Numerous examples of synergistic action of cellulases and xylanases and other enzymes in the degradation of lignocellulose were presented in the recent extensive review by Van Dyk and Pletschke (2012). Cellulose is a particularly difficult polymer to degrade, as it is insoluble and present as hydrogen-bonded crystalline fibres (Mansfield et al. 1999). It is generally accepted that three types of enzymes are required to hydrolyse cellulose into glucose monomers, namely exo-1,4-β-glucanases i.e. cellobiohydrolase (EC 3.2.1.91 and EC 3.2.1.176), endo-1,4-β-glucanases (EC 3.2.1.4) and β-glucosidases i.e. cellobiases (EC 3.2.1.21), as reviewed by Van Dyk and Pletschke (2012). Endoglucanases cleave cellulose chains in the middle and rapidly reduce the degree of polymerisation, whereas cellobiohydrolases attack the ends of cellulose chains.

In contrast to the hydrolysis of lignocellulosic biomass for e.g. biofuels production, in food processing the aim of plant cell wall degradation is not always complete hydrolysis of the cell wall constituents to monosaccharides. With targeted enzymatic modification of the plant cell walls, the quality of the end product, for example texture and sensory properties, as well as the nutritional properties of the product can be affected (Harris and Smith 2006; Lebesi and Tzia 2012; Nordlund et al. 2013). Cell walls also affect the release of potentially health-promoting components, such as phenolic compounds, which are bound to cell wall structures (Mateo Anson et al. 2009; Bunzel et al. 2001), and it has been shown that the bioavailability of ferulic acid in bran-supplemented bread can be increased by enzymatic bran pre-treatment (Mateo Anson et al. 2011). In baking the aim of the use of cell wall degrading enzymes is opening up of the cell wall structures and solubilisation of AX without too extensive depolymerisation (Courtin and Delcour 2002). Thus, specificity and selectivity are important criteria for the selection of enzymes for a specific application. Commercial enzyme products are typically mixtures of several hydrolytic activities that in theory enable the synergistic action of enzymes and thus effective plant cell wall degradation, but this might be a disadvantage when aiming for more specific targets.

1.3 Wheat bran: properties and processing strategies for food applications

Bran is the fraction of the outer layers of a grain which is removed in the milling process during the production of refined flour. The annual world production of wheat is currently about 650–690 million tons (Earth Policy Institute 2013), and the bran fraction accounts for 11–15% of the grain weight (Hemery et al. 2007). Most of the bran is used for animal feeding. Bran is also used in food products to increase the DF content of the end product or as a filler to reduce its energy density. Wheat bran also contains substantial amounts of protein and phytochemicals, which further motivates its use in food products. However, it is generally known that the use of unprocessed bran is usually detrimental for product quality, and it may also contain contaminants that need to be taken in consideration when used in foods. Numerous processing methods have been developed and are currently studied as potential means to facilitate the use of bran fractions in different types of food products.

1.3.1 Composition and nutritional properties of wheat bran and DF

Several different tissues can be distinguished in wheat (*Triticum aestivum L.*) grain from the centre to the periphery, i.e. the embryo, the endosperm (composed of the starchy endosperm and the single cell layered aleurone layer), the seed coats (composed of the nucellar epidermis and the testa), and the pericarp (composed of the tube cells, the cross cells, the hypodermis, and the epidermis). Wheat bran is composed of the pericarp, seed coats and aleurone layer, as well as variable amounts of remnants of starchy endosperm and germ depending on the separation efficiency of the milling process (Kamal-Eldin et al. 2009) (Figure 2). The main chemical constituents of wheat bran are cell wall carbohydrates (AX, β-glucan, cellulose), starch, fructan, lignin, protein, fat and minerals (Table 1). Wheat bran is also a source of vitamins, phenolic acids, alkylrecinols, plant sterols and lignans (Hemery et al. 2007). Variation in composition arises from differences in the genetic and agricultural backgrounds of wheat, as well as in the milling processes (Kamal-Eldin et al. 2009). In particular there can be wide variation in starch content, which directly affects the ash and fibre content of bran.

The structure and chemical composition of the cell walls vary in different bran layers. For example, the cell walls of the aleurone layer consist mainly of sparsely substituted AX and β-glucan, whereas the bran pericarp contains cellulose, lignin and highly substituted AX (Antoine et al. 2003). Most of the bran protein, lipids and phytochemicals are concentrated in the aleurone fraction (Hemery et al. 2007). The chemical, structural, and physical properties significantly affect the nutritional and technological functionality of the different bran layers, which can be separated using modern fractionation techniques (Hemery et al. 2007).

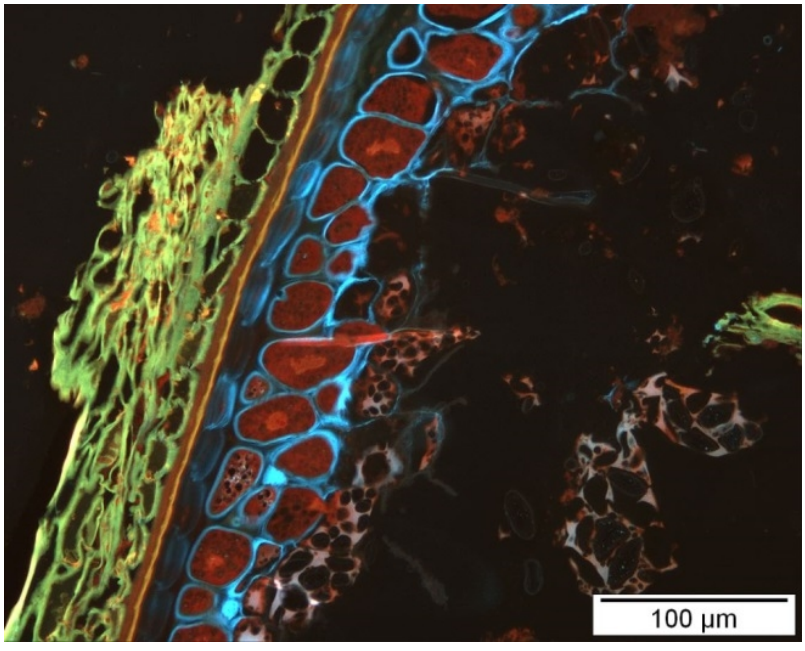

Figure 2. Microstructure of wheat bran stained with Acid Fuchsin and Calcofluor White. The β-glucan-rich aleurone cell walls appear in blue, the pigment strand (between the pericarp and the aleurone layer) in orange, the pericarp layer in light green and yellowish, and proteins in red (Courtesy of VTT Technical Research Centre of Finland, Ulla Holopainen).

Table 4. Main constituents of wheat bran.

Component	Content (% bran dry matter)
Arabinoxylan	19–30
Cellulose	9.3–12.
Klason lignin	3.0–4.9
Fructan	2.8–3.7
β-glucan	1.2–2.6
Starch	8.8–29
Protein	14–17
Fat	5.6–6
Ash	4–6.6

Values obtained from Kamal-Eldin et al. (2009); Maes and Delcour (2002); Swennen et al. (2006).

A major portion (40–50%) of wheat bran is dietary fibre (DF), i.e. those compounds of edible plants that are not digested in the human small intestine. The main DF polymers in wheat bran are AX, cellulose, lignin, fructan and β-glucan (Table 4). The importance of DF as a health protective component has long been recognized, but its analysis and definition based on chemical and physical properties has been a topic of intense debate and research, as reviewed by Raninen et al. (2011). DF is mainly formed from cell wall constituents and includes carbohydrate polymers with ≥ 3 or ≥ 10 monomeric units, depending on the definition, occurring naturally in food or those derived by modification and synthetic means (European Commission 2008; FAO/WHO 2009). Lignin and associated plant substances are also included in DF when existing naturally in the material. Dietary guidelines generally recommend a daily intake of 25–35 g of DF, or 3 g DF/1000 kJ (Raninen et al. 2011). Furthermore, EFSA has accepted several health claims related to the consumption of DF or wheat bran. A minimum of 3 g and 6 g of DF per 100 g of the product is required for claims of “source of fibre” and “high fibre”, respectively. Related to the consumption of wheat bran, the positive effect on human health can be due to the increase of faecal bulk and the reduction of intestinal transit time. To substantiate these claims, the daily wheat bran fibre consumption should be more than 10 g.

The substantial content of vitamins, phenolic acids, alkylrecinols, plant sterols, lignans and other potentially health-promoting components makes wheat bran a particularly interesting ingredient for food production. However, the accessibility of the compounds may be limited as they are trapped or encapsulated by complex cell wall structures. Wheat bran also contains phytate, mostly located in the aleurone layer. Phytate is able to bind minerals, and may thus reduce their availability in foods containing bran. Bran may also contain contaminants, such as mycotoxins, pesticide residues, heavy metals, etc., as well as yeasts and bacteria. However, it has been shown that these unwanted features can largely be overcome by using targeted processing and fractionation techniques (Hemery et al. 2007; Katina et al. 2007; Katina et al. 2012; Mateo Anson et al. 2011; Servi et al. 2008).

1.3.2 Technological properties of wheat bran and DF and methods for their modification

As in the case of many other DF-rich materials, addition of wheat bran usually causes significant and typically unwanted changes in the texture, flavour and colour of foods (Ortiz and Lafond 2012). Products to which wheat bran is generally added include bread and other baked products, extruded snacks and breakfast cereals, pasta and noodles. Depending on the product, increased hardness, brittleness and roughness is associated with bran addition. Wheat bran is known to have an adverse effect on volume, softness, flavour and appearance of bread and other baked products at 5–10% or higher levels (Pomeranz et al. 1977; Lebesi and Tzia 2011; Coda et al. 2014). Wheat bran addition to light coloured products such as crackers or rice cake leads to darker, browner or speckled appearance. Fur-

thermore, on the basis several studies it has been shown that cereal bran addition reduces volumetric expansion and increases hardness of extruded snacks (Robin et al. 2012).

The reasons behind the adverse effects of bran have not been fully elucidated, but both physical and chemical mechanisms have been suggested (Gan et al. 1992; Guy 2001; Lai et al. 1989; Moraru and Kokini 2003; Noort et al. 2010). One of these is the fact that bran addition decreases the concentration of the main structure-forming components, starch and gluten. The mechanisms behind the adverse effects of bran are generally related to its insoluble nature and coarseness, and its ability to bind large amounts of water and alter viscosity. These properties are affected by particle size, porosity, cell wall architecture, chemical composition, and molecular structure of the DF polymers. Processing methods aiming to improve the technological functionality of bran in baked, extruded and other products generally aim to modify these properties. The methods used for the modification of bran and other DF-rich plant materials include various enzymatic, chemical, mechanical, hydrothermal and thermo-mechanical methods (Guillon and Champ 2000). In addition to modification of the physicochemical properties, the processability and stability of bran have been targeted by inactivating endogenous microbes, enzymes or enzyme inhibitors of bran by e.g. heat treatments or fractionation techniques (de Kock et al. 1999; Hemery et al. 2007). The physicochemical properties of bran are strongly interrelated, and a change in one property typically also alters other physicochemical properties. However, some general relationships between the physicochemical properties and technological functionality of bran can be distinguished, as summarized in Table 5 and discussed further below.

Table 5. General relationships between physical, chemical, physicochemical and technological properties of bran and examples of methods for their modification.

Physical/ physicochemical property	Determinant physical / chemical properties	Technological impacts of modification of the property	Examples of methods used for modification of the property
Particle size	Surface area, number of particles	Reduction of particle size • positive/negative impact in bread (Noort et al. 2010; Lai et al. 1989; Coda et al. 2014) • positive impact in extruded products (Blake 2006; Alam et al. 2013)	• Grinding (Noort et al. 2010; Zhu et al. 2010) • Ball milling (Van Craeyveld et al. 2009)
Solubility of dietary fibre (DF)	Cell wall architecture, level of branching of DF polymers	Increase in DF solubility • positive impacts in baked products (Katina et al. 2012; Lebesi and Tzia 2012) • positive impact in extruded products (Pai et al. 2009)	• Enzyme treatments (Lebesi and Tzia 2012) • Fermentation (Katina et al. 2006; 2012) • Chemical alkaline treatment (Pai et al. 2009) • Grinding (Zhu et al. 2010) • Extrusion (Ralet et al. 1990; Wang et al. 1993)
Hydration properties	Particle size, porosity, cell wall architecture, level of branching of DF polymers	Decrease of bran water binding capacity • positive impacts in baked products (Lebesi and Tzia 2012)	• Enzyme treatments (Lebesi and Tzia 2012) • Grinding (Noort et al. 2010; Sanz Penella et al. 2008) • Extrusion, hydrothermal treatments (Caprez et al. 1986)
Viscosity of soluble phase	Concentration, molecular weight, degree of substitution and substitution pattern of soluble DF	High viscosity of dough/melt • positive impact in baking (Courtin and Delcour 2002; Lebesi and Tzia 2012) • positive or negative impact in extrusion (Pai et al. 2009)	• Enzyme treatments (Lebesi and Tzia 2012), • Chemical alkaline treatment (Pai et al. 2009), • Extrusion (Wang et al. 1993)

Particle size

The average particle size of native wheat bran is generally about 800–1000 µm (Auffret et al. 1994; Kamal-Eldin et al. 2009; Noort et al. 2010; Sanz Penella et al. 2008). Particle size is an important parameter for the use of bran, affecting both its physiological effects and technological functionality (Hemery et al. 2011; Robin et al. 2012; Zhang and Moore 1999). Particle size determines the surface area of the particles and may thus affect reactions that are dependent on the available surface area and surface characteristics. Reduction of particle size increases the total surface area and the number of the particles, and several studies have reported that decreasing the particle size of plant-based substrates may enhance their enzymatic hydrolysis (Dasari and Berson 2007; Mahasukhonthachat et al. 2010; Niemi et al. 2012; Silva et al. 2012). Reduction of particle size may also affect the release of components from the particles, and an increase in the level of soluble DF is often observed after intensive milling (Hemery et al. 2011; Zhu et al. 2010). Particle size is also a factor affecting hydration properties of bran and viscosity of doughs (Sanz Penella et al. 2008; Noort et al. 2010).

In food products, large bran particles may cause a gritty mouthfeel (Ortiz and Lafond 2012). Although reduction of particle size has been shown to alleviate this problem (Coda et al. 2014), it also alters other functional properties of the fibre particles. In baking, the impact of bran particle size is a controversial issue, as some studies indicate that reduction of bran particle size improves baking performance, such as dough mixing properties and loaf volume (Lai et al.1989; Moder et al. 1984), whereas others report the opposite (Zhang and Moore 1999; Noort et al. 2010). Coda et al. (2014) compared the baking performance of brans with mean particle sizes of 750, 400, 160 and 50 µm, and reported that the least detrimental particle size was 160 µm. The negative impact of particle size reduction has been ascribed to increased interaction surface and liberation of reactive components due to cell breakage (Noort et al. 2010). In extrusion, decreasing fibre ingredient particle size has been reported to increase the radial expansion of extrudates containing DF (Lue et al. 1991; Blake 2006; Alam et al. 2013). However, particle size reduction has not improved expansion when the size differences or bran addition levels have been low (Robin et al. 2011a; Blake 2006; Alam et al. 2013).

Particle size reduction equipment for cereal materials (wet and dry) in food and feed processing include hammer, roller and attrition mills, but blade, pin, ball and cryogenic mills are also used, especially for specialty products (Mahasukhonthachat et al. 2010). These mills differ in the effective operating force and the extent of frictional heat generation during grinding. Particle size of bran might also decrease as a consequence of other processes involving mechanical shear, e.g. extrusion (Wang et al. 1993; Robin et al. 2011a).

Solubility of DF

Solubility has a fundamental role in DF functionality. Fibres are generally classified as soluble or insoluble in water, although when considered soluble they might also

be present as a colloidal suspension as opposed to a true solution (Ortiz and Lafond 2012). Insoluble and soluble DF have typically very different impacts in cereal products. Insoluble fibre particles may physically interrupt the food macrostructure, causing potential weak points in the three-dimensional food matrix (Ortiz and Lafond 2012). Insoluble fibres can further negatively influence food texture by their water retention and swelling properties (Thebaudin et al. 1997). Soluble DF, on the contrary, is more easy to incorporate in foods and is characterized by its ability to increase viscosity in solution and to form gels and/or act as an emulsifier (Elleuch et al. 2011).

Solubility is related to the chemical structure of the polymers, and also to the extraction conditions used. In general, linear regions in polysaccharides are able to form strong interchain interactions allowing the formation of ordered crystalline structures that are insoluble in water (Ortiz and Lafond 2012). Branching limits the number of interchain interactions, and thus polysaccharides with some irregularities in their structure (in the backbone or as side chains) tend to be soluble (Guillon and Champ 2000). Wheat bran contains 1.5–4.0% soluble DF and 35–48% insoluble DF (Vitaglione et al. 2008). The main DF component in wheat bran is AX, and thus the chemical nature of AX has a major impact on bran functionality. Based on their solubility, wheat bran AXs are divided into two fractions, i.e. extractable (WEAX) and unextractable (WUAX) in water. The content of WEAX in wheat bran is approximately 0.3–0.9% of bran DM (Ward et al. 2008).

Processing of cereal bran with cell wall degrading enzymes has been shown to facilitate the addition of bran to baked products, and the beneficial effects of these processes have been related to the solubilisation of bran AX (Coda et al. 2014; Figueroa-Espinoza et al. 2004; Lebesi and Tzia 2012). Fermentation with microbes is another method in which enzymatic hydrolysis plays an important role, as the beneficial impact of fermentation on the quality of high fibre bread has been ascribed largely to the solubilisation of AX due to the action of endogenous hydrolytic enzymes naturally present in cereal materials (Katina et al. 2012). The solubilisation of AX and DF might also accompany mechanical or thermo-mechanical treatments, such as mixing, grinding or extrusion (Cleemput et al. 1997; Dornez et al. 2007; Ralet et al. 1990; Wang et al. 1993; Zhu et al. 2010).

In terms of extruded products, it has been reported that soluble DF, such as pectin, inulin or guar gum, generally performs better than fibres that are mostly insoluble such as wheat bran (Brennan et al. 2008; Yanniotis et al. 2007), and it has been suggested that increasing the solubility of DF prior to extrusion could be a means to improve the functionality of DF in extruded products (Robin et al. 2012). However, only a few studies have examined this possibility. Pai et al. (2009) showed that increasing the content of soluble DF in corn bran with concomitant reduction of insoluble DF by a chemical alkaline treatment resulted in higher expansion as compared to untreated bran.

Hydration properties

Insoluble fibres which also form the majority of wheat bran DF can hydrate and physically entrap water, but still be present in the food matrix as discrete particles (Ortiz and Lafond 2012). Hydration properties are considered important in terms of DF functionality, and the detrimental effect of addition of insoluble DF to both baked and extruded products has been partly attributed to the competition for water between the DF and other components (Gan et al. 1992; Robin et al. 2012; Moraru and Kokini 2003). A number of terms and methods are used interchangeably to describe and quantify the ability of a fibre to retain water, including *water uptake, hydration, adsorption, absorption, retention, binding, or holding* (Guillon and Champ 2000; Thebaudin et al. 1997). According to a common definition, the water binding capacity (WBC) refers to the quantity of water that is retained by the soaked sample following the application of an external force (pressure or centrifugation), whereas the water holding capacity (WHC) means the quantity of water that is absorbed into the fibres without the application of external forces after soaking (except for gravity and atmospheric pressure) (Thebaudin et al. 1997). Swelling capacity is also included in the hydration properties and is defined as the volume occupied by a known weight of sample after soaking under the conditions used (Guillon and Champ 2000; Robertson et al. 2000).

The ability of bran to retain water is mainly determined by its particle size, porosity and DF polymer chain length (Tungland and Meyer 2002). By modifying the physical properties of the fibre matrix, processes such as grinding, drying, heating or extrusion cooking all affect the hydration properties (Thibault et al. 1992). In general, particle size reduction of bran and other DF preparations decreases their water binding capacity (Noort et al. 2010; Auffret et al. 1994; Zhu et al. 2010). When particle size increases, so does the trapped volume due to imperfect packing and consequent apparent water binding (Thebaudin et al. 1997). However, grinding may also increase the hydration properties of DF preparations as a consequence of the increase in surface area (Elleuch et al. 2011). Furthermore, when added to bread dough, the water absorption of the dough has been found to increase along with reduction of bran particle size (Sanz Penella et al. 2008; Noort et al. 2010).

In baked products the reduction of water binding capacity of bran or the increase in the level of available water by enzymatic hydrolysis has been related to improved baking properties (Lebesi and Tzia 2012; Katina et al. 2006,). Insoluble DF such as WUAX are known to bind more water than their soluble counterparts (Courtin and Delcour 2002), and thus the solubilisation of DF by hydrolytic enzymes can reduce its water holding capacity (Yu and Perret 2003; Lebesi and Tzia 2012). On the other hand, treatment with hydrolytic enzymes has also been found to increase the bran swelling capacity and decrease batter a_w (Lebesi and Tzia, 2012). In terms of extruded products, several researchers have theorized that the binding of water to DF molecules might reduce the amount of water available for starch gelatinization, eventually reducing radial expansion (Yanniotis et al. 2007; Moraru and Kokini 2003). However, Robin et al. (2011b) reported that addition of

wheat bran resulted in increase in the level of available water. It can be concluded that interactions related to the hydration properties of DF, availability of water and the impacts of these factors on the quality of baked and extruded products are complex and partly unresolved.

Viscosity in solution

Soluble DF is able to thicken or form gels in fluids due to entanglement of the polysaccharide chains within the fluid. Viscosity depends on intrinsic characteristics of the polysaccharide (amount of space occupied by the polymer, generally characterised by intrinsic viscosity), its concentration, the solvent and temperature (Guillon and Champ 2000). Generally, as the molecular weight or chain length of DF increases, the viscosity in solution also increases (Tungland and Meyer 2002). The viscosity of AX in solution is also determined by its degree of substitution and substitution pattern (Courtin and Delcour 2002).

Addition of bran or other DF ingredients can have an essential impact on the viscosity and other rheological properties of the dough (Sanz Penella et al. 2008; Wang et al. 2002). These properties are affected by bran particle size and solubility of DF (Collar et al. 2006; Noort et al. 2010; Rosell et al. 2010; Sanz Penella et al. 2008). The beneficial impact of the use of xylanolytic enzymes in baking is largely related to the increase in dough viscosity due to the increase in the content of WEAX (Courtin and Delcour 2002). The increase in dough viscosity by an endoxylanase treatment of bran was also related to the improved baking properties of oat and rice bran (Lebesi and Tzia 2012). However, too extensive degradation of WEAX to low MW oligo- or monosaccharides can have a detrimental effect on bread due to the decrease in dough viscosity (Courtin and Delcour 2002), and thus the enzyme selection and dosing are important factors determining the impact of the use of xylanases in baking. In extrusion, melt viscosity is an important factor affecting expansion, and the effects of soluble and insoluble DF on expansion have also been related to their effects on melt viscosity (Moraru and Kokini 2003; Pai et al. 2009). The improved expansion due to solubilisation of corn bran DF by an alkaline treatment was related to favourable changes in melt viscosity (Pai et al. 2009).

2. Aims of the study

Wheat bran is a nutritionally appealing and vastly available raw material, but is currently under-utilized as a food ingredient due to its technological and sensory challenges. Treatments with hydrolytic enzymes such as xylanases have been studied as a means to modify the properties of bran, but very little focus has been given to the impact of water content on the enzyme function and efficiency of these processes. Processing at reduced water content could be economically beneficial, especially when targeting dry end products. However, reduction of water content of an enzymatic process presents several challenges that need to be studied in order to be able to develop new low-water processes.

The present work aimed to examine and develop techniques to utilize hydrolytic enzymes at reduced water content in order to increase the technological functionality of wheat bran. More specifically, the aims were:

1. To determine and control the interactions between water content and physical and physicochemical properties of bran-water mixtures.
2. To examine the effects of water content on the reaction kinetics, mode of action and interplay of exo- and endogenous cell wall degrading enzymes, especially xylanases.
3. To develop practical applications for enzymatic treatment at low water content in order to improve the technological functionality of wheat bran, and to study the effects of the process on the technological properties of bran in a food application.

3. Materials and methods

A general outline of the materials and methods used in the work is presented in this section. Detailed descriptions can be found in the original Publications I–IV.

3.1 Raw materials

3.1.1 Cereal raw materials

Two different brans ground to different levels of fineness were used in the study (Table 6). The bran in Publications I and II was obtained from mixed wheat varieties, and the grains were peeled by friction using the Bühler Peeling technology to remove 2–3% of the grain outer layers before bran removal in order to reduce the level of contaminating microbes and enzymes in the bran. The bran made from the peeled grains was ground in an impact sieve mill to finer particle size before use. In Publications III and IV, commercial native wheat bran obtained from Finnish spring wheat was produced using a conventional roller mill and ground by TurboRotor technology to three different levels of fineness. The Turborotor grinding process is based on high air throughput, that keeps the product airborne during the grinding. The intensity of size reduction is controlled by the number of grinding components and by the rotation speed and rate of air flow. Rye endosperm flour was used as a base material for the expanded extrudates in Publication IV. Average particle size and DF, AX and starch content of the wheat brans and rye flour, analysed as described in Publications I–IV, are presented in Table 6.

Table 6. Cereal raw materials used in the study. Dietary fibre (DF), arabinoxylan (AX) and starch content expressed as % of bran dry matter.

	Bran from peeled grains	Native bran				Rye endosperm flour
		Unground	Coarse	Fine	Ultrafine	
Publication	I and II	III and IV				IV
Grinding	Impact mill	-	Turborotor technology			-
Average particle size (µm)	113±9	1001±9	702±59	327±9	81±2	nd
Total DF	49.5	48.0	48.9	47.9	48.4	11.8
soluble DF	-[nd]	3.1	3.5	4.1	4.6	9.6
Total AX	21.7±0.6	20.6±0.4	20.5±0.3	20.3±0.6	20.6±0.2	-[nd]
Water extractable AX	0.6±0.1	0.5 ±0.1	0.5 ±0.1	0.6 ±0.1	0.8 ±0.1	-[nd]
Starch	11.6±0.1	16.3±0.1	16.7±0.1	16.8±0.2	16.5±0.1	84.7±0.3
Ash	6.7±0.1	6.4±0.1	6.4±0.1	6.3±0.1	6.3±0.1	0.6±0.1

-[nd] not determined

3.1.2 Enzymes

Commercial hydrolytic enzymes, Depol 761P (Biocatalysts Ltd, Cardiff, UK), a xylanase preparation derived from *Bacillus subtilis*, and Veron CP (AB Enzymes GmbH, Darmstadt, Germany), a cellulolytic enzyme preparation with hemicellulase side activities from *Trichoderma reesei*, were used either individually or in combination for the bran treatments. The activity profile of the enzymes is presented in Table 7. Depol 761P was selected based on a screening study (Petersson et al. 2013) and its activity profile: xylanase with only few side activities (Table 7). Veron CP enzyme preparation was selected because of its high cellulase (filter paper) and endoglucanase activities that were not found in Depol 716P (Table 7). All activity measurements were performed at pH 5, 50 °C.

Table 7. Activity profile of the enzyme preparations used in the study (nkat/g).

Activity	Depol 761P	Veron CP	Substrate	Reference
Endoxylanase	28660	14610	1% birch glucurone xylan	Bailey et al. (1992)
Polygalacturonase	1317	8469	0.4% polygalacturone acid	Bailey and Pessa (1990)
β-glucanase	1625	75760	1% barley β-glucan	Zurbriggen et al. (1990)
α-amylase	44	94	p-nitrophenyl maltoheptaoside	Megazyme Ceralpha method
β-xylosidase	2	257	5mMp-nitrophenyl-β-D-xylopyranoside	Poutanen and Puls (1988)
Cellulase / filter paper	-[a]	53[b]	filter paper	IUPAC (1987)
Endoglucanase	-[a]	18974	1% hydroxyethyl cellulose	IUPAC (1987)
Mannanase	-[a]	3022	0.5% locust bean gum	Stålbrand et al. (1993)
β-glucosidase	-[a]	528	1 mM p-nitrophenyl-β-D-glucopyranoside	Bailey and Linko (1990)
α-arabinosidase	-[a]	530	2 mM p-nitrophenyl-α-L-arabinofuranoside	Poutanen et al. (1987)
Ferulic acid esterase	-[a]	-[a]	4 mM ethyl ferulate	Forssell et al. (2009)
Endoprotease	-[a]	-[a]	azurine-crosslinked casein	Protazyme AK tablet method, Megazyme International Ireland

[a]no activity detected. [b]activity expressed as filter paper units/g.

3.2 Enzymatic treatments of bran

Different reactor and mixing systems used in the study are summarized in Table 8 and shown in Figure 3. The enzyme powders were mixed with bran before the addition of pre-heated water. All incubations were performed at 50 °C, which was the temperature recommended by the supplier of the Depol 761P enzyme preparation. The enzyme preparations were dosed according to their xylanase activity at 20 or 200 nkat/g bran dry matter (DM) (treatments with Depol 761P, Publications I–IV) or 100 nkat/g (treatments with Veron, CP, Publication IV). For the treatments with a combination of the two enzymes the dosages as xylanase activity were 200 nkat Depol 761P and 100 nkat Veron CP /g bran DM. Corresponding treatments without addition of enzymes were performed for each bran incubation process.

Table 8. Summary of the bran treatments.

	Reactor/ mixing type	Treatment water content (%)	Incubation time	Stirring during incubation	Publication
Low-water treatments	Horizontal mixing with z-blades	20–70	1–24 h	60–63 rpm	I and II
	Twin-screw extrusion[a]	37–60	0 or 4 h	no	III and IV
	Vertical mixing with K-blade[a]	37–60	0 or 4 h	no	III
High-water treatments	Vertical double-blade mixing	80 and 90	1–24 h	160 rpm	I and II
	Shaking	92	4 h	120 rpm	III

[a] Used for pre-mixing (3 min) and forming the material before stationary incubation.

In Publications I and II, the enzymatic treatments of bran at water contents of 20–70% were performed in a farinograph mixing bowl (Brabender Farinograph, mixer type S300 with z-blades) using continuous mixing (60–63 rpm). The bran was placed in the mixer, and pre-heated water was added by spraying during 1–3 min with rotating blades in order to obtain an even distribution of water. The treatments at water contents of 80 and 90%, due to the liquid form of the mixture, were performed with continuous double-blade mixing (speed 160 rpm) in a sealed vertical steel container placed in a water bath (50 °C). The reactions were stopped by cooling down and freezing the sample immediately. Subsequently, part of the frozen sample was freeze dried and ground in a laboratory mill (0.5 mm sieve) for later analyses.

In Publications III and IV, the enzymatic treatments at water contents of 37–60% were performed using two different pre-mixing and forming methods, blade-mixing and extrusion, followed by stationary incubation (i.e. without stirring), whereas the treatments at high water content (92%) were performed with continuous shaking. For the extrusion-aided treatments, a pre-conditioned (20% moisture) bran mixture was fed to the extruder (APV MPF 19/25, Baker Perkins Group Ltd, Peterborough, UK) at a rate of 26 g/min. The barrel temperature was 50 °C and screw speed was 65 rpm. Water was pumped to the barrel at an appropriate rate in order to obtain the intended moisture content in the bran mixture. The residence time inside the barrel was about 3 min. Bran mixture was collected from the die exit (diameter 3 mm) for 2 min and either immediately frozen in liquid nitrogen or transferred to incubation (4 h in sealed containers) or to drying, which was performed either in an oven (samples spread on metal trays and dried with air circulation at 50°C for 18–20 h) or by freezing the sample in liquid nitrogen for subsequent freeze drying. The incubated samples were also immediately dried by oven drying or by freeze drying. For the treatments without extruder treatment (hereafter referred to as 'blade-mixed treatments') at water contents of 37–60%, the pre-conditioned bran sample was pre-heated in a heating chamber at 55 °C for 12 min, after which it was brought to the intended moisture content by spraying pre-heated water while mixing with a Kenwood mixer (K-blades, speed setting 2) for 3 min at 55 °C. The bran mixture was divided into samples which were either frozen immediately in liquid nitrogen or incubated further in sealed containers at 50 °C for 4 h and then frozen in liquid nitrogen. The treatments at high water content (92%) were performed in centrifuge tubes with continuous shaking (120 rpm), after which the reaction was stopped by freezing the sample in liquid nitrogen.

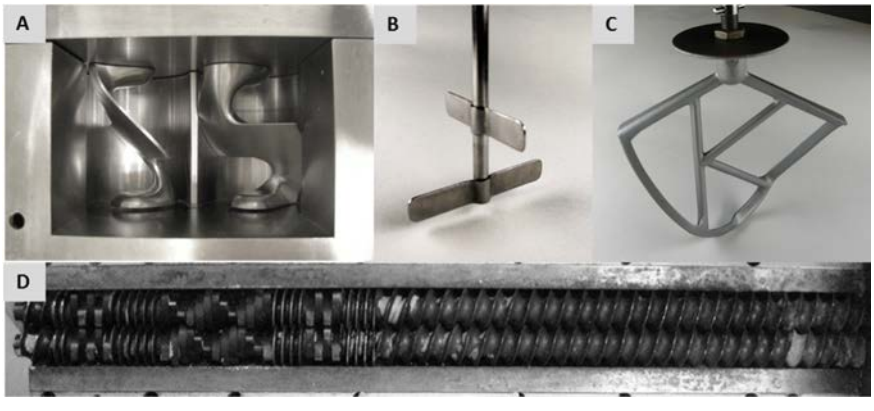

Figure 3. Mixing equipment used in the study. Horizontal z-blades (Farinograph) (a), vertical double-blade (b), vertical K-blade (c), twin-screw extruder (d).

3.3 Analysis of bran samples

3.3.1 Consistency, water activity and pH of bran-water mixtures

Changes in textural properties of the bran sample during the first 60 min of the treatments in Publication I were followed as Farinograph torque values reflecting the resistance of the bran-water mixture to the mixing blades. The water activity at the end of 24 h bran treatment was determined from a fresh sample using AquaLab CX2 (Decagon Devices Inc., USA). The pH of treated bran was measured by solubilising 0.2 g of the freeze dried sample in 10 ml of distilled water and stirring for 15 min prior to measurement.

3.3.2 Particle size, microstructure and hydration properties of bran

Average bran particle size was measured by laser light diffraction using either a Coulter LS320 particle size analyser (Coulter Corporation, Miami, FL, USA) or a Mastersizer 3000 (Malvern, Worcestershire, UK). Samples were measured either from dry bran dispersion (Publication I and untreated brans in Publication III) or after dispersing the samples in water (Publication II and treated samples in Publication III) or in ethanol (Publication IV). Mean or median particle sizes were calculated from the volumetric distribution of the particles using the Fraunhofer optical model.

For the microscopy analyses in Publication II, the bran samples were pretreated as described by Dornez et al. (2011) and stained and imaged using exciting light as described by Andersson et al. (2011). For imaging the sample, sections (2 µm) were stained either with Light Green and Lugol's iodine solution, which stain protein green and starch purple, respectively, or with Acid Fuchsin and Calcofluor White, which stain protein red and β-glucan-rich cell walls light blue, respectively.

Water binding capacity (WBC, termed as approximate WHC in Publication I) of the bran was analysed by dispersing the bran sample in water, followed by 60 min incubation (Publication I) or 30 min shaking (Publication II) at room temperature, and centrifugation. After removing the supernatant, the weight addition (plus the water contained initially in the sample) per gram of bran sample dry matter was the WBC of the sample. Water holding capacity (WHC) and its kinetics were determined using the Baumann apparatus (Baumann 1966) as described in Publication II using a sample size of 75 mg (Publication II) or 50 mg (Publications III and IV). The water uptake was recorded for 25 or 30 min at room temperature.

3.3.3 Analysis of arabinoxylan and monosaccharides

For the analysis of WEAX in Publications I and IV, dry bran sample was extracted with distilled water for 15 min in cold water (4 °C) in order to avoid enzymatic activity

during the extraction. After centrifugation, the supernatant was boiled and centrifuged again. In Publication III, the wet (frozen) bran samples were dispersed and extracted with glass beads as described in Publication III. The contents of AX and pentose monosaccharides in bran water extracts (i.e. WEAX), were determined by a colorimetric phloroglucinol method (Douglas, 1981) using xylose as a standard (Publications I, III and IV). Free pentose sugars were corrected by a factor of 0.88 to anhydro sugars. The degree of solubilisation (DS) was calculated by dividing the WEAX content of the sample by the total AX content of the bran. For the quantification of total AX (Publications I and III), bran sample was mixed with 0.5 M H_2SO_4 and boiled for 20 or 30 min and centrifuged, followed by the colorimetric determination (Douglas 1981).

For the analysis of water-extractable monosaccharide composition (Publication II), the dry bran sample was extracted as described above and the supernatant was hydrolysed with 3.75 M H_2SO_4 at 100° C for 2 h. The hydrolysate and monosaccharide standards were analysed as their alditol acetates as described by Blakeney et al. (1983) by gas chromatography (GC) using an Agilent 6890 GC equipped with a flame ionization detector (Agilent, Palo Alto, CA, USA). The column was a DB-225 [30 m × 0.32 mm; film thickness 0.15 µm (Agilent)]. The monosaccharides were identified according to their retention times and quantitated with corresponding standard curves. Free hexose sugars were corrected by a factor of 0.9 to anhydro sugars, and pentose sugars by factor of 0.88. For the analysis of total monosaccharide composition, dry bran sample was hydrolysed with H_2SO_4 at 100 °C as described in Publication II and the supernatant was acetylated and analysed by GC.

3.3.4 Molecular weight analysis of WEAX by HP-SEC

The apparent WEAX molecular weight (MW) distribution of bran in Publications I and III was analysed by high performance size exclusion chromatography (HP-SEC). In Publication I, the samples were extracted in boiling water, and starch and β-glucans present in the water extract were hydrolysed by a saccharifying enzyme solution (Optidex L-400, Genencor International). In Publication III, the analysis of the apparent MW distribution of WEAX was performed by also removing other poly- and oligomeric compounds from water extracts by an enzymatic procedure, followed by EtOH precipitation, using a method modified from that of Andersson et al. (2009) as described in Publication III. The liquid chromatograph with Alliance 2690 separation module and M-2414 refractive index detector consisted of three columns (7.8 × 300 mm) µHydrogel 500, µHydrogel 250 and µHydrogel 120 (Waters Inc., Milford, MA, USA). The suitability of the conditions of the HP-SEC analysis was previously checked using a dual angle laser light scattering detector (Precision Detectors, USA) that is sufficiently sensitive to detect aggregates (not observed) and by using flow rates of 0.2 ml/min and 0.5 ml/min. The results were similar at both flow rates, indicating that the sample does not hydrolyse during the analysis. Pullulan standards and maltopentaose were used for calibration. In Publication III, the average MW of the sample was calculated between 32.7 min (the

elution point of the largest standard) and 53.0 min (corresponding to a MW of approx. 2 kDa).

3.3.5 Analysis of residual endoxylanase activity in bran

For the calculation of the percentage of residual endoxylanase activity in the enzyme-treated bran samples in Publication I, the bran samples were extracted with phosphate buffer (25 mM, pH 6.0) during 60 min at room temperature, and the endoxylanase activity in the supernatant was analysed by Xylazyme AX Tablet assay (Megazyme, Ireland) using a reaction time of 60 min. The initial activity was calculated as the sum of the activity of the added xylanase plus the endogenous activity analysed for the bran.

3.3.6 Sequential extraction and analysis of proteins

Proteins of the bran samples were examined in Publication II using a sequential buffered extraction procedure. Salt-soluble proteins were extracted at 4 °C with 0.5 M Tris-HCl buffer (pH 8) containing 1 M NaCl and the residual protein fraction was extracted from the unsoluble sediment at 50 °C with a mixture containing 2% sodium dodecyl sulphate (SDS), 10% glycerol, 1.5% dithiothreitol (DTT), and 0.05 M Tris-HCl pH 8 buffer (repeated once). The different protein fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and visualized with a Criterion stain-free imaging system (Bio-Rad, Hercules, CA, USA) as described in Publication II. The protein content of all extracts was analysed by a commercial kit (RC DC Protein Assay, Bio-Rad).

3.4 Production and analysis of expanded extrudates

In Publication IV, bran-supplemented expanded extrudates were produced from mixtures of bran and rye endosperm flour (20:80 ratio) using a twin screw extruder (APV MPF 19/25, Baker Perkins Group Ltd, Peterborough, UK) with a 3 mm diameter die. Rye endosperm flour was selected as base material because of its good expansion properties and nutritional profile (DF content 11.8%). Extrusion parameters were selected based on pre-trials performed with the flour base material to obtain maximum expansion (feed rate 60 g/min, screw speed 450 rpm, temperatures at the barrel zones 1 to 4 set at 80, 90, 120 and 130 °C). The water feed rate was adjusted to attain a moisture content of 16%. The extrudates were collected in trays and dried at 50°C for 30 min in an oven drier with air circulation. The extrusions were performed in duplicate.

For the analysis of macrostructural parameters, the extrudates were cut into 20 pieces each of 5 cm length using a band saw (Scheppach, Germany). Expansion rate (ER), specific length and piece density of each sample were calculated as described by Alam et al. (2013).

Mechanical properties of the extrudates were measured by applying uniaxial compression using a texture analyser (TA.XT plus, Stable Micro Systems Ltd., United Kingdom) containing a 30 kg load cell and a 25 mm aluminium probe under 70% strain with a test speed of 1 mm/s. All extrudates were cut into 10 mm pieces (radial section) and equilibrated at 43% relative humidity (RH) at 21 °C prior to analysis. Measurements were made for 20 replicates. Exponent software version 6.0.7.0 (Stable Micro Systems Ltd., United Kingdom) was used to obtain values for calculation of the hardness indicators, F_{max} (the maximum point of the force-deformation curve) and crushing force (F_{cr}), i.e. average puncturing force (van Hecke et al. 1998), and crispiness index (C_i) (Heidenreich et al. 2004).

For imaging of the radial cross-sections of the extrudates, the samples were cut into 10 mm pieces and examined with a SteREO Discovery.V8 stereomicroscope (Carl Zeiss MicroImaging GmbH, Germany) and imaged using a DP-25 single chip colour CCD camera (Olympus Life Science Europa GmbH, Germany) and the Cell^P imaging software (Olympus).

For the analysis of total and soluble DF contents, the extrudates were ground in a laboratory mill with a 0.5 mm sieve and analysed by AOAC method no. 2009.01 (McCleary et al. 2013).

3.5 Statistical analysis

All bran treatments were made in duplicate, and each sample was analysed at least in duplicate. Thus all the results were calculated as means of at least four analysis results. In Publication III, the parameters of macrostructure and mechanical properties of the extrudates were calculated as means of 35–40 results. Data were subjected to analysis of variance using IBM SPSS Statistics 19 (IBM Corporation, Somers, NY, USA), and significant differences ($P < 0.05$) between individual means were identified by the Tukey's test (Publications II, III and IV). All replicates (analytical and treatment replicates) were considered as equivalent replicates in the variance analysis. In Publication IV, correlations between different variables were determined by subjecting the mean result values to the 2-tailed Pearson's bivariate correlation analysis.

4. Results and discussion

4.1 Impact of water content on xylanase action and properties of bran during continuous mixing (Publications I and II)

4.1.1 Consistency, microstructure and pH of bran-water mixtures

The impact of water content, ranging from 20 to 70%, on the action of xylanase and on the properties of bran-water mixtures during continuous mixing was studied using a horizontal mixer (Farinograph) with z-blades. The WHC of the bran used was 3.6 g water/g bran DM, from which it can be calculated that bran could hold all the added water up to a water content of 78%. Thus, at the water contents between 20 and 70% there was no free bulk water, which was reflected in the appearance and consistency of the mixtures (Table 9). When bran was mixed in the Farinograph at different water contents between 20 and 70%, the mixture transformed from granular powder to a paste at a water content of 40%. At this 'transition' moisture content, the bran-water mixture formed a very compact, plastic-like mass during the 24 h treatment (Figure 4). According to the Farinograph resistance values recorded during the first 60 minutes of the treatments (Table 9), the viscosity of the paste increased with increasing water content up to a water content of 50%, and then at higher water contents the viscosity again decreased. Addition of xylanase increased the resistance values, especially at a water content of 40%, but did not affect the water activity or the visual appearance of the mixtures (Table 9). For comparison, the bran was also treated with a traditional high-water treatment using vertical blade-mixing, since the free water present in the bran mixtures prevented the use of the Farinograph as a mixing device at water contents of 80 and 90% because of leakage of water. A solids content of 15% (i.e. water content of 85%) is generally considered to be the upper limit of efficient mixing of lignocellulosic slurries in conventional stirred-tank reactors (Hodge et al. 2009; Kristensen et al. 2009). In the current study the use of a blade mixer for wheat bran slurries of water content below 80% was not possible because of too high viscosity of the mixtures.

Table 9. Water activity and appearance of bran after 24 h treatment with continuous mixing without added xylanase, and resistance values of bran mixtures after 60 minutes with different xylanase dosages of Depol 761P (compiled from Publication I).

Water content	Water activity	Appearance	Resistance value at 60 min (FU)		
			No added enzyme	Depol 761P xylanase 20 nkat/g	Depol 761P xylanase 200 nkat/g
20	0.83	powder	60	60	– [b]
30	0.89	powder	110	150	200
40	0.93	paste	240–270	330–400	400–550
50	0.96	paste	280–310	310–340	310–340
60	0.97	paste	140	130	– [b]
70	0.98	paste	40	40	– [b]
80	≥0.98[a]	slurry	– [b]	– [b]	– [b]
90	> 0.98[a]	liquid	– [b]	– [b]	– [b]

[a] With the measurement system used it was not possible to determine the accurate water activity of samples with high amounts of free water. [b] Not determined.

Figure 4. Bran mixture at a water content of 40% in a Farinograph mixer after 24 h treatment with xylanase enzyme preparation Depol 761P (20 nkat/g) (Publication I).

Examination of the microstructure of the bran by staining protein and cell wall structures with specific dyes showed that the layered cell wall structure of the bran treated at 40% water content with or without added xylanase preparation was much more degraded as compared to the bran treated at 90% water content (Figure 5). In the bran treated at 40% with no added enzymes, the structure of aleurone cells had broken, and seed coats with pigment strand and nucellar epidermis had separated from the aleurone layer. The compact consistency of the sample at a water content of 40% presumably enhanced the impact of mixing shear and caused the physical degradation of bran cell walls. The use of xylanase enzyme preparation Depol 761P (200 nkat/g) caused further degradation of the bran cell wall structure and the separation of pigment strand and nucellar epidermis from

each other, whereas in the samples treated at 90% water content the layer of pericarp, pigment strand and nucellar epidermis still remained attached to the aleurone cell layer and intact aleurone cells were still detectable, especially in the sample treated with no added xylanase. Processing of bran also caused a significant release of proteins from the aleurone cells of the bran particles, especially after the treatments at 40% water content, but the change was also detectable (although to a lesser extent) at 90%. The bran micrographs stained with Light Green and Lugol's iodine solution further showed that starch granules present in the bran sample were not significantly affected by any of the treatments (data not shown).

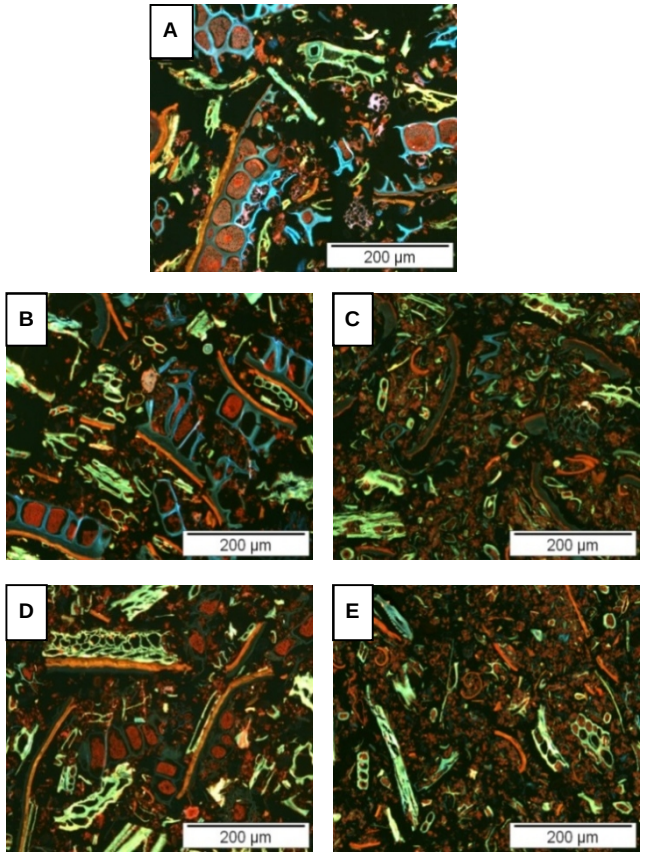

Figure 5. Microstructure of untreated bran (a) and bran samples treated for 24 h without added enzymes at 90% water content (b) and without added enzymes at 40% (c), and bran samples treated for 24 h with xylanase at 90% (d) and with xylanase at 40% water content (e). Micrographs, prepared from fresh (frozen) bran samples, were stained with Acid Fuchsin and Calcofluor White. β-Glucan-rich endosperm and aleurone cell walls appear in blue, pigment strand (between the pericarp and aleurone layers) in orange, pericarp layer in light green and yellowish, and proteins in red and reddish brown (Publication II).

The initial pH of the bran-water mixture was 6.8, and during the first 16 h of incubation the pH remained between 6.2 and 6.8 regardless of water content (Publication I). After 24 h, however, pH decreased to 4.7 at the highest water content (90%), indicating the growth and metabolic activity of acid-producing bacteria, such as lactic acid bacteria. Bran material naturally contains rather high amounts of yeasts and bacteria, but peeling of grain before bran removal is known to decrease the microbial load of bran (Katina et al. 2007). The use of bran from peeled grains and the relatively high temperature of the treatment (50 °C) were assumed to restrict the interference of microbes, but apparently some microbial growth occurred after long incubation times at the high water content. By contrast, in the treatments at decreased water contents the low a_w probably restricted the activity of these microbes, as the pH did not significantly change at the lower water contents (Publication I).

4.1.2 Action of xylanase during continuous mixing

The effect of water content on the action of xylanase on bran during continuous horizontal mixing with z-blades (Farinograph) was examined by analysing the content of WEAX after 24 h treatment (Publication I). WEAX content did not increase during the treatment at the lowest water content studied (20%), but remarkable increase in WEAX content occurred already at a water content of 30% (Figure 6). This indicates that the minimum required water content for the action of xylanase in the bran-water mixture was between 20 and 30%. The availability of water is better indicated by water activity, and most enzymes generally require a_w ≥0.85 for catalytic activity (Simpson et al. 2012). This was also the case in the current study, as a_w was 0.83 at a water content of 20% and increased to 0.89 at 30% (Table 9). Lee (1970) also reported that the limiting water content for hydrolytic enzyme reactions in wheat flour was about 30% of water, although the a_w at this point was higher (0.958) than in the current study. The range of approximately 25–30% of moisture has been defined as the saturation point below which the major part of the water in lignocellulose is present as primary or secondary bound water (Felby et al. 2008), and similar values (28–30%) have also been reported for wheat flour, as reviewed by Lee (1970).

Solubilisation of AX was highest at the water contents of 40 and 90% (6.9% WEAX of bran DM at both water contents, corresponding to DS of 32%), whereas at intermediate water contents (50–80%) the solubilisation was lower (Figure 6). Effects of treatment water content and the use of xylanase on AX solubilisation were also studied as a function of incubation time and xylanase dosage (20 and 200 nkat/g) at water contents of 30, 40, 50 and 90% (Publication I). No unexpected differences were observed in the rate of AX solubilisation with different xylanase dosages at different water contents. Generally the higher dosage caused significantly higher WEAX content and most of the WEAX was hydrolysed within 4 h incubation time, after which the solubilisation rate clearly reduced. An exception was the faster initial AX solubilisation at 90% water content during the first hour,

and the relatively higher increase in AX solubilisation with the higher enzyme dosage at the water content of 30% as compared to the other water contents studied (Publication I).

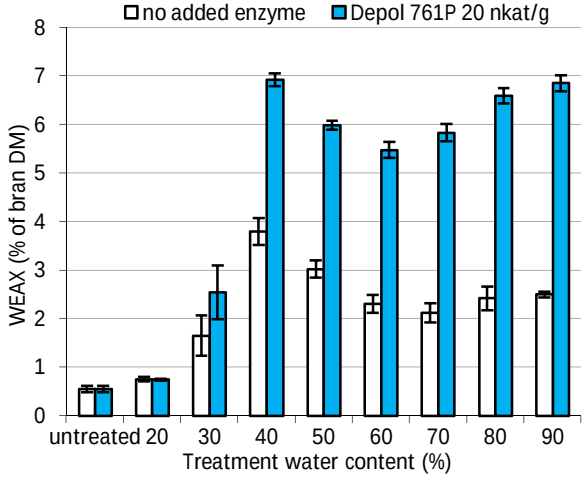

Figure 6. Effect of treatment water content and Depol 761P xylanase (20 nkat/g) on the WEAX content of wheat bran after 24 h incubation (Publication I).

The enhanced AX solubilisation at 40% water content was interesting, because decreasing the water content below 85% is known to decrease enzyme action on lignocellulosic biomass (Kristensen et al. 2009; Modenbach and Nokes 2013). There are only a few previous publications in which the modification of plant-based substrates with hydrolytic enzymes has been studied at solids contents above 40%, i.e. below the water content of 60% (Moore et al. 2006; Steinmacher et al. 2012), except for the use of enzymes in baking and hydrolysis of starch in screw reactors (Baks et al. 2008; Linko 1989). The reasons for the decreased enzyme action at increased solids content are generally related to the lack of available water, difficulties with mixing, insufficient mass and heat transfer, and increased concentration of inhibitors (Modenbach and Nokes 2013). It is probable that increased amount of available water and decreased viscosity at the water contents between 60 and 90% have improved the mass transfer and diffusion of components, and thus improved AX solubilisation. In this respect, the enhanced AX solubilisation at the water content of 40% was unexpected. However, the increase in AX solubilisation at the water content of 40% was evidently caused not only by the action of added xylanase but also by other factors, as even without added xylanase the solubilisation was also notably higher at the water content of 40% (3.8% WEAX in bran DM) than e.g. at 90% (2.5% WEAX) (Figure 6). It could be concluded that the compact consistency of the material at 40% water content probably enhanced AX solubilisation by physical breakdown of bran cell walls due

to shear forces, which was also indicated by the microscopic analysis. It has been shown that mechanical size reduction may cause solubilisation of DF and AX in wheat bran (Hemery et al. 2011; Zhu et al. 2010; Van Craeyveld et al. 2009).

The content of WEAX in selected bran samples was analysed by GC (Table 10), and the results were well in accordance with those determined by the colorimetric method (Publication I). The fact that some of the arabinose residues might have originated from arabinogalactan peptides (AGP) was not taken into account in the calculations of AX levels. No data is available concerning the content or presence of AGP in bran, but in wheat flour the contents of WE-AGP have been reported to be around 0.15–0.38% of flour DM (Andersson et al. 1994; Loosveld et al. 1997), which is very low compared to the level of AX in bran (generally in the range of 13–30%). Thus, it can be concluded that the increase in the content of water extractable pentose sugars (Publication I), or of arabinose and xylose (Publication II), was due to solubilisation of AX.

The analysis of A/X ratio (Publication II) provided new information about the role of enzymes in the solubilisation of AX during the treatment at the water content of 40%. The A/X ratio of the water extract of bran treated for 24 h at 40% was 0.32 (Table 10), which was in the range of values reported in the literature for enzymatically solubilised wheat bran AX (0.27–0.32) (Beaugrand et al. 2004; Swennen et al. 2006), whereas AX oligosaccharides produced by mechanical treatment have been reported to have much higher A/X ratios (0.65–0.72) (Van Craeyveld et al. 2009). The result suggested that although physical shear enhanced AX solubilisation at 40%, the origin of WEAX was presumably still due to enzymatic action on AX fragments with low arabinose substitution, occurring mostly in the aleurone (A/X 0.3–0.5) and in the nucellar epidermis (A/X 0.1), rather than to direct mechanical solubilisation of AX from the more substituted outer tissues (A/X≥1.0) (Antoine et al. 2003; Barron et al. 2007; Van Craeyveld et al. 2010). During the treatments at both water contents with and without added enzymes, the A/X ratio of the bran water extract decreased almost identically with increasing WEAX content, suggesting that AX was solubilised from the same bran tissues regardless of the processing conditions used (Publication II). The shear forces and the physical breakdown of bran particles could have enhanced the action of endo- and exogenous enzymes by improving the availability of the substrate, e.g. by increasing its surface area. It has been shown that decreasing the particle size of plant materials may enhance their enzymatic hydrolysis (Silva et al. 2012; Niemi et al. 2012; Mahasukhonthachat et al. 2010; Dasari and Berson 2007). The impact of bran particle size was further studied in Publication III and is discussed in section 4.2.2.

Table 10. Content and A/X ratio of WEAX in bran after 4 and 24 h treatment with Depol 761P xylanase at water contents of 40 and 90% as analysed by GC (Publication II) and the level of residual endoxylanase activity in bran water extract (Publication I).

Treatment water content (%)	Treatment time	WEAX[a] (% of bran DM)	A/X[a]	Endoxylanase activity (% of initial[b])
90	4 h	9.1±0.6	0.40±0.02	70±2
	24 h	11.1±0.5	0.36±0.01	64±4
40	4 h	8.5±1.1	0.41±0.02	45±3
	24 h	12.1±0.5	0.32±0.01	10±1

[a] xylanase dosage 200 nkat/g, [b] xylanase dosage 20 nkat/g.

The mode of action of xylanase was further studied by analysing the residual endoxylanase activity and the apparent MW distribution of WEAX in purified bran water extracts. The recovery of xylanase activity in water extracts of enzyme-treated (200 nkat/g) bran samples was only 10% of the initial activity after 24 h treatment at the water content of 40% (Table 10). The recovery was higher both at lower and higher water contents than 40%, and the highest recovery after 24 h treatment, 64%, was detected in bran treated at 90% water content. This was unexpected in view of the degree of AX solubilisation, but it is possible that the compact structure of the material and efficient binding of enzyme to the substrate at the water content of 40% prevented the extraction of the enzyme from the freeze dried sample in the enzyme activity assay used, thus giving lower activity values.

The analysis of the apparent MW distribution of purified bran water extract indicated that the amount of large WEAX polymers was higher after treatments with continuous mixing at 40% than at 90%, both with and without added xylanase (Publication I). This suggests that although the degree of AX solubilisation was the same (when added xylanase was used) or even higher (without added xylanase) at the low water content as compared to the high water content treatments, the depolymerisation efficiency of xylanase was higher at the water content of 90% than at 40% when continuous mixing was used. According to Sibakov et al. (2013), the use of a water content of 90% for enzymatic hydrolysis of β-glucan resulted in rapid breakdown into short oligosaccharides, whereas low water content (50%) enabled a more easily controlled depolymerisation of high MW β-glucan. Van der Veen et al. (2005) also reported that increased dry matter content in the hydrolysis of maltodextrins resulted in increased formation of condensation products so that both the quantity and the length of the product polymers increased. Similarly, Hardt et al. (2013) reported that high solids concentrations in wheat gluten hydrolysis resulted in a higher weight fraction of peptides with a high molecular mass than low solid concentrations. This was ascribed to high viscosity

and mass transfer limitations (Hardt et al. 2013), and the same attributes probably caused the decreased depolymerisation efficiency of xylanase in the current study.

4.1.3 Physicochemical properties of bran treated with continuous mixing

Solubility of carbohydrates

The impact of enzyme treatment of bran with continuous mixing at high (90%) and low (40%) water content on the solubilisation of carbohydrates was followed by analysing the monosaccharide composition of the water extracts of freeze dried bran samples after acid hydrolysis (Publication II). After 24 h treatment at a water content of 40% the total water-extractable monosaccharide content (19.0% of bran DM) was higher than after the treatment at a water content of 90% (10.0% of bran DM). This was mainly due to the glucose content, which increased at 40% but did not significantly change at 90% after 4 h treatment. An increase in the soluble glucose content at 40% indicates the degradation of starch, cellulose, or β-glucan, probably due to the physical degradation of the bran. The total monosaccharide composition of bran (glucose 24.0±0.8%, galactose 1.16±0.04%, mannose 0.63±0.04%, AX 22.8±0.6%) was not affected by any of the treatments, except for the glucose content, which decreased to 21.3±1.3% after 24 h treatment at 90% both with and without added xylanase. The decrease in glucose content during the treatment at the water content of 90% was probably caused by metabolic activity of contaminating microbes, as was also indicated by the decrease of pH after 16 h treatment at the water content of 90% (section 4.1.1).

Solubility and molecular weight of bran proteins

The impact of bran treatment with continuous Farinograph mixing on the solubility and molecular weight of bran proteins was analysed by sequential extraction and electrophoretic analysis of salt-extractable and SDS+DTT-extractable (residual) proteins (Publication II) and by the spectrophotometric quantification of protein in the extracted fractions (Figure 7). The physicochemical characteristics of bran proteins are not yet well established, although the protein content of bran is relatively high, generally around 15–17% (Kamal-Eldin et al. 2009; Maes and Delcour 2002). The spectrophotometric quantification indicated that the concentration of salt-extractable proteins decreased during the bran treatments, especially during the treatments at 40% water content, whereas the concentration of SDS+DTT-extractable residual protein increased during the bran treatments (Publication II). The results of the SDS-PAGE analysis (Figure 7) were in agreement with the quantification results. In the salt-soluble fraction the proteins above 25 kDa had disappeared or appeared as lighter bands in the treated bran samples. In the residual protein fraction of the treated bran samples, protein “dust” occurred in the area above 100 kDa, whereas in the untreated bran no protein was observed in this area. Furthermore, especially in the case of the samples treated at 40%, a

large amount of protein had remained in the wells of the electrophoresis gel, indicating the formation of large protein-containing aggregates. The SDS+DTT-extractable fractions also contained small proteins of molecular weight 10–20 kDa not detectable in the initial bran (Publication II).

Protein aggregation and depolymerisation were probably caused by the high shear exerted on the bran especially during the treatment at a water content of 40%. Because electrophoresis was performed in denaturing conditions, which leads to the reduction of disulphide bonds during preparation of samples for SDS-PAGE, the high molecular weight protein aggregates were presumably caused by the formation of covalent bonds during the bran treatments. It is known that heating and shearing may cause degradation or aggregation of proteins by disulphide or covalent bond formation, for example in extrusion processing, and several authors have reported a decrease in protein extractability after extrusion, as reviewed by Anderson and Ng (2000). Bran is known to contain endogenous proteases, but they generally activate below pH 5 (Loponen et al. 2004) and thus the depolymerisation observed in the residual fraction was probably not caused by endogenous proteases, since the pH decreased below 5 only after 24 h treatment at 90% (Publication I). The observed decrease in the solubility of bran proteins supports the hypothesis that the decreased level of residual endoxylanase activity in the water extract of bran treated at 40% was caused by limited extractability of enzyme proteins from the bran sample. The recent study of Nordlund et al. (2013) showed that bioprocessing by sequential treatments with cell-wall hydrolysing enzymes and yeast fermentation caused release of protein from aleurone cells, assessed as a higher content of soluble protein in bran and a higher hydrolysis rate *in vitro*. The solubilisation and digestion of bran-associated proteins were explained both by the yeast fermentation and the treatment with the hydrolytic enzyme mixture, which also contained low endoprotease activity. In the current study, the enzyme preparation used was free of endoprotease activity, and the use of the enzyme had no visible effect on the electrophoretic patterns of proteins at either water content. Similarly, the use of xylanase had no significant impact on the content of protein in the salt-extractable and residual fractions analysed by the spectrophotometric quantification.

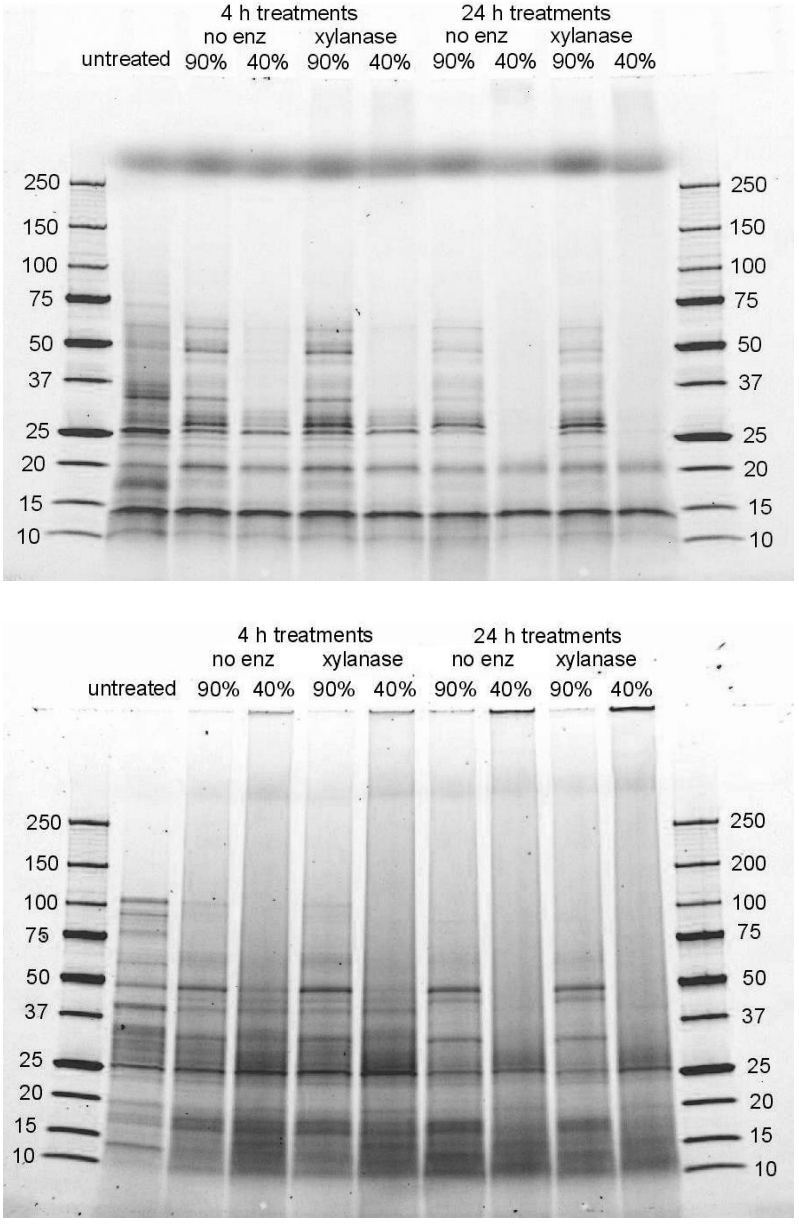

Figure 7. SDS-PAGE patterns of salt extractable (a) and SDS+DTT extractable (b) (residual) proteins of untreated bran and bran samples treated at water contents of 40% and 90% with and without Depol 761P xylanase (200 nkat/g) (Publication II).

Hydration properties and particle size of xylanase-treated bran

Analysis of the hydration properties of brans treated at 40% and 90% (Publication II) showed that the treatments at 40% water content resulted in lower WHC and WBC of the freeze dried sample than the corresponding treatments at 90% water content (Table 11). This may be due to the significant decrease of the bran particle size (especially upon grinding the freeze dried processed sample) during the treatment at 40% (Table 11). Reduction of particle size of bran and other DF preparations generally decreases WBC due to the decrease in the trapped volume as a consequence of structure collapse (Noort et al. 2010; Zhu et al. 2010; Thebaudin et al. 1997). During the treatment at 40% water content, transformation of the bran-water mixture into a compact, plastic-like mass probably enhanced the impact of the mechanical shear during the treatment in the Farinograph mixer, and caused more severe particle size reduction than the blade-mixing treatment at the water content of 90%.

The WHC of the bran increased at 90% after 4 h treatment without enzymes. This might be due to swelling of the bran in water. The use of freeze drying preserved the sample with minimal structural damage. The use of xylanase decreased WHC at both water contents as compared to the corresponding treatments with no added enzymes (Table 11), which was expected as WUAX is known to bind more water than WEAX (Courtin and Delcour 2002). The presumed degradation and solubilisation of other DF components, such as β-glucan, probably also affected the WHC in the same way as the solubilisation of AX by binding less water.

Hydration properties were measured using two different methods; WHC with the Baumann method and WBC with a centrifugation method. In the Baumann method no external force is used and the measurement is based on the principle of the diffusion of a liquid by capillary action, and thus it also describes the kinetics of water movement. However, the samples did not significantly differ in the kinetics of water holding, as observed from the estimated slopes of the WHC curves (Publication II). WBC of the bran changed during the treatments in a similar manner as the WHC (with minor exceptions), but WBC was always 0.6–0.9 units lower than the WHC of the same sample (Table 11), probably because WHC also includes the proportion of water loosely associated with the fibre matrix, whereas WBC includes only strongly absorbed water. WBC might have greater practical significance than WHC, because food manufacturing processes typically utilise some form of physical stress such as mixing, stirring, kneading or homogenization (Tungland and Meyer 2002). On the other hand, in the centrifugation method, the water-soluble components of the sample are lost in the supernatant, and thus the result describes only the hydration properties of the insoluble solids and may also depend on the g-force used (Chaplin 2003).

Table 11. Mean particle size (µm), water binding capacity (g water / g bran DM) and water holding capacity (g water / g bran DM) of untreated bran and bran samples treated at 40% and 90% water content (determined from freeze dried and ground samples). Values marked with different letters within the same column are significantly different (P < 0.05) (Compiled from Publication II).

	Treatment water content (%)	Treatment time	Mean particle size (µm)	Water binding capacity (g water /g bran DM)	Water holding capacity (g water /g bran DM)
		untreated	113 a	2.8 a	3.6 b
With no added enzyme	90	4 h	110 a	3.0 a	3.9 a
		24 h	94 b	3.0 a	3.6 ab
	40	4 h	56 c	2.5 b	3.2 c
		24 h	37 d	2.3 bc	3.0 d
With Depol 761P xylanase (200 nkat/g)	90	4 h	65 c	2.1 c	2.9 d
		24 h	60 c	2.3 bc	2.9 d
	40	4 h	37 d	1.7 d	2.4 e
		24 h	26 e	1.3 e	2.0 f

4.2 Impact of properties of bran-water mixture on xylanase action during stationary incubation (Publications III and VI)

In the first part of the work (Publications I and II), the efficient xylanase action at a water content of 40% was attributed to the compact consistency of the bran-water mixture that enhanced physical degradation of bran during continuous mixing. The role of the physical form of the substrate mixture (continuous mass vs. powdery/granular material), and the impact of bran particle size on xylanase action were further studied by comparing two different pre-mixing and forming methods, blade-mixing and extrusion, on xylanase action during stationary incubation of wheat bran (Publication III).

4.2.1 Physical form and particle size of bran after pre-mixing by extrusion and blade-mixing

The consistency of bran-water mixtures by vertical blade-mixing and extrusion was examined at water contents of 37–60% (Table 12). In the extrusion treatments, the bran mixture was forced through a small die after intensive mixing in the extruder barrel, causing the formation of uniform ‘sticks’ of moist bran mass (Figure 8). When using vertical blade-mixing with the coarsely ground bran, the material remained in the form of moist granular material at all water contents studied (37–60%)

(Table 12, Figure 8). By contrast, in the case of the fine bran, the blade-mixed bran-water mixture remained in the form of a moist granular powder up to a water content of 48%, but at 54% it formed a continuous, plastic mass during the 3 min blade-mixing. The higher tendency of the ultrafine bran to form a continuous mass might be explained by its lower water holding capacity, which was 3.3 g water /g bran DM as compared to 3.6 g/g bran DM for the coarse bran (Publication III). When a material binds less water, more water remains to act as a plasticizer. Furthermore, when the bran and water were mixed using an extruder, the reduction of particle size caused a reduction in the torque values of the extruder, especially at water contents of 54–60% (data not shown), indicating a reduction of viscosity with decreasing bran particle size. Dasari and Berson (2007) and Viamajala et al. (2009) also reported a reduction in viscosity as a result of decreasing particle size. Reduction in viscosity due to smaller particle size may allow for higher solids loading (i.e. lower water content) in enzymatic processing (Dasari and Berson 2007).

The particle size of the coarse bran had decreased during the extrusion, especially at the low water contents (Table 12). Reduction in particle size was presumably due to the higher shear forces, indicated by higher torque values (data not shown) exerted on the bran mixture at low water content. Particle size was reduced similarly or even slightly more in the samples with no added enzyme (data not shown). By contrast, when bran was processed with continuous mixing in the Farinograph mixer (Publication I), the particle size decreased more with the use of xylanase, and the samples with xylanase also increased the torque values (Table 9). WEAX is known to increase viscosity in solution, depending on its molecular weight. In the current study, however, the samples were heterogeneous mixtures with high contents of insoluble material, which also affects the rheological properties e.g. by its water absorption properties and friction caused by particles. The different impact of xylanase addition in the two processing systems, extruder and Farinograph, may be due to the essentially different time scales of the measurement (one hour in the Farinograph and about 3 minutes in the extruder). Contrary to the extruder-treated samples, the particle sizes of the blade-mixed samples (892–938 µm for coarse and 84–85 µm for ultrafine bran) were slightly higher than those of the untreated brans measured with the dry method (702 and 81 µm), which is probably due to swelling of the blade-mixed brans by the water of the wet method used for the particle size analysis.

Table 12. Appearance and particle size of coarse and ultrafine bran after extrusion and blade-mixing. Values marked with different letters within the same bran type (coarse/ultrafine) are significantly different (P < 0.05).

		Coarse (702 µm)		Ultrafine (84 µm)	
		Appearance	Particle size	Appearance	Particle size
37	Extruded	solid	348 ± 13 a	solid	55 ± 2 a
	Blade-mixed	granular	902 ± 35 d	granular	84 ± 5 c
42	Extruded	solid	-[a]	solid	-[a]
	Blade-mixed	granular	-[a]	granular	-[a]
48	Extruded	solid	603 ± 21 b	solid	76 ± 2 b
	Blade-mixed	granular	938 ± 31 d	granular	84 ± 5 c
54	Extruded	solid	-[a]	solid	-[a]
	Blade-mixed	granular	-[a]	solid	-[a]
60	Extruded	solid	770 ± 32 c	solid	85 ± 3 c
	Blade-mixed	granular	892 ± 41 d	solid	85 ± 5 c

[a] not determined

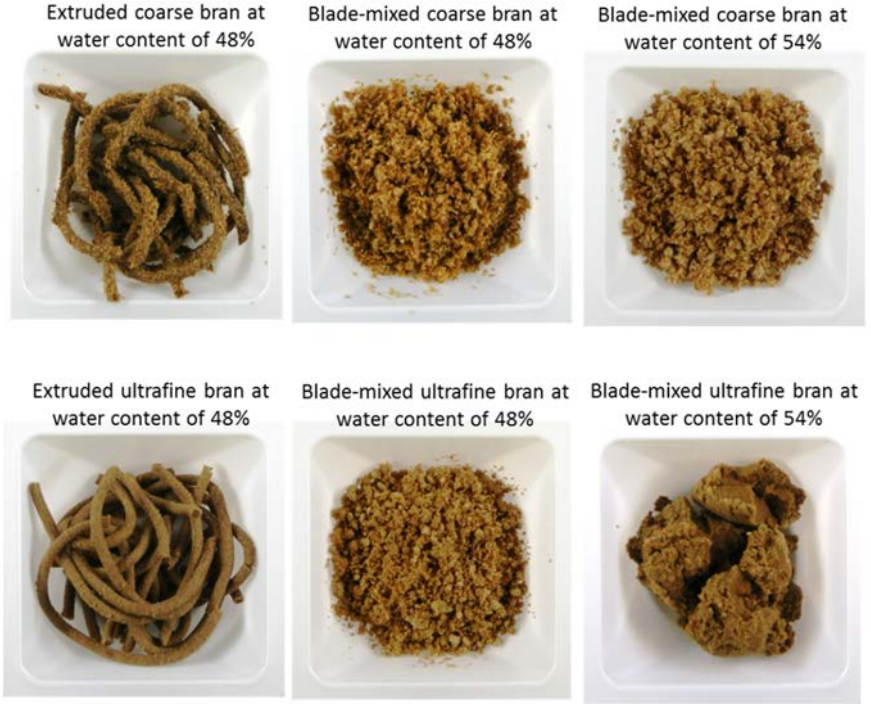

Figure 8. Coarse and ultrafine wheat bran-water-xylanase mixtures after blade-mixing or extrusion at water contents of 48 and 54% (before incubation).

4.2.2 Action of xylanase after pre-mixing by extrusion and blade-mixing

Impact of pre-mixing method and particle size on AX solubilisation

The impact of treatment water content, pre-mixing method and bran particle size on xylanase action was evaluated by analysing the amount of WEAX in the processed bran samples (Figure 9). The level of WEAX after the treatments varied between 2.6% (blade-mixed coarse bran treated at a water content of 37%) and 6.3% (extruded ultrafine bran treated at a water content of 60%) of bran DM, corresponding to DS of 13–30%. In both fine and coarse brans the solubilisation of AX was higher after the extrusion-aided treatments than after the corresponding blade-mixed treatments, especially below the water content of 54% (Figure 9). With the coarsely ground bran, the solubilisation of AX increased rather linearly with the increase of water content in the blade-mixed treatments. However, in the case of the fine bran, there was a notable increase in the AX solubilisation between 48% (WEAX 4.7% of bran dm) and 54% (WEAX 5.9%) water content, which was the water content at which the bran-water mixture of the ultrafine bran formed a continuous, plastic mass during the blade mixing (Table 12, Figure 8). A similar observation was also made earlier when studying the solubilisation of bran AX during continuous mixing (Publication I). The enhanced enzyme activity in the extrusion-aided treatment below the water content of 54%, as compared to the blade-mixed treatments at the same water content, might similarly be due to the formation of plastic mass by the pressure and mechanical shaping in the extruder. It has previously been pointed out that the absence of continuous free water phase may cause the bulk to behave as a wet granular material when portions of the ‘‘void’’ volume contain air rather than liquid, which is detrimental to efficient mixing, and consequently to efficient enzyme action (Viamajala et al. 2009). Hence, the continuous consistency of the material formed during the extrusion probably enhanced enzyme action, for example by improving diffusion, which is considered a major factor affecting enzymatic reaction rates especially at high solids concentration (Lavenson et al. 2012). However, the particle size reduction observed in the extruder (Table 12) could also have enhanced AX solubilisation, but the reduced particle size of the coarse bran was still much higher than that of the blade-mixed ultrafine bran (84–85 µm), which showed lower AX solubilisation after blade-mixed treatment than the coarse bran in the extruder. This confirms that the enhanced AX solubilisation in the extruder-aided treatment was not merely caused by the particle size reduction in the extruder.

The impact of bran particle size on the solubilisation of AX was further studied using two additional bran particle sizes, i.e. unground bran (Dv50 = 1001 µm) and fine bran (Dv50 = 327 µm), in addition to the coarse (Dv50 = 702 µm) and ultrafine (Dv50 = 81 µm) brans. Reduction of bran particle size enhanced solubilisation of bran AX, but in most points studied the differences between different brans were statistically significant only when comparing the ultrafine bran (Dv50 = 81 µm) with the coarse (Dv50 = 702 µm) and unground bran (1000 µm) (Publication III). The

results showed that the impact of bran particle size on AX solubilisation with added xylanase was similar in all the processing methods and water contents studied, including a treatment at a water content of 92% with continuous shaking, and it was also similar without added enzymes (Publication III). The only notable exception was in case of the blade-mixed treatments at a water content of 54%, when the difference between the ultrafine and coarsely ground bran was higher than in the other water contents (Figure 9). The enhanced AX solubilisation of the ultrafine bran was most probably caused by plasticization of the ultrafine bran mixture in these conditions, as already discussed.

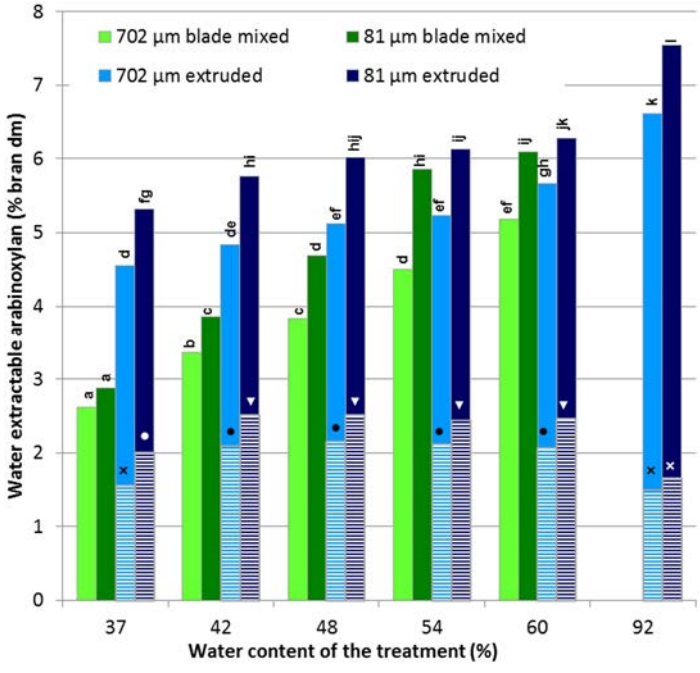

Figure 9. The amount of water extractable arabinoxylan after blade-mixing and extrusion-aided treatments (including 4 h stationary incubation) at water contents of 37–60% and after shaking treatments at a water content of 92%. The solid columns represent samples treated with Depol 761P xylanase (200 nkat/g) and the patterned columns represent samples without added enzymes. Values marked with different letters within the samples with xylanase and with different symbols within the samples without added enzymes are significantly different ($P < 0.05$) (Compiled from Publication III).

When solubilisation of AX at the low-water content (37–60%) was compared to a high-water (92%) system with continuous shaking, the solubilisation of AX with xylanase was higher in the high water content treatment (WEAX 6.4% of DM for coarsely ground bran) than in the corresponding low water treatments in the ex-

truder (4.4–5.7% at water contents of 37–60%) (Figure 9). By contrast, when xylanase action was studied at water contents of 40 and 90% using continuous mixing (Publications I and II), the solubilisation of AX was similar at both water contents (Table 10). This was probably due to the continuous mixing, whereas in the extrusion-aided treatments there was no mixing during the incubation. As recently reviewed by Lavenson et al. (2012), efficient mixing and mass transfer are generally considered to be essential for the efficient performance of enzymatic reactions, and in that respect even higher differences would be expected because of the lack of mixing during the low water content incubations. Probably the efficient initial mixing was sufficient to facilitate the enzyme action during the extrusion-aided treatment. It has been reported that effective initial mixing to promote good enzyme distribution and continued, but not necessarily continuous mixing is necessary in order to facilitate high biomass conversion rates at high solids concentration (Roche et al. 2009).

Contrary to the treatments with added xylanase, in the blank treatments with no added enzymes the solubilisation of AX was notably higher in the extrusion-aided processes at the water contents of 42–60% than in the high water content (92%) treatments (Figure 9). The solubilisation of AX was most probably caused by the action of endogenous hydrolytic enzymes of the bran material (Dornez et al. 2009), and the results suggest that low moisture content could be favourable for the solubilisation of AX by endogenous bran enzymes. This would be logical, as the natural environment and activation of the endogenous enzymes is not necessarily highly aqueous.

Impact of water content and mixing method on molecular weight distribution of WEAX

The HP-SEC analysis of WEAX indicated that the apparent average MWs of WEAX in the untreated brans were 158 kDa for coarse and 143 kDa for ultrafine bran (Table 13), which were very close to the values obtained by Zhang et al. (2011) for wheat bran WEAX using a different HP-SEC method (152 kDa). When bran was treated without added enzymes at a water content of 37%, the MW of AX of the processed brans did not markedly change from that of the untreated brans, but at a water content of 48% the MW was already significantly lower (79 and 76 kDa for coarse and ultrafine bran, respectively), and at 60% water content the MW was further decreased to the same level as after the shaking treatment at high water content (66–68 kDa). These results indicated that without added enzymes the depolymerisation of WEAX increased with increasing water content, presumably due to the action of endogenous bran enzymes, although the solubilisation of AX without added enzymes was favoured by the low water content process (extrusion-aided treatment) (Figure 9). Similarly, when bran was processed with continuous mixing, solubilisation of AX without added enzymes was higher and less depolymerisation occurred at low (40%) water content than at high (90%) water content (Publication I). These results may indicate that endogenous enzymes causing AX solubilisation preferably act at lower water activity, whereas the

enzymes that further depolymerize solubilised AX require more water. However, due to the higher shear exerted on the bran at low water content, this needs to be further confirmed.

In contrast to the treatments without enzymes, with added xylanase the MW of WEAX was not dependent on the used water content when comparing the extrusion-aided and shaking treatments (WEAX range 48–56 kDa with no statistically significant difference) (Table 13). However, with continuous mixing the depolymerisation of bran WEAX with xylanase was lower at the water content of 40% than at 90% (Publication I). The different results are probably caused by the different processing methods (extrusion and shaking vs. Farinograph-mixing and blade-mixing). However, the difference may also be due to the different purification procedures used for preparation of the samples for HP-SEC analysis. In Publication III, the removal of other components than AX in the sample was ensured by a more complete enzymatic purification method. The presence of high MW components in the bran treated at 40% and analysed in Publication I may thus be caused by incomplete removal of interfering components, such as proteins, which also showed aggregation as indicated by the SDS-PAGE analyses (Figure 7).

In order to learn how well the determined MW represented the total WEAX of the bran sample, the content of WEAX in the HP-SEC sample was also analysed (Publication III), since the method used for the MW analysis applies only to the WEAX fraction precipitating at 65% EtOH. When compared to the total level of WEAX in the original sample, the precipitated WEAX amounts in the HP-SEC samples corresponded to 24–35% of the total WEAX when bran had been processed with added xylanase, whereas without added enzymes and in the untreated bran, the analysed MW of WEAX represented as much as 57–86% of the total WEAX (Table 13). The rest of the WEAX were smaller oligosaccharides which were not present in the MW chromatograms. The contents of WEAX in HP-SEC samples are important because the level of WEAX in the original sample and the analysed MW are not necessarily comparable as such, especially in the case of enzymatic treatments. It is known that both the level of AX solubilisation (WEAX content as compared to water insoluble AX) and the MW of the WEAX are important factors affecting the technological properties of cereal AX (Courtin and Delcour 2002). In many food applications, the preferred xylanolytic reaction is the solubilisation of insoluble bran AX without intensive depolymerisation of WEAX (Courtin and Delcour 2002). In this study the xylanase enzyme dose used (200 nkat/g) was relatively high, since most of the solubilised AX was hydrolysed to oligosaccharides.

Table 13. Average apparent molecular weight (MW) of water extractable AX (WEAX) precipitated at 65% EtOH and its content (as % of total WEAX) in the brans after different treatments. Values marked with different letters are significantly different (P < 0.05) (Compiled from Publication III).

			Coarse bran		Ultrafine bran	
		Treatment water content (%)	Average MW (kDa)	% of total WEAX	Average MW (kDa)	% of total WEAX
Untreated bran			158 g	75	143 f	83
Without added enzymes	Extrusion-aided treatment	37	131 e	85	133 e	86
		48	79 d	60	76 cd	68
		60	68 b	57	68 b	58
	Shaking treatment	92	66 b	57	68 bc	67
With Depol 761P xylanase 200 nkat/g	Extrusion-aided treatment	37	51 a	25	52 a	32
		48	52 a	27	51 a	31
		60	56 a	24	52 a	31
	Shaking treatment	92	51 a	29	48 a	35

4.2.3 Impact of incubation time and drying on modification of bran by the extrusion-aided pre-mixing process

In order to study the impact of incubation time (0 or 4 h) and drying on bran after the extrusion-aided pre-mixing process, coarse (Dv50 = 700 µm) and ultrafine (Dv50 = 80 µm) bran were treated at a water content of 48% either without enzymes followed by direct freeze or oven drying, or with Depol 761P xylanase preparation followed by 4 h stationary incubation and drying by both techniques (Publication IV). In order to gain better understanding of the effects of the use of incubation and different types of enzymes, the fine bran was additionally treated by different combinations of the process parameters (4 h incubation without enzymes and direct drying with Depol 761P) and by two different enzyme preparations, Depol 761P and Veron CP, and their combination.

The effects of different process variations on the level of WEAX, bran particle size and WHC are shown in Table 14. The particle size reduction caused by the processing was higher than that observed in Publication III (Table 12), obviously due to the regrinding after drying, since in Publication III the bran samples produced by the extrusion-aided pre-mixing process were analysed without any drying step. Extrusion treatment without enzymes with direct drying increased the WEAX content of both coarse and ultrafine bran, indicating that some AX was

solubilised by the extrusion process itself, probably due to the shear exerted on the bran mixture. However, when the fine bran sample was further incubated for 4 h the WEAX content increased to 2.7%, indicating the action of bran endogenous enzymes since there was no shear during the stationary incubation. With Depol enzyme preparation, significant increase in the WEAX content (to 4.2%) occurred already without incubation, indicating that the added enzymes started to act immediately during mixing in the extruder, despite the relatively low water content used (48%). When incubated with Depol for 4 h, the WEAX content further increased to levels (4.8–4.9 and 5.6–5.7% in the coarse and ultrafine brans, respectively) that were slightly lower than those analysed in Publication III from the fresh samples (5.1 and 6.0% of bran DM, respectively) after similar treatment (Figure 9).

The fine bran treated with the combination of Veron and Depol enzyme preparations had a slightly higher WEAX content (6.2%) than the brans treated with Depol (5.7%) or Veron (4.3%) alone. This was obviously due to the different doses of endoxylanase and other enzyme activities in the treatments. Depol treatment contained mainly endoxylanase (200 nkat/g), whereas Veron treatment contained 100 nkat/g endoxylanase and additionally 130 nkat/g endoglucanase and 465 nkat/g β-glucanase, as well as higher levels of other side activities (Publication IV). The enzyme dose was highest in the combination treatment since it contained both enzyme preparations dosed at the same level as in the individual enzyme treatments. The use of multiple hydrolytic enzyme activities is generally considered beneficial in degradation and solubilisation of DF due to the synergistic action of different enzymes specific for certain cell wall components (Faulds and Williamson 1995; Petersson et al. 2013). In the current study, however, instead of studying the synergistic action of the enzymes, the dosages were selected aiming to obtain brans with different levels of AX degradation, in order to elucidate the impact of AX solubilisation on the functionality of bran in extrusion.

Brans were dried either by freeze drying or by oven drying. Freeze drying is known to cause minimal structural damage to the products, whereas oven drying is considerably cheaper. When bran was treated without enzymes, the higher content of WEAX in the oven dried brans was probably caused by the action of the bran endogenous enzymes in the beginning of the oven drying, whereas the use of liquid nitrogen for the samples that were freeze dried obviously stopped the enzyme reactions immediately. However, when the brans were treated with Depol 761P, the drying method did not have a significant impact on the content of WEAX, probably due to the high enzyme activity already during the extruder mixing and incubation, which probably obscured the possible impact of the short continuation of the enzyme action in the oven.

Similarly to the results of the bran treatment with continuous mixing (Table 11), WHC of the bran was generally reduced during the extruder-aided bran treatments with stationary incubation, especially with added enzymes, and it was always lower in the ultrafine brans than in the coarse brans (Table 14). However, the WHC of the coarse bran was significantly lower after oven drying than after freeze drying, both with and without the use of enzymes. It has also been reported previously that freeze dried DF ingredients are capable of holding more water than

those dried in an oven (de Escalada Pla et al. 2012; Massiot and Renard 1997). This can be explained by the fact that forced heated air dehydration (oven drying) can collapse the structure of DF, whereas freeze drying preserves their structural features, resulting in higher porosity that allows more water entrapment. However, the drying method did not have an impact on the WHC of the fine bran, probably because it already underwent significant structural collapse due to severe grinding conditions both before and after the treatments.

Table 14. Properties of the untreated and modified (0 h = treated without incubation, 4 h = treated with 4 h incubation, OD = oven dried, FD = freeze dried) coarse and ultrafine bran ingredients. Values marked with different letters within the results are significantly different ($P < 0.05$). For median particle size, the statistical analysis was performed separately for fine and coarse bran, and thus the letters indicate significant differences ($P < 0.05$) within each bran type (Publication IV).

			WEAX (% bran dm)		Median particle size (µm)		WHC (g water /g bran dm)	
			Coarse	Ultrafine	Coarse	Ultrafine	Coarse	Ultrafine
Untreated bran			0.5 a	0.8 b	702 a	84 a	3.7 a	3.3 b
Treated with no added enzymes	0 h	OD	1.4 de	1.6 e	279 b	68 ab	3.0 c	3.1 bc
	0 h	FD	1.1 c	1.3 cd	318 b	61 ab	3.7 a	3.1 bc
	4 h	OD	-	2.7 f	-	69 ab	-	3.0 c
Treated with Depol 761P	0 h	OD	-	4.2 g	-	70 ab	-	2.6 d
	4 h	OD	4.8 h	5.6 i	205 c	52 ab	2.5 d	2.4 d
	4 h	FD	4.9 h	5.7 i	285 b	45 b	3.1 bc	2.4 d
Treated with Veron CP	4 h	OD	-	4.3 g	-	62 ab	-	2.4 d
Treated with Veron CP +Depol 761P	4 h	OD	-	6.2 j	-	57 ab	-	2.4 d

4.3 Impact of enzymatically modified bran on the quality of bran-enriched expanded extrudates (Publication IV)

The technological functionality of enzymatically modified dry bran ingredients was examined in bran-supplemented expanded extrudates (Publication IV). Bran ingredients were produced at the water content of 48% by different variations of the extrusion-aided modification process followed by oven or freeze drying, and the effects of the distinct properties of the bran ingredients (Table 14) on the quality of rye endosperm-flour based expanded extrudates were examined at a bran supplementation level of 20%.

4.3.1 Structure of bran-supplemented extrudates

The impact of bran modifications and particle size on the macrostructure of endosperm-flour based extrudates was analysed by measuring the expansion rate (ER), specific length and piece density of the extrudates, which represented the radial, longitudinal and volumetric expansion, respectively. Addition of untreated or modified brans caused a significant reduction in the ER and piece density and an increase in the specific length of the extrudates as compared to that of the control extrudate without bran (Table 15), which is in accordance with information from the literature on the effects of wheat bran or insoluble DF addition in cereal extrudates (Robin et al. 2012; Yanniotis et al. 2007; Brennan et al. 2008; Lue et al. 1991; Karkle et al. 2012). Addition levels up to 50% of wheat bran have previously been studied and it has been reported that the negative effects of insoluble DF on extrudate quality, such as expansion volume and density, increase with increasing DF addition level, as reviewed by Robin et al. (2012).

ER remained generally rather similar when modified brans were used as compared to the use of untreated brans, but the ER was generally higher when bran of fine particle size was used as compared to the use of coarse bran. Indeed, there was a significant ($P < 0.01$) negative correlation between ER and bran particle size (Table 16). Smaller fibre particle size has also previously been reported to favour radial expansion and reduce the density of rye bran extrudates (Alam et al. 2013) and corn meal extrudates containing sugar beet fibre (Lue et al. 1991) or corn bran (Blake 2006). It has been suggested that coarse particles may cause early rupture of gas cells before their optimal expansion, or that reduction of particle size may improve expansion by providing more nucleation sites, and thus more air cells, than coarse fibre particles (Lue et al. 1991). Bran particle size also affected the cell structure as observed from the radial cross-section images obtained by stereomicroscopy (Figure 10). In the samples with coarse bran, the cells were small and the large bran particles were clearly visible, whereas in the samples with fine bran, the cell size distribution was less homogeneous due to the presence of some large cells, and the bran particles were less visible. It was also observed that particle size and WHC of the bran were significantly correlated (Table 16), and it is thus possible that the effect of fibre particle size on expansion and structure was not only related to their physical dimensions, but also to their different hydration properties and their impact on melt rheology, as previously pointed out by Sozer and Poutanen (2013).

Table 15. Macrostructural and mechanical properties of the extrudates with and without modified (0 h = treated without incubation, 4 h = treated with 4 h incubation, OD = oven dried, FD = freeze dried) coarse and ultrafine bran ingredients. Values marked with different letters within the same parameter are significantly different ($P < 0.05$) (Compiled from Publication IV).

			Expansion rate (%)		Specific length (m/kg)		Piece density (kg/m^3)		Crushing force (N)		Crispiness index (×10^3)	
Control (no bran)			452 a		54 a		130 c		18.3 c		5.8 bc	
			Coarse	**Fine**	**Coarse**	**Fine**	**Coarse**	**Fine**	**Coarse**	**Fine**	**Coarse**	**Fine**
Untreated bran			354 gh	390 bcd	68 c	62 b	169 a	155 b	22.5 a	21.8 ab	3.4 a	3.9 a
Treated with no added enzymes	0 h	OD	371 ef	401 b	78 efg	79 efgh	132 c	113 e	16.6 cd	15.3 def	6.3 cd	7.8 de
	0 h	FD	351 h	404 b	74 de	69 cd	155 b	126 cd	20.6 b	18.3 c	4.2 ab	5.8 bc
	4 h	OD	-	371ef	-	82 ghi	-	128 cd	-	15.1 def	-	10.8 gh
Treated with Depol 761P	0 h	OD	-	399 bc	-	83 ghi	-	108 e	-	16.3 de	-	9.7 fg
	4 h	OD	355 gh	404 b	84 hij	83 ghi	136 c	106 e	15.4 def	13.2 g	8.4 ef	12.1 hi
	4 h	FD	367 fg	384 cde	80 fgh	74 ef	133 c	129 c	15.9 de	13.8 fg	9.6 fg	12.9 i
Treated with Veron CP	4 h	OD	-	372 ef	-	89 j	-	116 de	-	14.6 efg	-	10.7 gh
Treated with Veron CP +Depol 761P	4 h	OD	-	379 def	-	86 ij	-	116 de	-	14.8 defg	-	11.7 hi

It has previously been reported that soluble DF generally produces higher radial expansion than insoluble DF (Pai et al. 2009; Yanniotis et al. 2007; Brennan et al. 2008), but in the current study no correlation was found between radial expansion and WEAX content of the bran (Table 16). In accordance, clear differences were not observed when comparing the radial cross-sectional images of the samples with treated brans to those of the corresponding untreated brans. This could be due to the relatively low differences in the chemical composition of the flour-bran mixtures in the current study as compared to those of the previous studies. The content of insoluble DF in the extrudates varied between 8.3 and 9.7% and the content of soluble DF (including oligosaccharides) between 6.5 and 7.9% (Publication IV). For example, in the study of Pai et al. (2009), who studied the impact of alkali-solubilised corn bran on extrusion, the differences in soluble DF content ranged from 1.6 to 64%, whereas other studies have mainly compared the impacts of addition of bran to the impacts of oligosaccharides or gums with no insoluble DF and essentially different chemical composition to that of the bran (Brennan et al. 2008; Yanniotis et al. 2007). The total DF content in all extrudates was between 15.8 and 16.4% (data not shown), indicating that the bran treatments had only minor or no impact on the total DF content of the bran.

In contrast to the less significant effects on the ER, it was noted that the bran treatments had a clear impact on the volumetric and longitudinal expansion. Compared to the untreated bran, the piece density decreased and the specific length increased when the modified brans were used. Melt viscosity and the level of available water are considered to be important factors affecting expansion, and the different effects of insoluble and soluble DF on expansion have also been related to these properties (Robin et al. 2011a; Moraru and Kokini 2003; Pai et al. 2009). In the current study, specific length was significantly correlated with both WEAX content and WHC (Table 16). It appears probable that the increase in longitudinal expansion was caused by altered melt viscosity due to increase in WEAX content and/or by increasing level of available water in the system due to reduced WHC. WEAX is known to affect viscosity in solution, depending on its molecular weight (Courtin and Delcour 2002). Robin et al. (2011a, 2011b) reported that addition of wheat bran resulted in an increase in water activity and decrease in the glass transition temperature of the melt, which would decrease the starch viscosity at constant temperature. Thus, it is also possible that in the current study, the increase in the level of available water (decrease in bran WHC) might also have promoted longitudinal expansion due to decreased melt viscosity by reducing melt glass transition temperature. However, the above mentioned possible mechanisms behind the observed effects of bran WHC and WEAX content on expansion remain to be tested experimentally.

Table 16. Pearson's correlation matrix for physicochemical properties of the bran ingredients and the macrostructural and mechanical properties of the bran-supplemented extrudates (Compiled from Publication IV).

	WHC	Particle size	Expansion ratio	Specific length	Piece density	Crushing force	Crispiness index
WEAX	-0.864**	-0.454	0.127	0.709**	-0.617*	-0.799**	0.898**
WHC	1	0.666**	-0.355	-0.710**	0.775**	0.858**	-0.866**
Particle size		1	-0.682**	-0.355	0.755**	0.636*	-0.608*
Expansion ratio			1	-0.045	-0.655*	-0.319	0.285
Specific length				1	-0.716**	-0.808**	0.747**
Piece density					1	0.852**	-0.764**
Crushing force						1	-0.927**
Crispiness index							1

** Correlation is significant at the 0.01 level.
* Correlation is significant at the 0.05 level.

4.3.2 Mechanical properties of bran-supplemented expanded extrudates

Bran addition increased the hardness of the extrudates as shown by the increased crushing force (F_{cr}) from that of the control extrudate (Table 15). By contrast, when the treated brans were used, the hardness generally decreased below that of the control (extrudate without bran), and it was decreased most when the brans were incubated with Depol 761P. Furthermore, the extrudates with modified fine bran were generally less hard than the corresponding coarse bran extrudates. Crispiness, analysed by measuring the crispiness index (C_i), decreased from that of the control when untreated coarse or fine bran was added, whereas bran treatment with enzymes produced more crispy extrudates than control, untreated bran or bran treated without enzymes with direct drying. C_i was also increased even when Depol 761P enzyme preparation was used without 4h incubation (Table 15).

Hardness and crispiness of cereal extrudates are mainly determined by their cellular structure, formed during the expansion of the extrudate, and by the phase properties and composition of the solid matrix (Moraru and Kokini 2003). It has been reported that addition of DF increases the hardness and decreases the crisp-

iness of expanded extrudates due to higher cell density and shorter cell diameter (Robin et al. 2011a; Karkle et al. 2012; Yanniotis et al. 2007; Jin et al. 1995). The samples with coarse bran had more homogenous and smaller cell size than the samples with fine bran, as visually observed from the stereomicroscopy images (Figure 10), but the improvements in the mechanical properties of the extrudates with treated brans were not clearly reflected in the cellular structure. However, the improved mechanical properties correlated with decreased piece density and increased specific length, as well as with increased WEAX content and decreased WHC of the bran. Decreased hardness and increased crispiness also correlated with decreased bran particle size, as also reported previously for rye bran (Alam et al. 2013). However, the properties of the extrudates prepared with untreated fine bran with mean particle size of 84 µm were significantly inferior to those of the extrudates made with modified coarse brans with particle size of 205–318 µm. Thus it can be concluded that the reduction of bran particle size after the bran treatments was not the primary reason for improved mechanical properties of the extrudates with modified bran. Rather, the extrudates with modified brans had improved mechanical properties probably due to the effects of the increased WEAX content and decreased WHC of the brans on the extrudate expansion. The differences in the extrudates due to the drying method can also be attributed to the slightly different impacts that the drying methods had (or did not have, as in case of the Depol 761P treatments) on the WEAX content and WHC of the brans. However, due to the complexity of the phenomena governing the expansion and texture formation, the precise mechanism behind the effect of the bran treatments on expansion and mechanical properties of the extrudates remains to be further elucidated. In addition to the analysed bran features, the changes in other bran properties, such as MW of WEAX and other DF components, as well as the solubility and molecular properties of bran proteins, may provide valuable information in this respect.

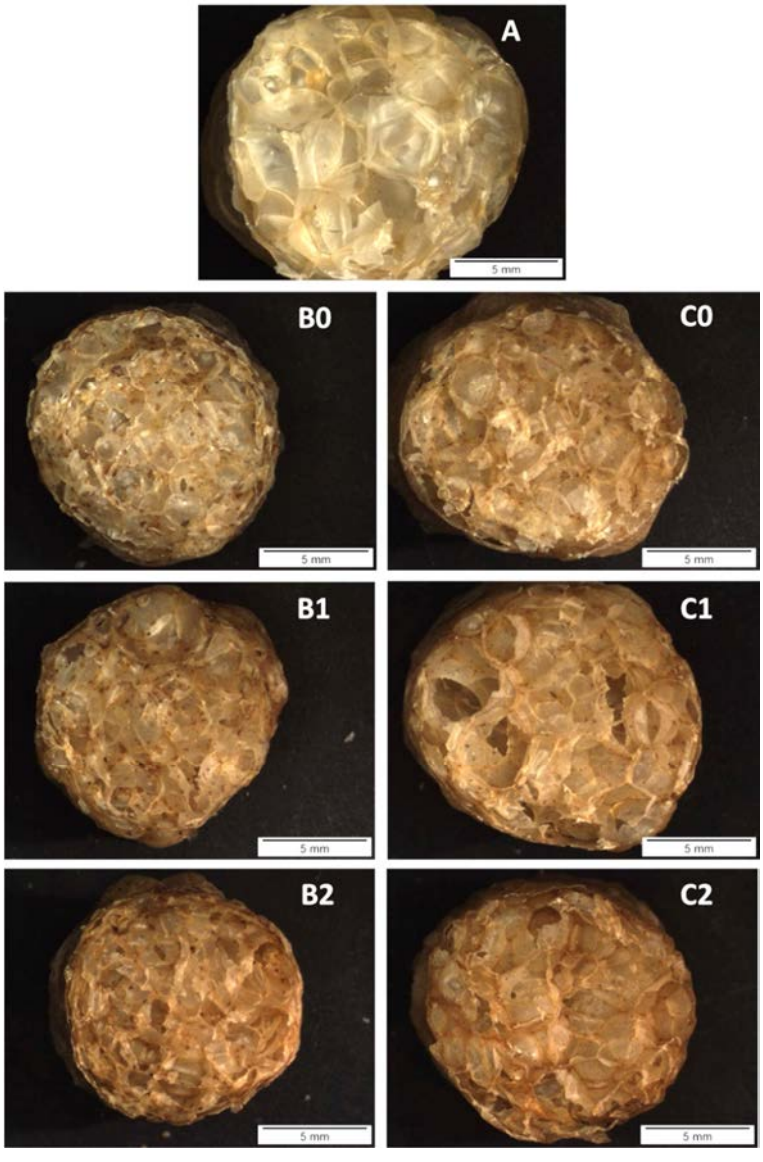

Figure 10. Stereomicroscope images of radial sections of the extrudates. Control extrudate with no added bran (A), extrudates supplemented with 20% of untreated coarse bran (B0), coarse bran treated with no added enzymes and direct oven drying (B1), coarse bran incubated 4 h with Depol 716P enzyme preparation (B2), untreated ultrafine bran (C0), ultrafine bran treated with no added enzymes and direct oven drying (C1), ultrafine bran incubated 4 h with Depol 716P enzyme preparation (C2) (Publication IV).

5. Conclusions

Enzymatic modification of plant-based materials at reduced water content could offer several advantages over processing at high water content, including reduced processing volumes and reduced downstream processing costs. In this work, the impact of water content on enzymatic modification of wheat bran was examined with the aim of increasing the technological functionality of wheat bran in food applications.

The minimum required water content for the action of xylanase on wheat bran, indicated by AX solubilisation, during continuous mixing was between water contents of 20 and 30%, corresponding to a_w of 0.83–0.89. Xylanase action was significantly enhanced at a water content of 40% (a_w 0.93), at which the granular material was transformed to a continuous paste. Furthermore, it was shown that the use of an extruder for pre-mixing and forming of bran-water mixture increased the action of xylanase during stationary incubation at a water content of ≤54%, as compared to pre-mixing with a blade-mixer. The results indicated that the formation of a continuous paste is important for efficient enzyme action at low water content, probably due to improved diffusion, and that it is possible to increase the enzyme action by changing the granular structure of the material to a continuous paste using an extruder, without increasing the water content. The results also showed that without added enzyme, the solubilisation of AX was higher at low (40–60%) than at high (90–92%) water content, suggesting that low water content may be favourable for the action of endogenous bran hydrolytic enzymes. Due to the higher shear exerted on the bran at low water content and the possible solubilisation of AX by mechanical mechanisms, the effect of water content on the action of endogenous enzymes needs to be further confirmed.

The mode of action of xylanase was examined by analysing the apparent MW of WEAX. During the treatment with xylanase, the MW distribution of WEAX precipitated with 65% EtOH was not affected by the water content or processing method at a water content of > 40%. However, since oligosaccharides were not included in the analysis, the impact of water content on their amount and MW needs to be further studied. When bran was treated with continuous mixing, the A/X ratio of the bran water extract decreased similarly at both water contents of 40% and 90%, suggesting that AX was solubilised from the same bran tissues regardless of the processing conditions studied.

The results showed that reduction of particle size, either prior to the treatment by grinding or during the treatment by intensive mixing and shear, may be used as a means to enhance enzyme action and AX solubilisation. At reduced water content, the shear exerted on the bran-water mixture caused reduction of bran particle size and might have enhanced AX solubilisation by mechanical mechanisms. However, it was also shown that reduction of bran particle size by grinding enhanced the action of xylanase, presumably due to improved substrate availability as a result of increased surface area, and the effect was rather similar at all water contents and in all process conditions studied. Furthermore, small particle size favoured the transformation of the bran-water mixture from granular mass to a continuous paste, which also enhanced enzyme action.

The study showed that both structural and physicochemical properties of bran were affected by the treatment water content. When processed with continuous mixing, the bran treated at a water content of 40% was characterized by higher solubilisation of DF polysaccharides, smaller average particle size, lower WHC and more changes in bran proteins than the treatment at a water content of 90%. The more intensive changes in the properties of bran treated at low water content with or without enzyme were related to the compact consistency and thus higher impacts of shear exerted on the bran-water mixture. Solubility of DF, bran particle size and its hydration properties are amongst the most important properties of bran affecting its technological functionality in food applications. In this work, the technological functionality of modified bran was demonstrated in bran-supplemented expanded extrudates. Modification of bran by hydrolytic enzymes by a low-moisture process increased the crispiness and reduced the hardness and piece density of the bran-enriched expanded extrudates. The improvements in extrudate properties were attributed to the increased WEAX content and decreased WHC of the modified brans, since the mechanical properties analysed significantly correlated with these properties.

The results of the work showed that enzymatic solubilisation of bran AX and improved technological functionality of bran can be achieved by enzymatic modification at a water content of 40–50%, which is well below the point of absence of free bulk water (70–80%). The consistency of the reaction mixture, mixing method and bran particle size were found to be important factors affecting the intensity of the modification process at reduced water content. It was shown that the use of an extruder-aided pre-mixing process enabled efficient xylanase action on wheat bran at low water content without the requirement for continuous mixing, and the processing method may also be applicable to other biomass sources. The results encourage further development of processes at reduced water content for the enzymatic modification of plant raw materials. The role of water in enzymatic processing is however complex, and numerous aspects of process economy need to be further elucidated in order to develop overall industrially feasible low-water processes.

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PUBLICATION I

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Impact of water content on the solubilisation of arabinoxylan during xylanase treatment of wheat bran

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ABSTRACT

Arabinoxylan (AX) has a major impact on the functional properties of wheat bran, and it has been shown that technological properties of bran can be improved by using endoxylanases. Enzymatic treatments are typically conducted at high water content. However, in industrial applications, low water content may be advantageous, especially when targeting dry end products. The aim of the study was to examine the impact of water content, ranging from 20 to 90%, on the efficiency of endoxylanase treatment of wheat bran. Interestingly, AX solubilisation was highest at the water contents of 40 and 90%. At water contents 50–80%, AX solubilisation was lower than at 40 and 90%. Furthermore, at low water content, less depolymerisation was detected. At water content of 40%, the bran-water mixture was transformed from powder-like into compact mass. Probably the compact consistency of the material enhanced AX solubilisation by increased breakdown of bran cell walls due to shear forces or via enhanced enzyme binding to the substrate. The results show that solubilisation of bran AX can also be efficiently performed at low water content.

1. Introduction

Bran is an important by-product of the cereal industry, and comprises the outer tissues of the grain. Bran is rich in dietary fibre (DF), and is composed mainly of cell wall material. Different cell wall components, such as cellulose, arabinoxylans (AX), beta-glucans, proteins and phenolic compounds, are associated with each other both non-covalently and covalently, constituting a complex network (Iiyama et al., 1994).

Several studies have shown that whole grain consumption and intake of DF reduce the risk of chronic diseases (Mellen et al., 2008, de Munter et al., 2007). Many of these beneficial effects have been ascribed to cell wall polysaccharides such as AX and beta-glucans. The use of whole grain or bran in food applications, however, is limited due to challenges in technological and sensory properties of the bran fraction. The complex nature of the cell wall also partly restricts the bioavailability of potentially health promoting compounds, such as phenolic compounds (Vitaglione et al., 2008).

The technological and nutritional functionality of bran can be improved by enzymatically modifying the complex insoluble network structure of the cell walls (Anson et al., 2009; Katina et al., 2006). Endoxylanases (EC 3.2.1.8) are hydrolytic enzymes that cleave the backbone of AX, decreasing the degree of polymerisation, increasing solubility of the polymer and releasing xylo-oligosaccharides of varying composition. In cereal processing, xylanases are increasingly used; e.g. in the baking industry because of their ability to improve textural properties of bread (Courtin and Delcour, 2002). Cereal materials also contain endogenous xylanolytic activity, which plays an important role for example in fermentation processes (Katina et al., 2007). Xylanase specificity, substrate selectivity and synergistic action with other enzymes have been studied using varying cereal AX fractions and bran components as substrate (Beaugrand et al., 2004; Benamrouche et al., 2002; Courtin and Delcour, 2001; Faulds et al., 2006; Moers et al., 2005). Xylanases have been applied for example for solubilisation of rye AX (Figueroa-Espinoza et al., 2004), increasing the amount of soluble DF (Napolitano et al., 2006) and for production of oligosaccharides (Swennen et al., 2006; Yuan et al., 2006).

In the majority of the studies, the enzyme reactions have been conducted in highly aqueous systems, typically over 90% water content. However, high water content may not be feasible in industrial scale applications, especially if the product will be dried after the enzymatic treatment. On the other hand, water content also affects the mass transfer and rheological properties of the

Abbreviations: AX, arabinoxylan; DF, dietary fibre; FU, farinograph resistance unit; dm, dry matter; HP-SEC, high performance size exclusion chromatography; Mw, molecular weight; WEP, water extractable pentosans; DS, degree of solubilisation.

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reaction mixture, which may pose technological challenges at lower water contents.

Enzymes need water for their catalytic activity. Water affects enzyme reactions and stability in various ways, influencing enzyme structure via non-covalent bonding and disruption of hydrogen bonds, facilitating reagent diffusion, and influencing the reaction equilibrium (Hari Krishna, 2002). Too low water content generally reduces enzyme activity, but the minimum hydration level for activity varies a lot. Some enzymes can function even in solvent-free reaction conditions on solid substrates, expressing extraordinary reaction patterns (Hobbs and Thomas, 2007). Despite the abundant research in the area of enzymatic modification of bran and AX, there are only a few publications dealing with the effect of water content on the efficiency of these treatments (Napolitano et al., 2006; Sørensen et al., 2006).

The objective of this study was to demonstrate the influence of water content of bran-water mixtures on the solubilisation of AX during xylanase treatment of wheat bran. The relationship between the water content and the physical state (rheology, water activity) of bran-water mixtures in relation to the AX solubilisation and remaining extractable xylanase activity was also evaluated.

2. Experimental

2.1. Materials

The bran was obtained from mixed wheat varieties (Mühle Rüningen GmbH & Co. KG, Braunschweig, Germany). Before bran removal, the grains were peeled to remove 2–3% of the grain outer layers in order to reduce the level of contaminating microbes and enzymes on the surface layers of the grains. The chemical composition was (% of dm): DF 49.5 (including pentosans 21.7, fructan 3.6, beta-glucan 2.8), protein 19.5, starch 11.6, fat 4.8, ash 6.7. The bran was ground in batches of about 4 kg by passing each batch three times through a mill (Hosokawa Alpine, 100 UPZ, Retsch GmbH, Germany; mill sieve size 0.3 mm). After grinding, the mean particle size was about 100 μm, and 90% of the particles were smaller than 390 μm as determined by Coulter Particle size analyser dry module (Coulter Corporation, USA).

A commercial *Bacillus subtilis* xylanase preparation, Depol 761P (Biocatalysts, UK), was used for bran treatments. According to the manufacturer, it is especially suitable for extraction of soluble fibre from wheat bran. The xylanase activity of Depol 761P was 137 000 EU/g, as analysed by the by Xylazyme AX Tablet assay as described in section 2.3.4.

2.2. Enzymatic treatment of wheat bran

Bran was treated with or without added enzyme according to the scheme shown in Fig. 1A. The enzyme preparation was dosed according to its xylanase activity at levels of 97 or 970 EU/g bran. The enzyme powder was mixed with bran before water addition. The water content of a treatment is expressed as the total water content, i.e. the water content of bran was taken in account. The treatments at ≤70% water contents were performed in a farinograph mixing bowl (Brabender Farinograph, mixer type S300 with z-blades, Fig. 1B). The mixer bowl was heated by water circulation (50 °C) and the mixing speed was 60–63 rpm. 125 g of ground bran (with or without enzyme addition) was placed in the mixer, and pre-heated water was added by spraying during 1–3 min while the blades were rotating to obtain an even distribution of water. The mixing bowl was sealed tightly to avoid evaporation of moisture. At higher water contents (80 and 90%), due to the liquid form of the mixture, the reactions were performed in a covered steel container (0.5 l) with double-blade mixer (speed 160 rpm) placed in a water bath (50 °C).

The experiments were done in duplicate. The reaction time varied from 1 to 24 h, after which a sample was taken and reaction stopped by cooling down and freezing the sample immediately. Each sample was divided in two pieces: one part of the sample was used as such for water activity measurement, and the rest of the sample was freeze dried for other analyses. Before analyses, the freeze dried samples were ground with a laboratory mill (0.5 mm sieve). The remaining moisture contents of freeze dried samples were determined by oven drying (1 h at 130 °C).

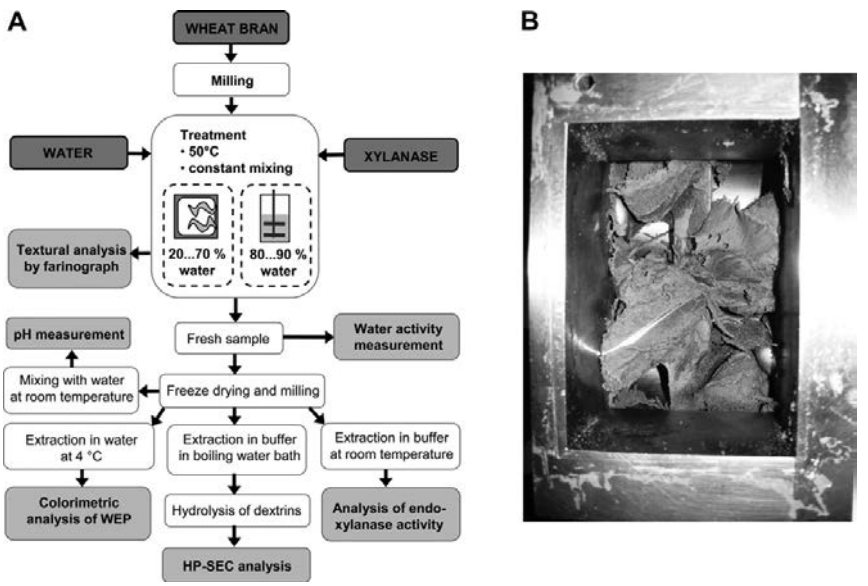

Fig. 1. A) Process scheme for the enzymatic treatments and analyses. B) Bran mixture at water content of 40% in a farinograph mixer after 24 h incubation with xylanase (97 EU/g).

2.3. Analysis methods

2.3.1. Chemical analyses

Analyses of the bran were made as follows: protein content by Kjeldahl method (AACC, 2003b, no. 46–11A), total DF by enzymatic-gravimetric method (AOAC, 1990 method no. 985.29), beta-glucan by AACC (2003a) method no. 32–23, fructan by AOAC (2003) method no. 999.03, fat by AOAC (2000) method no. 922.06, digestible starch by Megazyme method (McCleary et al., 1994) and ash by burning at 550 °C. For the quantification of water extractable pentosan (WEP), 0.25 g of the freeze dried sample was mixed with 8 ml of 4 °C distilled water and shaken with glass pearls for 15 min at 4 °C. After centrifugation, the supernatant was boiled for 10 min in order to inactivate the residual enzyme activity, and centrifuged again. The amount of pentose sugars in the water extracts was determined by a colourimetric phloroclucinol method (Douglas, 1981) using xylose as a standard. For the quantification of total pentosan, a dry sample of 0.1 g was mixed with 5 ml of 0.5 M H_2SO_4 and boiled for 20 min and centrifuged, followed by the colourimetric determination (Douglas, 1981).

2.3.2. Water holding capacity, water activity, consistency and pH

The approximate water holding capacity (approx. WHC) of bran was analysed by a method modified from Quinn and Paton (1979). 0.5 g bran was weighed in a centrifuge tube and the tube with the contents was tared. An adequate amount of water was added and mixed by vortex. The bran suspension was allowed to swell for 60 min at room temperature and centrifuged. The supernatant was discarded and the tube was weighed. The weight difference per gram of bran dm was taken as the approx. WHC. Changes in textural properties during the first 60 min of bran treatments were followed as farinograph torque values reflecting the resistance of the bran-water mixture against the mixing blades. The water activity at the end of 24 h bran treatment was determined from a fresh sample using AquaLab CX2 (Decagon Devices Inc., USA). The pH of treated bran was measured by solubilising 0.2 g of the freeze dried sample in 10 ml of distilled water and stirring for 15 min prior to measurement.

2.3.3. HP-SEC analysis

The effect of bran treatments on the molecular weight (Mw) distribution of water extractable compounds was analysed by high performance size exclusion chromatography (HP-SEC). 0.2 g of freeze dried sample was mixed in 5 ml of 50 mM Na-phosphate buffer (pH 4.7), boiled for 30 min and centrifuged. Starch and beta-glucans were hydrolysed by adding 50 μl of 1:25 or 1:50 diluted Optidex L-400 (Genencor International) saccharifying enzyme solution to 1000 μl of supernatant and incubating the samples with mixing at 60 °C for 4 h. After incubation, the samples were boiled for 30 min and cooled down. Optidex has been pre-tested with commercial rye and wheat arabinoxylans to confirm that under the conditions applied, it affects neither AX molecular weight nor concentration. The liquid chromatograph with Alliance 2690 separation module and M-2414 refractive index detector, consisted of three columns (7.8 × 300 mm) μHydrogel 500, μHydrogel 250 and μHydrogel 120. All the equipment was purchased from Waters Inc. (Milford, MA, USA). The eluent was 0.2% H_3PO_4 at a flow-rate of 0.5 ml/min. The columns were at 60 °C and the injection volume was 100 μl. Pullulan standards (Waters Inc., Milford, MA, USA) ranging from 788 000–5900 Da, maltoheptaose and maltopentose were used for calibration.

2.3.4. Endoxylanase activity assay

The endoxylanase activity was analysed by Xylazyme AX Tablet assay (Megazyme, Ireland). For the extraction of enzymes, 0.15 g of dry sample was mixed with 10 ml of phosphate buffer (25 mM, pH 6.0) and shaken during 60 min at room temperature. After centrifugation, 0.5 ml of suitably diluted supernatant was equilibrated in 50 °C water bath for 5 min before the addition of an AX substrate tablet. After 60 min, the reaction was terminated by adding 5 ml of Trizma base solution (2% w/v) and mixed. After 6 min at room temperature, the slurry was mixed again and filtered (Whatman No.1). The absorbance of the filtrate at 590 nm was measured against a substrate/enzyme blank prepared according to the assay instructions. Activity levels were expressed in endoxylanase units (EU) per gram. One EU is the amount of endoxylanase needed to yield an absorbance (at 590 nm) of 1.0 per 60 min of incubation, under the conditions of the assay. For the calculation of the percentage of remaining endoxylanase activity in the enzyme treated bran samples, the initial activity was calculated as the sum of the activity of the added xylanase plus the endogenous activity analysed for of the bran.

3. Results

3.1. Consistency and properties of bran mixtures at different water contents

The approx. WHC of bran was 2.8 g water/g bran dm. At water contents of 20 and 30%, the bran was in the form of powder, and the water activity at the end of 24 h incubation was 0.83 and 0.89, respectively (Table 1). At the water content of 40%, the bran-water mixture was transformed into a very compact, plastic-like mass (Fig. 1B). Increasing water content from 40 to 70% increased the water activity from 0.93 to 0.98, while the appearance of the reaction mixture was like a paste. At the highest water contents, 80 and 90%, the water activity was ≥0.98, and the bran-water mixture was a slurry-type dispersion. Addition of xylanase did not affect the water activity or the appearance of the mixture.

The rheological properties of bran-water mixtures, measured as farinograph resistance units (FU), varied with water content (Table 2). Without added enzyme, the resistance value increased with increasing water content up to the water content of 50%, and then at higher water contents, the resistance value again decreased. Addition of exogenous xylanase increased the resistance values, especially at the water content of 40%.

The initial pH of the bran-water mixture was 6.8. During the first 16 h of incubation, only minor pH changes were noticed, and the pH remained between 6.2 and 6.8 regardless of water content (Table 3). After 24 h, however, at the highest water content, 90%, pH decreased to 4.7.

Table 1
Water activity (measured at 24–27 °C) and appearance of bran mixtures at different water contents. Water activity and appearance were not affected by the addition of xylanase. The water activities are expressed as means of four analysis results (duplicate measurements for each bran sample). The standard deviations were less than 1% of the mean.

Water content (%)	Water activity (after 24 h treatment)	Appearance (after 24 h treatment)
20	0.83	Powder
30	0.89	Powder
40	0.93	Paste
50	0.96	Paste
60	0.97	Paste
70	0.98	Paste
80	≥0.98[a]	Slurry
90	>0.98[a]	Liquid

[a] With the used measurement system it was not possible to determine the accurate water activity of samples with high amount of free water.

Table 2
The resistance values of bran mixtures after 1 h incubation. The values are approximate values obtained graphically from farinograph curves.

Water content of bran mixture (%)	Resistance value at 60 min (FU)		
	No added enzyme	With xylanase 97 EU/g	With xylanase 970 EU/g
20	60	60	–[a]
30	110	150	200
40	240–270[b]	330–400[b]	400–550[b]
50	280–310[b]	310–340[b]	310–340[b]
60	140	130	–[a]
70	40	40	–[a]

[a] Not determined.
[b] The range of the values refers to the fluctuation of the resistance curve.

3.2. Remaining endoxylanase activity of enzyme treated bran

The total initial endoxylanase activity of the treatment was 982 EU/g bran, which was calculated by summing the endogenous xylanase activity of the bran, 12 EU/g, and the added xylanase activity dosage, 970 EU/g. The recovery of xylanase activity in water extracts of enzyme treated bran samples varied with the treatment time and water content (Table 3). After 24 h treatment at the water content of 40%, with the initial xylanase dosage of 970 EU/g, only 10% of the initial activity could be detected in the bran extract. The recovery was higher both at lower and higher water contents, and the highest recovery after 24 h treatment, 64%, was detected in bran treated at 90% water content.

3.3. Effect of water content and xylanase treatment on the content of water extractable pentosans

The effect of water content and use of xylanase on bran was examined by analysing the content of WEP after 24 h treatment (Fig. 2A). Without addition of exogenous xylanase, at the lowest water content studied, 20%, no significant increase in WEP content was noticed. However, already at the water content of 30%, remarkable increase in WEP content could be noticed. The highest degree of solubilisation (DS, calculated by dividing the WEP content of the sample by the total pentosan content of the bran, which was 21.7% of dm) was monitored at 40% water content (3.8% WEP of total dry matter, corresponding to DS of 18%). When the water content was increased further, the WEP content was again reduced. With added xylanase (dosage 97 EU/g), WEP content reached the highest value both at 90 and 40% water contents (6.9% WEP of total dm at both water contents, DS 32%), and between water contents of 40 and 90%, the WEP content was lowest at 60% water content (5.5% WEP, DS 25%).

However, the impact of added xylanase on the increase in WEP content in the enzyme treatments varied with water content. The contribution of added xylanase was calculated as a percentage (of the difference between the WEP content of control and enzyme treatments) from the WEP content of the enzyme treated sample. At the water content of 90% with added xylanase, 63% of the increase in WEP content was caused by added enzyme, while at the water content of 40%, that was 45%. At the water content of 30, 50, 60, 70 an 80%, with added xylanase, the contribution of added xylanase on the increase in WEP content was 35, 50, 58, 64 and 63%, respectively.

Effect of treatment water content and xylanase addition was also studied as a function of incubation time and xylanase dosage (Fig. 2B). Without added enzyme, the content of WEP was very low after 1 h treatment at all water contents studied (30, 40, 50 and 90%). After 4 h, water content had an impact on the rate in which the WEP content increased, as observed from the (estimated) slopes of the curves in Fig. 2B, and the rate was highest at the water content of 40% and lowest at the water contents of 90 and 30%. With the xylanase dosage of 97 EU/g, increase in the WEP content during the first hour was fastest at the water content of 90%, followed by the water content of 50% (Fig. 2B). This was also the case when higher enzyme dosage (970 EU/g) was used. However, between 4 and 24 h, at the water contents of 50 and 90%, the rates in which the WEP content increased were approximately the same regardless of enzyme dosage, whereas at the lower water contents (30 and 40%), the rates were relatively more increased with higher enzyme dosage. After 24 h treatments with the enzyme dosage of 970 EU/g, WEP content was highest at the water content of 40% (11.8% WEP, DS 54%), followed by the water content of 90% (10.6% WEP, DS 49%) and 50% (9.5% WEP, DS 44%).

Table 3
pH of bran samples and remaining endoxylanase activity of water extracts of bran samples treated at 30, 40, 50 and 90% water contents with xylanase (970 EU/g). The results are expressed as means of four analysis results (duplicate analyses for each bran sample). The standard deviations were less than 5% of the mean in pH results. The standard deviations of the endoxylanase activity results are indicated in parentheses.

Water content (%)	pH				Endoxylanase activity (% initial)			
	1 h	4 h	16 h	24 h	1 h	4 h	16 h	24 h
30	6.8	6.7	–[a]	6.4	77 (±5)	55 (±2)	–[a]	33 (±5)
40	6.6	6.4	6.3	6.3	81 (±3)	45 (±3)	13 (±2)	10 (±1)
50	6.6	6.4	–[a]	6.3	85 (±1)	67 (±2)	–[a]	63 (±4)
90	6.5	6.5	6.2	4.7	80 (±3)	70 (±2)	66 (±2)	64 (±4)

[a] Not determined.

3.4. HP-SEC analysis

The Mw distribution chromatograms of water extractable compounds (prepared as described in chapter 2.3.3.) of bran treated for 4 and 24 h at 40 and 90% water contents are presented in Fig. 3. After treatment with low enzyme dosage of 97 EU/g and short treatment time (4 h), there were no significant differences between the elution profiles of the samples treated at different water content. However, when longer treatment time or higher enzyme dosage was used, the amount of large polymers eluting before 53 min (corresponding to Mw of >1500 Da) was higher after treatments at 40% than at 90%. In untreated bran, there was a sharp peak of polymers eluting at 52.2 min (corresponding to Mw of 2000 Da), and this peak was still detectable after enzyme and control treatments at 40%, but not after treatments at 90%. The largest polymers (Mw exceeding 50 000 Da) were detected after treatment at 40% without added enzymes.

4. Discussion

In the present work, the impact of water content and xylanase treatment was studied by analysing the physical state of bran-water mixtures, remaining xylanase activity and depolymerisation of water extractable compounds, in relation to AX solubilisation. The solubilisation of AX was evaluated by analysing the changes in the content of water extractable pentose sugars. It is well known that AX represents about 70% of the non starch polysaccharides in wheat bran (Maes and Delcour, 2002), hence, also the pentose sugars are mostly derived from AX. Pentose sugars may also originate from arabinogalactan peptide, which is easily extractable (Fincher et al., 1974). No data is available about the content or presence of AGP in bran, but in wheat flour, the contents of WE-AGP have been reported to be around 0.15–0.38% dry basis (Andersson et al., 1994; Loosveld et al., 1997). As the reported values of AGP in wheat (though available only for flour fractions) are very low

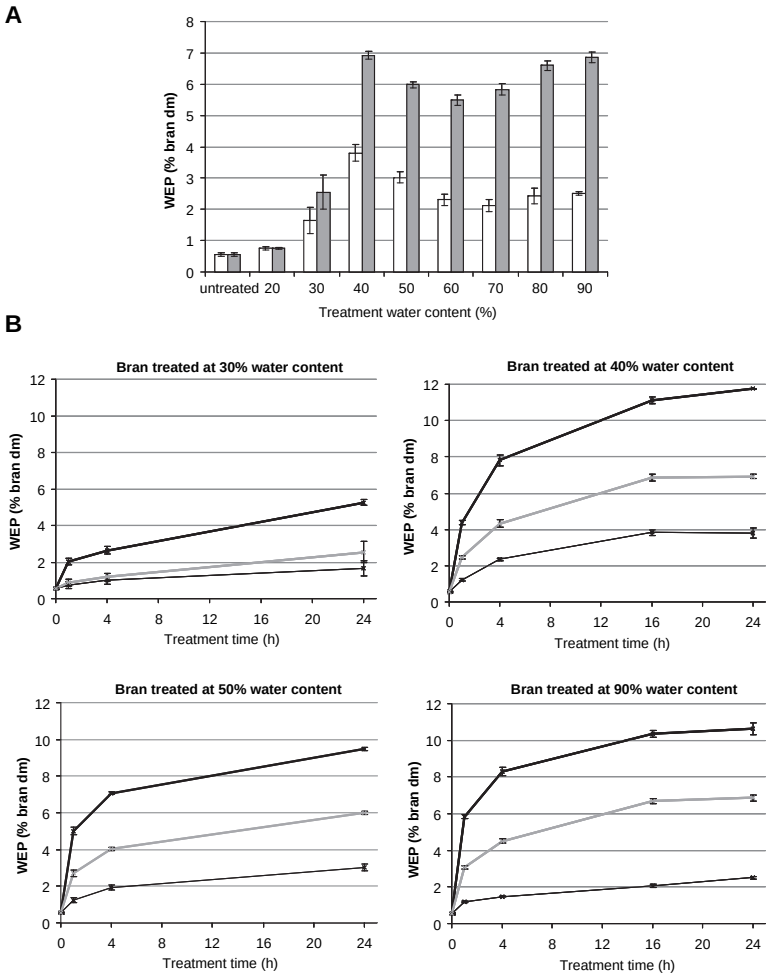

Fig. 2. A) Effect of treatment water content and xylanase addition of 97 EU/g on the content of water extractable pentosans (WEP) of wheat bran after 24 h incubation. □ no added enzyme ▒ xylanase 97 EU/g. B) Effect of incubation time and xylanase addition of 97 and 970 EU/g on the content of WEP of bran treated at the water contents of 30, 40, 50 and 90%. The content of WEP was analysed after 1, 4 and 24 h, and at the water contents of 40 and 90%, also after 16 h incubation. The results are expressed as means of duplicate analyses for each bran sample. —— no added enzyme —— xylanase 97 EU/g —— xylanase 970 EU/g.

compared to the level of AX in bran, which has been reported to be in the range of 13–30% in common wheats (Gebruers et al., 2008; Kamal-Eldin et al., 2009), it can be concluded that the increase in the content of WEP, reported in the present study, was due to solubilisation of AX.

Enzymatic solubilisation of wheat bran AX was demonstrated to proceed efficiently even at low water contents. Interestingly, the solubilisation was highest at the water contents of 90 and 40%, whilst at intermediate water contents (50–80%) the solubilisation was lower. However, at the water content of 40%, the solubilisation of AX was most probably enhanced also by other factors than by the hydrolysing activity of the added xylanase.

There are only a few previous publications dealing with the effect of water content on the efficiency of enzymatic modification of bran and AX (Moore et al., 2006; Napolitano et al., 2006; Sørensen et al., 2006). Moore et al. (2006) have successfully treated wheat bran with hydrolytic enzymes at water contents of 30–43% for improving the bioaccessibility of antioxidants. Most of the previous studies, however, have reported decreased enzymatic function at decreased water contents. The yields of enzymatic degradation of AX in a wheat derived fermentation residue were found to decrease as a function of the substrate dry matter level ranging from 2.5 to 10 wt% dm (Sørensen et al., 2006). In a study of enzymatic solubilisation of DF from durum wheat fibre at substrate concentrations of 3.3–33.3%, a concentration of substrate of 6.6% gave the most satisfactory results, whilst at the substrate concentration of 33%, very low enzyme hydrolytic activity was detected (Napolitano et al., 2006). According to the results of several publications collected by Kristensen et al. (2009), it has also been shown that in the enzymatic conversion of lignocellulosic biomass to fermentable sugars, increasing substrate concentration leads to decreased conversion. The main cause of this phenomenon has been suggested to be the inhibition of enzyme adsorption by hydrolysis products (Kristensen et al., 2009), but other possible

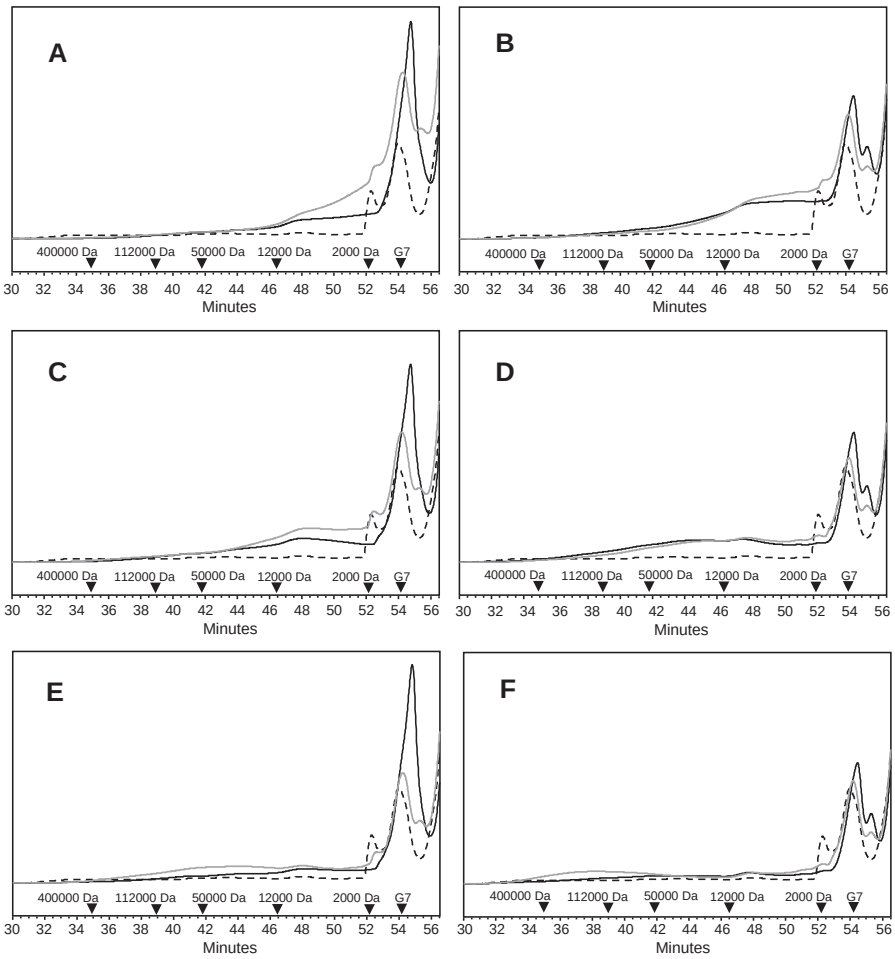

Fig. 3. Mw distribution of water extractable compounds (prepared as described in chapter 2.3.3.) of bran treated with A) xylanase 970 EU/g for 24 h, B) xylanase 970 EU/g for 4 h, C) xylanase 97 EU/g for 24 h, D) xylanase 97 EU/g for 4 h, E) no added enzymes for 24 h, F) no added enzymes for 4 h - - - - untreated bran —— treated at 40% water content ▬▬ treated at 90% water content.

explanations have also been proposed, as reviewed by Kristensen et al. (2009). These previous studies have investigated only the area above the water content of 60%. Similarly, in the present study the solubilisation of AX was decreasing with decreasing water content in the area between 60 and 90% water contents. At even lower water content of 50 and 40%, however, the solubilisation again increased.

When studying the effects of water content in a biological system, it is important to note that water content can rarely be considered as an independent variable, because water affects directly a number of other factors, such as the viscosity and consistency of the studied material. Consequently, in this study the experiments had to be performed in two different kinds of mixing devices because of the very different consistency of the tested samples. The free water present in the bran mixtures at the water contents of 80 and 90% prevented the use of farinograph as a mixing device because of leaking of water. Similarly, the use of a blade mixer for samples of water content less than 80% was not possible because of too high viscosity of the mixtures. Thus, the possible impacts of these two different mixing methods on the AX solubilisation need to be taken into account when interpreting the results.

The solubilisation of AX with added xylanase was highest and approximately the same at water contents of 40 and 90%, but the solubilisation without added enzymes was notably higher at the water content of 40%. Thus, the increase in AX solubilisation at the water content of 40% was evidently caused not only by the action of added xylanase alone, but also by other factors. During the treatments at the water content of 40%, the bran material was transformed to a compact, plastic-like mass. The compact structure of the material was also observed by the measured resistance values. At the water content of 40%, AX was notably solubilised also without added xylanase, which suggests the presence and action of endogenous enzymes in the bran material. However, it is possible that the compact structure of the material at 40% water content might have enhanced the AX solubilisation by physical breakdown of bran cell walls due to shear forces. The degradation of cell wall structures might have increased AX solubilisation, e.g. by causing the breakdown and release of AX molecules that were initially

bound to other cell wall structures, or by facilitating the action of hydrolytic enzymes by exposing new, initially inaccessible AX substrates. In the present study it was calculated that the contribution of added xylanase to the AX solubilisation was 63% at the water content of 90%, while at the water content of 40%, it was only 45%. However, it is difficult to estimate to what extent the solubilisation without added enzymes was caused by the mechanical work input alone, or by the possible synergistic action of both mechanical breakdown of the components and the concomitantly enhanced action of bran endogenous enzymes. It has also previously been postulated that wheat flour AX can be solubilised by disaggregation of AX chains by a temperature increase or mechanical work input (Cleemput et al., 1997; Dornez et al., 2007). Wheat and rye bran AX have also been solubilised mechanically by intensive ball milling process (Van Craeyveld et al., 2009).

The property of bran to absorb water is important in terms of physical properties of bran-water mixtures, and thus, also in terms of AX solubilisation. The approx. WHC of bran was 2.8 g water/g bran dm, which means that bran can hold all the added water up to a water content of 74%. It is likely that the increase in the amount of free water has improved the mass transfer and diffusion of components at the water contents between 70 and 90%, and thus improved the AX solubilisation. At the water contents between 20 and 70%, there was no free water, which reflected the appearance and consistency of the mixtures. Below the water content of 40%, the material was powder-like and AX solubilisation was the lowest. It is presumable that the enzyme diffusion at water contents below 40% is restricted by the absence of a continuous water phase.

Interestingly, the endoxylanase activity was significantly decreased during the treatment at the water content of 40%, whereas at 90% the enzyme activity decreased much less. This was unexpected in view of the degree of AX solubilisation. One possible explanation is that the compact structure of the material and efficient binding of enzyme to the substrate at the water content of 40% may have prevented the extraction of the enzyme from the freeze dried sample in the enzyme activity assay used.

The pH of bran samples did not change remarkably during the 24 h treatments, except for the bran treated at the high water content of 90%. Between 16 and 24 h, the pH of bran treated at 90% decreased from above 6 to less than 5. The decrease in pH indicates the growth and metabolic activity of acid-producing bacteria, such as lactic acid bacteria. It is known that the bran material contains naturally quite high amounts of yeasts and bacteria (Katina et al., 2007). The bran in this study was obtained after grain peeling, which is known to decrease the amount of microbial load of bran (Katina et al., 2007). The relatively high temperature of the treatment (50 °C) was also assumed to further restrict the interference of microbes. However, apparently some microbial growth was present after long incubation times at the high water content. On the contrary, in the treatments at decreased water contents, the low water activity has probably restricted the activity of these microbes, as the pH did not significantly change at the lower water contents. Microbes play an important role in the safety issues of bran processing, and as suggested by this study, water content may also be used as a process parameter to control the growth and metabolic activity of microbes.

The Mw distribution of the water extracted compounds of bran (supposed to mainly be composed of AX) was also affected by the water content of the reaction, especially when long treatment time or high enzyme dosage was used. In the elution profiles of all the samples, the largest peak was eluting after 53 min, corresponding to small oligosaccharides having Mw < 1500. However, in this work the interest was in comparing how the water content affects the Mw profiles of the larger sized molecules eluting before 53 min (Mw > 1500). In many food applications, the preferred xylanolytic reaction is the solubilisation of unsoluble bran AX without intensive depolymerisation of WEAX (Courtin and Delcour, 2002). Both with and without added xylanase, the amount of large polymers was higher after treatments at 40% than at 90%, and the peak of 2000 Da polymers of untreated bran were still left after the treatments at 40% water content, whereas at 90%, the peak vanished. This suggests that although the degree of AX solubilisation was the same both at the low and high water content treatments, the depolymerisation efficiency of xylanase was stronger at the high water content. However, the differences in the Mw profiles may partly result from the physical breakdown of the components at the water content of 40%. The results of the present study indicate that the Mw distribution of water extractable polymers may be affected by the water content of the solubilisation process.

In conclusion, AX could be solubilised at the water content of 40% at least as effectively as at the conventionally used high water content, but at intermediate water contents, the solubilisation was lower. It appears that the texture of the reaction mixture is an important factor in AX solubilisation. The impact of water content on enzyme binding is an interesting topic for further research. Further studies are also needed to confirm the possible effects of mechanical energy input and the role of endogenous enzymes on AX solubilisation. The amount of process water is an important factor in the development of new sustainable enzyme processes, and it is evident that from an ecological and economical point of view, it would be advantageous to be able to produce the same amount of dry product using less water. However, the overall industrial feasibility of a production process depends of course on several factors that may be affected by the water content of the process material. For example, the physical structure of low water content material may pose challenges and require specific process equipment.

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PUBLICATION II

Treatments with xylanase at high (90%) and low (40%) water content have different impacts on physicochemical properties of wheat bran

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ORIGINAL PAPER

Treatments with Xylanase at High (90 %) and Low (40 %) Water Content Have Different Impacts on Physicochemical Properties of Wheat Bran

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Abstract The aim of the work was to elucidate the impacts of treatment with xylanase at high (90 %) and low (40 %) water contents on the structural and physicochemical properties of wheat bran. The bran treatments at 40 % water content, both with and without added xylanase, resulted in a smaller average bran particle size, more changes in bran microstructure, and higher solubilization of polysaccharides than the corresponding treatments at 90 %. Also, the water holding capacity of bran (3.6±0.1 g water/g bran dm), determined by Baumann method, decreased more already after 4-h xylanase treatments at 40 % (2.4±0.1) than at 90 % (2.9±0.2). The solubility of salt-extractable bran proteins decreased during the treatments, especially at 40 %, also without added xylanase. Protein aggregation was detected in the SDS+DTT-extractable bran fraction, which also contained small proteins of 10–20 kDa not detectable in the untreated bran. The use of xylanase had only minor effect on bran proteins as compared to the treatments without added xylanase. The results indicate the large role of mechanical shear on the bran properties at 40 % water content. The low arabinose/xylose ratio (0.32) in the bran water extract after 24-h xylanase treatment at 40 %, however, suggests that the solubilization of arabinoxylan was caused by enzymatic action, and not by mechanical degradation. Arabinose/xylose ratio of the bran water extract decreased similarly during all the treatments, suggesting similar solubilization pattern of arabinoxylan at both water contents. The study showed that bran properties can be significantly modified by adjusting the water content and mechanical energy used in processing.

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Introduction

Cereal bran comprises the outer layers of grain which are separated in the milling process during the production of refined flours. Wheat bran is high in dietary fiber (DF) and a good source of phytochemicals, but its use in food applications is limited by technological and sensory challenges. The reasons behind the adverse effects of bran in food processes are not fully elucidated, but both physical and chemical mechanisms have been suggested (Gan et al. 1992; Lai et al. 1989; Noort et al. 2010). The well-documented evidence about the positive health effects of the consumption of DF and whole grain foods has increased the interest to study and improve the technological and nutritional properties of bran and other DF-rich plant materials.

The technological functionality as well as the physiological effects of bran is dependent on its physicochemical characteristics, which are influenced by particle size, cell wall architecture, chemical composition, and molecular structure of the DF polymers of bran (Noort et al. 2010; Auffret et al. 1994; Izydorczyk 2009). These properties affect, for example, the hydration characteristics of the material, including water binding capacity (WBC), water holding capacity (WHC), and solubility, which are important for both the technological applicability and the physiological function of DF (Thebaudin et al. 1997; Chaplin 2003). In wheat, the main DF component is arabinoxylan (AX), and especially AX solubilization is considered essential for the baking performance of bran and motivates the use of AX-degrading enzymes in breadmaking processes (Figueroa-Espinoza et al. 2004; Courtin and Delcour 2002). Desired

modifications of nutritional and technological properties can be achieved by the use of hydrolytic enzymes, such as endoxylanases (Figueroa-Espinoza et al. 2004; Katina et al. 2006; Anson et al. 2009). Endo-β-1,4-xylanases depolymerise and solubilize AX by cleaving the β-xylosidic bond between two D-xylopyranosyl residues linked in β-(1,4). Enzyme function and AX hydrolysis can also be facilitated by thermal or mechanical methods, such as extrusion, high shear treatment, and intensive ball milling (Figueroa-Espinoza et al. 2004; Gajula et al. 2008; Van Craeyveld et al. 2009). In addition to DF, bran also contains a relatively high amount of protein (Kamal-Eldin et al. 2009). Bran protein has been very little studied, but it is obvious that bran processing also influences the functional properties of the protein fraction.

In cereal processing, water content is an important parameter, influencing for example diffusion of components, rheological properties of the material as well as enzyme activity. Enzymatic treatment at reduced water content could be economically beneficial, especially when targeting dry end products. We have previously studied the impact of water content, ranging from 20 to 90 %, on the solubilization of AX during xylanase treatment of wheat bran (Santala et al. 2011). Interestingly, AX solubilization was detected to be highest at the water contents of 40 and 90 %. In the current study, the aim was to elucidate further the factors affecting the solubilization of AX at the low (40 %) and high (90 %) water content treatment of bran (with and without xylanase) by assessing the subsequent structural and physicochemical changes in bran properties, with focus on water-extractable polysaccharides and bran proteins.

Materials and Methods

Bran

The bran was obtained from mixed wheat varieties (Mühle Rüningen GmbH & Co. KG, Braunschweig, Germany). Before bran removal, the grains were peeled to remove 2–3 % of the grain outer layers in order to reduce the level of contaminating microbes and enzymes on the surface layers of the grains. The chemical composition was: (percentage of dm): DF 49.5 (including AX 22.8, fructan 3.6, and ß-glucan 2.8), protein 19.5, starch 11.6, fat 4.8, and ash 6.7. The bran was ground in batches of about 4 kg by passing each batch three times through a mill (Hosokawa Alpine, 100 UPZ, Retsch GmbH, Haan, Germany; mill sieve size, 0.3 mm). After grinding, the mean particle size was 110 μm, and 90 % of the particles were smaller than 450 μm as determined by Coulter LS320 particle size analyzer wet module (Coulter Corporation, Miami, FL, USA).

Xylanase Enzyme Preparation

A commercial *Bacillus subtilis* xylanase preparation Depol 761P (Biocatalysts Ltd, Cardiff, UK) was used for the bran treatments. According to the manufacturer, it is especially suitable for extraction of soluble fiber from wheat bran. The activity profile of the enzyme preparation was as follows: xylanase (1 % birch glucurone xylan as substrate) 28,660 nkat/g (Bailey et al. 1992), polygalacturonase (0.4 % polygalacturone acid) 1,317 nkat/g (Bailey and Pessa 1990), β-glucanase (1 % barley β-glucan) 1,625 nkat/g (Zurbriggen et al. 1990), α-amylase (*p*-nitrophenyl maltoheptaoside) 44 nkat/g (Megazyme Ceralpha method), and β-xylosidase (5 mM *p*-nitrophenyl-β-D-xylopyranoside) 2 nkat/g (Poutanen and Puls 1988). The preparation was found to be free from cellulase (filter paper) (IUPAC 1987), endoglucanase (1 % hydroxyethyl cellulose) (IUPAC 1987), mannanase (0.5 % locust bean gum) (Stålbrand et al. 1993), β-glucosidase (1 mM *p*-nitrophenyl-β-D-glucopyranoside) (Bailey and Linko 1990), α-arabinosidase (2 mM *p*-nitrophenyl-α-L-arabinofuranoside) (Poutanen et al. 1987), endo-protease (azurine-crosslinked casein, Protazyme AK tablet method, Megazyme International Ireland), and ferulic acid esterase (4 mM ethyl ferulate) (Forssell et al. 2009) activities. All activity measurements were performed at pH 5, 50 °C.

Enzymatic Treatment of Wheat Bran

Bran was treated with or without added enzyme using two different mixing systems which have also been described previously (Santala et al. 2011). The enzyme preparation was dosed according to its xylanase activity at a level of 200 nkat/g bran, which corresponds the dosage used previously (970 EU/g bran) (Santala et al. 2011). The enzyme powder was mixed with bran before water addition. The treatments at 40 % water contents were performed in a farinograph mixing bowl (Brabender Farinograph, mixer type S300 with z-blades, Brabender, Duisburg, Germany). The mixer bowl was heated by water circulation (50 °C), and the mixing speed was 60–63 rpm. One hundred twenty-five grams of ground bran (with or without enzyme addition) was placed in the mixer, and preheated water was added by spraying for 1–3 min while the blades were rotating to obtain an even distribution of water. The mixing bowl was sealed tightly to avoid evaporation of moisture. At water contents of 90 %, due to the liquid form of the mixture, the reactions were performed in a covered steel container (0.5 l) with double-blade mixer (speed 160 rpm) placed in a water bath (50 °C). The water content of the treatment refers to the total water content, i.e., the water content of bran was taken into account.

The experiments were done in duplicate. The reaction time was 4 or 24 h, after which a sample was taken and

reaction stopped by cooling down and freezing the sample immediately. Subsequently, part of the frozen sample was freeze dried and ground with a laboratory mill (0.5 mm sieve) for later analyses.

Standard Analyses

The moisture content of the bran and the freeze dried bran samples was determined by oven drying (1 h at 130 °C). Analyses of the chemical composition of bran were made as follows: total protein content by the American Association of Cereal Chemists (AACC) method no. 46–11A (AACC 2003), total DF by AOAC method no. 985.29 (AOAC 1990), ß-glucan by AACC method no. 32–23 (AACC 2003), fructan by AOAC method no. 999.03 (AOAC International 2003), fat by AOAC method no. 922.06 (AOAC International 2003), digestible starch by Megazyme method (Mccleary et al. 1994), and ash gravimetrically by burning at 550 °C.

Particle Size Determination

The particle size distribution of bran samples was determined in duplicate by laser light (750 nm) diffraction using Coulter LS320 particle size analyzer wet module (Coulter Corporation, Miami, FL, USA). Seventy-five milligrams of freeze dried sample was dispersed in 1.5 mL of distilled water. The particle size measurement was performed after 15 min hydration including vortexing and incubation in an ultrasound bath (5 min) in order to prevent formation of aggregates. The results were calculated from the volumetric distribution of the particles using Fraunhofer optical model and geometric statistics.

Light Microscopy Analyses

Microscopy analyses were done using both freeze dried and fresh (frozen) samples. After defrosting at ambient temperature, the sample with 90 % water was first centrifuged to remove excess water. The bran samples were treated as described by Dornez et al. (2011) and sectioned to 2-μm sections in a rotary microtome HM 355 (Microm Laborgeräte GmbH, Walldorf, Germany). Sections were stained either with Light Green and Lugol's iodine solution, which stain protein green and starch purple, respectively, or with Acid Fuchsin and Calcofluor White, which stain protein red and β-glucan-rich cell walls light blue, respectively, as described by Andersson et al. (2011). The samples were imaged using exciting light (excitation 400–410 nm, emission >455 nm) and examined with an Olympus BX-50 microscope (Olympus, Tokyo, Japan). Micrographs were obtained using a PCO SensiCam CCD camera (PCO, Kelheim, Germany) and the Cell^P imaging software (Olympus).

Analysis of Monosaccharide Composition and Content

For the analysis of water-extractable monosaccharide composition, 0.25 g of freeze dried sample was mixed with 7 mL of 4 °C distilled water and shaken with glass pearls for 15 min at 4 °C. After centrifugation, the supernatant was boiled for 15 min and centrifuged again. The supernatant was hydrolysed with 3.75 M H_2SO_4. The hydrolysate and the monosaccharide standards (glucose, arabinose, xylose, galactose, and mannose) were analyzed as their alditol acetates as described by Blakeney et al. (1983) by gas chromatography (GC) using a Agilent 6890 GC equipped with a flame ionization detector (Agilent, Palo Alto, CA, USA). The column was DB-225 [30 m×0.32 mm; film thickness 0.15 μm (Agilent)]. Helium was used as carrier gas 1.2 mL/min. Split injection (1:3) was performed at 250 °C, and the detector was operated at 250 °C. The analytes were separated at 220 °C for 15 min. The monosaccharides were identified according to their retention times and quantitated with corresponding standard curves. Free hexose sugars were corrected by a factor of 0.9 to anhydro sugars, and pentose sugars by factor of 0.88. The fact that some of the arabinose residues might originate from arabinogalactan peptides (AGP) was not taken into account in the calculations of AX levels because the level of AGP in wheat bran is assumingly very low, as discussed previously (Santala et al. 2011). For the analysis of total monosaccharide composition, 50 mg of bran sample was mixed with 1.56 mL of 72 % (26 N) H_2SO_4 and incubated at room temperature for 30 min. After addition of 15.6 mL of water, the samples were boiled for 2 h and centrifuged. After that, the supernatants were acetylated and analyzed by GC as described above. All analyses were made in duplicate.

Protein Extraction and Analysis

Proteins of the bran samples were extracted using a sequential buffered extraction procedure modified from that of Loponen et al. (2007). Salt-soluble proteins were first extracted by mixing 50 mg of freeze dried sample with 1.5 mL of 4 °C 0.5 M Tris–HCl buffer (pH 8) containing 1 M NaCl, and shaken for 15 min at 4 °C. After centrifugation (21,100×*g*, 15 min, 4 °C), the extraction was repeated. The supernatants were combined and stored frozen until analysis. The residual protein fraction was extracted from the sediment at 50 °C water bath for 60 min, vortexing at 10-min intervals, using 1.5 mL of mixture containing 2 % sodium dodecyl sulphate (SDS), 10 % glycero1, 1.5 % dithiothreitol (DTT), and 0.05 M Tris–HCl pH 8 buffer. After centrifugation (21,100×*g*, 15 min, 21 °C), the extraction was repeated. The supernatants were combined and stored frozen until analysis.

The different protein fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–

PAGE) (Laemmli 1970). Two volumes of protein extracts were mixed with one volume of SDS-SB (20 % glycerol, 4 % SDS, 10 % β-mercaptoethanol, and 0.02 % bromophenol blue in 0.1 M Tris–HCl pH 6.8 buffer) and boiled for 10 min. Samples were run in Criterion TGX stain-free precast Tris–HCl gradient gels (4–20 %; Bio-Rad, Hercules, CA, USA) and visualized with a Criterion stain-free imaging system (Bio-Rad) where protein visualization is based on UV light-driven reaction of tryptophan residues in the presence of trichlorocompounds (Kazmin et al. 2002). The used protein standards were Precision Plus Unstained Protein Standards (Bio-Rad), ranging from 10 to 250 kD.

The protein content of all extracts was analyzed by a commercial kit (RC DC Protein Assay, Bio-Rad, Hercules, CA, USA) that is compatible for samples containing both reducing agents and detergents. In the assay procedure, sample proteins were precipitated and separated by centrifugation before the actual quantification, which is based on the Lowry protein assay (Lowry et al. 1951). Bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard protein. The analysis was made in duplicate.

Water Binding/Retention Capacity

WBC of bran samples was determined in duplicate by a simple centrifugation method modified from Sollars (1973). One hundred milligrams of freeze dried sample was weighed in an Eppendorf tube, and the tube with the contents was tarred. One milliliter of water was added and mixed by vortex. The bran suspension was shaken at room temperature (22–23 °C) for 30 min and centrifuged (12,100×*g*, 15 min). The supernatant was removed carefully, and the tube was weighed. The gained weight (plus the water contained initially in the sample) per gram of bran sample dry matter was the WBC of the sample.

Water Holding Capacity

WHC and its kinetics were determined using the Baumann apparatus (Baumann 1966). The Baumann apparatus consists of a small thermostated water tank that is connected to a horizontal graduated capillary tube filled with water. A sintered glass with Whatman grade 5 filter paper (Whatman, Little Chalfont, UK) was placed on top of the water tank, and after 5 min, the zero value was read from the graduated capillary tube. Seventy-five milligrams of dry sample was sprinkled for 30–40 s on the wetted filter paper, and the water uptake was recorded for 25 min at room temperature (22–23 °C). A glass lid was set on the sinter glass to minimize evaporative losses during the measurement. The results are expressed as milliliters of water uptake (plus the water contained initially in the sample) per 1 g of sample dry matter. Each sample was analyzed at least in duplicate.

Statistical Analysis

The results were calculated as means of at least four analysis results (duplicate analyses for each bran sample). Data were subjected to analysis of variance using the IBM SPSS Statistics 19 (IBM Corporation, Somers, NY, USA), and significant differences ($P<0.05$) between individual means were identified by the Tukey's test.

Results and Discussion

Impact of Processing on the Particle Size and Microstructure of Bran

Treatments at water content of 40 % resulted in notably smaller average bran particle size (upon grinding the freeze dried processed sample) than the corresponding treatments at 90 % water content (Table 1). The initial particle size of bran (mean diameter, 113 μm) did not significantly change during 4 h of treatment without added xylanase at water content of 90 %, while at 40 %, water content the average particle size decreased to 56 μm. After 24 h of treatment at 40 %, the average particle size was further reduced to 37 μm (35 % reduction as compared to the 4-h sample), while at 90 %, the particle size reduced to 94 μm (14 % reduction as

Table 1 Mean particle size (micrometer), water binding capacity (grams of water per gram of bran dm) and water holding capacity (grams of water per gram of bran dm) of untreated bran and bran samples treated at 40 and 90 % water content (determined from freeze dried and ground samples)

		4 h treatment				24 h treatment			
		No added enzyme		With xylanase		No added enzyme		With xylanase	
	Untreated	90 %	40 %	90 %	40 %	90 %	40 %	90 %	40 %
Mean particle size (μm)	113±9a	110±7a	56±3c	65±3c	37±3d	94±2b	37±4d	60±4c	26±2e
Water binding capacity (g water/g bran dm)	2.8±0.2a	3.0±0.1a	2.5±0.2b	2.1±0.1c	1.7±0.1d	3.0±0.2a	2.3±0.2bc	2.3±0.1bc	1.3±0.1e
Water holding capacity (g water/g bran dm)	3.6±0.1b	3.9±0.2a	3.2±0.2c	2.9±0.2d	2.4±0.1e	3.6±0.1ab	3.0±0.1d	2.9±0.2d	2.0±0.2f

The results are expressed as mean ($n=4$)±standard deviation. Values marked with different letters within the same row are significantly different ($P<0.05$)

compared to the 4-h sample). The use of xylanase reduced the particle size at both water contents, and already after 4-h treatment with xylanase, the mean particle decreased to 65 μm at 90 %, and to 37 μm at 40 % water content (Table 1).

Bran micrographs stained with Acid Fuchsin and Calcofluor White are presented in Fig. 1. The microscopy analyses were done using both freeze dried, ground samples and fresh (frozen) samples, but no differences were detected, and thus, only the images of fresh samples are shown. Light microscopy examination showed that processing of bran caused a significant release of proteins from the cells of the bran particles (Fig. 1). The change was most obvious after the treatments at 40 % (Fig. 1c, e), but detectable also at 90 % water content (Fig. 1b, d). The release of protein during endoxylanase treatment of wheat bran has also been noted by others (Benamrouche et al. 2002). Furthermore, the layered cell wall structure of the bran treated at 40 % water content was much more degraded, also when treated without added xylanase (Fig. 1c), as compared to the bran treated at 90 % (Fig. 1b). When bran was treated with xylanase at 90 %, the cell walls of the aleurone layer became thinner and lost their color, while seed coat and nucellar epidermis still remained attached to aleurone (Fig. 1d). In the bran treated at 40 % with no added enzymes, the structure of aleurone cells had broken, and the seed coat with nucellar epidermis had separated from aleurone cells (Fig. 1c). The use of xylanase at 40 % caused further degradation of the bran structures and the separation of seed coat and nucellar epidermis from each other (Fig. 1e).

The experiments at 40 and 90 % water content were performed in two different kinds of mixing devices because of the very different consistency of the bran–water mixtures. The difference in the consistency and the use of two different mixing systems brought about different shear forces, which obviously had an impact on the resulting bran properties. We have previously demonstrated that during the treatment at 40 % water content, the bran–water mixture transformed into a compact, plastic-like mass (Santala et al. 2011), which probably enhanced the impact of the mechanical shear during the treatment in the farinograph mixer. In the current study, the particle size measurement and

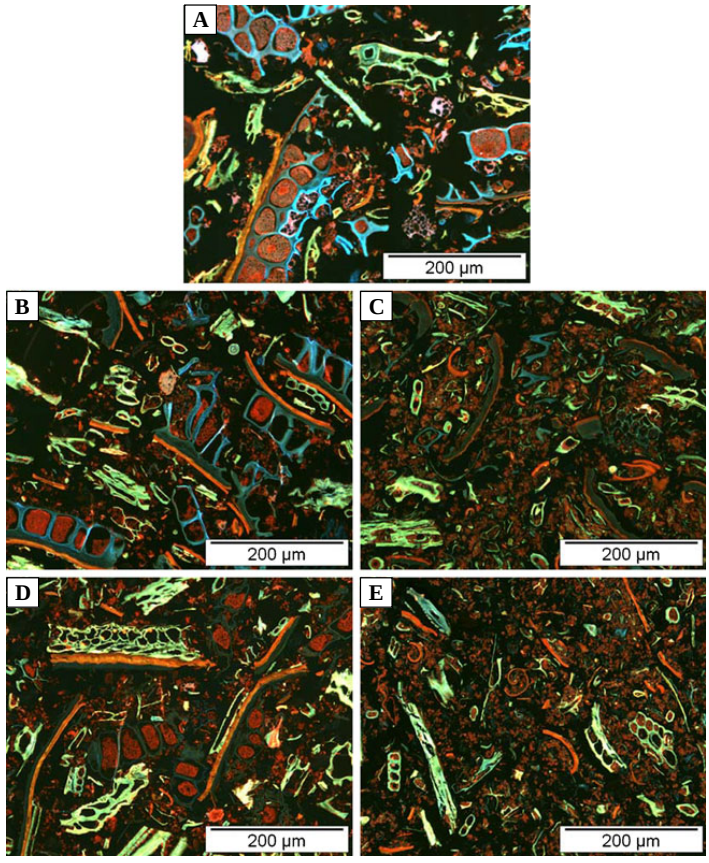

Fig. 1 Microstructure of untreated bran (**a**) and bran samples treated for 24 h without added enzymes at 90 % (**b**) and without added enzymes at 40 % (**c**), and bran samples treated for 24 h with xylanase at 90 % (**d**) and with xylanase at 40 % (**e**). Micrographs, prepared from fresh (frozen) bran samples, were stained with Acid Fuchsin and Calcofluor White. ß-glucan-rich endosperm and aleurone cell walls appear in *blue*, pigment strand (between pericarp and aleurone layer) in *orange*, pericarp layer in *light green and yellowish*, and proteins in *red and reddish brown*

microscopy analyses confirmed that the physical structure of the bran was significantly broken down by the treatments at 40 % water content, also without added enzymes, while smaller changes were detected in the bran treated at 90 %. The microscopy results also indicated that the bran components were broken down already during the treatments and not during the grinding of the freeze dried samples as no differences were detected between the micrographs prepared form fresh and frozen samples. The bran micrographs (prepared from fresh samples) stained with Light Green and Lugol's iodine solution further showed that starch granules were not significantly affected by any of the treatments (data not shown).

Total and Water-Extractable Monosaccharide Composition

The impact of treatment of bran at high (90 %) and low (40 %) water content on the solubilization of carbohydrates was followed by analyzing the monosaccharide composition of the water extracts of freeze dried bran samples after acid hydrolysis (Table 2). The total water-extractable monosaccharide content was higher after treatments at 40 % water content than at 90 %, especially after 24-h treatment without added enzymes, as the content of water soluble monosaccharides was 18.97 and 10.03 % of bran dm after treatments at 40 and 90 %, respectively (Table 2). This is mainly due to the glucose content, which increased at 40 % but did not significantly change at 90 % from 4 to 24 h. (Table 2). An increase in the soluble glucose content at 40 % refers to the degradation of starch, cellulose, or β-glucan. There was a trend of increase in the content of water-extractable mannose at 40 % and decrease at 90 % after 24-h treatments (Table 2). Mannose in the GC chromatograms originated probably from reduced fructose (fructan) because fructose as such cannot be detected by GC since fructose as a ketose produces mannitol and glucitol in the reduction step that was applied in the preparation of GC samples. Thus, fructose cannot be distinguished in GC analysis from glucose or mannose, which, respectively, forms glucitol and mannitol on reduction (Virkki et al. 2008). The increase in mannitol content at 40 % may thus be due to degradation of fructose, which bran contained 3.6 %.

The solubilization of AX, analyzed by the content of arabinose and xylose in the bran water extract, was rather similar at 40 and 90 % water content after 24-h treatment with xylanase (12.09 and 11.11 % of the bran dm, respectively; Table 2). However, without added enzyme, somewhat more solubilization was detected after the 24-h treatment at 40 than at 90 % water content (4.25 vs. 2.64 %). This indicates that the solubilization of AX at 40 % might not have been caused only by the action of the added xylanase alone, but also by other factors, such as the physical breakdown of bran cell walls by shear forces. The analysis of A/X ratio

Table 2 Monosaccharide composition (percentage bran dm) and A/X (arabinose/xylose) ratios of the water extracts of untreated bran and freeze dried bran samples after acid hydrolysis

		4 h treatment				24 h treatment			
		No added enzyme		With xylanase		No added enzyme		With xylanase	
	Untreated	90 %	40 %	90 %	40 %	90 %	40 %	90 %	40 %
Glucose (% bran dm)	3.64±0.17a	6.82±0.33b	8.43±0.38c	8.86±0.50c	10.69±1.23d	6.55±0.32b	13.58±0.45e	7.95±0.67bc	12.72±0.42e
Galactose (% bran dm)	0.48±0.03a	0.54±0.04ab	0.62±0.04bcd	0.63±0.04bcd	0.65±0.08 cd	0.57±0.03bc	0.60±0.02bcd	0.67±0.03d	0.65±0.02 cd
Mannose (% bran dm)	0.47±0.02bcd	0.37±0.03ab	0.41±0.03bcd	0.40±0.03abc	0.43±0.03bcd	0.27±0.11a	0.54±0.02d	0.39±0.10abc	0.53±0.03 cd
Ara+xyl (% bran dm)	0.66±0.03a	1.64±0.23ab	2.76±0.29b	9.12±0.56d	8.51±1.09d	2.64±0.16b	4.25±0.16c	11.11±0.43e	12.09±0.48e
A/X	0.99±0.02a	0.89±0.06b	0.72±0.02c	0.40±0.02e	0.41±0.02e	0.75±0.09c	0.59±0.01d	0.36±0.01e	0.32±0.01e
Sum[a]	5.24±0.24a	9.37±0.61b	12.22±0.66c	19.01±1.08d	20.28±2.39d	10.03±0.33bc	18.97±0.35d	20.12±1.02d	25.99±0.92d

The results are expressed as mean (n=4)±standard deviation. Values marked with different letters within the same row are significantly different (P<0.05). Free hexose sugars were corrected by a factor of 0.9 to anhydro sugars, and pentose sugars by a factor of 0.88

[a] The sum of the monosaccharides (percentage bran dm)

provided new information about the role of the enzymes in the solubilization of AX during the treatment at 40 % water content. The A/X ratio of the water extract of untreated bran was 0.99, and the ratio decreased with increasing treatment time and by the use of xylanase at both water contents (Table 2). It is known that xylanases preferably attack and solubilize AX fragments with low arabinose substitution (Benamrouche et al. 2002). This kind of AX occurs mostly in the aleurone (A/X 0.3-0.5) and in the nucellar epidermis (A/X 0.1), while the AX of outer tissues are more substituted (A/X≥1.0), and thus more resistant to enzymatic attack, as also indicated before (Van Craeyveld et al. 2010; Antoine et al. 2003; Barron et al. 2007). Interestingly, during the treatments at both water contents, the A/X ratio of the bran water extract decreased rather identically with increasing arabinose+xylose content (Table 2), suggesting that AX was solubilized from the same bran tissues regardless of the processing conditions studied. Furthermore, the A/X ratio of the water extract of bran treated for 24 h at 40 % was 0.32 (Table 2), which was in the range of the values reported in the literature for enzymatically solubilized wheat bran AX (0.27–0.32) (Beaugrand et al. 2004; Swennen et al. 2006). On the contrary, AX oligosaccharides produced by ball milling treatment have been reported to have much higher A/X ratio (0.65–0.72) (Van Craeyveld et al. 2009), suggesting that also some pericarp AX was rendered water-extractable by the ball milling treatment (Van Craeyveld et al. 2009). Thus, although the mechanical shear obviously enhanced the solubilization of AX during the treatments at 40 % water content, the mechanical energy input was probably not high enough to cause similar mechanical solubilization of AX as in the ball milling treatment (Van Craeyveld et al. 2009), and the solubilization of AX was also caused by enzymatic action at the 40 % treatments. The solubilization of AX during the treatments without added xylanase could have been due to the action of bran-associated (endogenous or microbial) enzymes. Instead of direct mechanical solubilization, the shear forces and the physical breakdown of bran particles have probably enhanced the action of endo- and exogenous enzymes by improving the availability of the substrate. The decrease of bran particle size during the treatment at 40 % (Table 1) increased the surface area of the substrate, which probably made the cell wall components more easily accessible to enzymes. It has been shown that the decrease of particle size may impact the hydrolysis of the substrate by enzymes (Mahasukhonthachat et al. 2010; Hemery et al. 2010).

The total monosaccharide composition of bran (glucose 24.0±0.8 %, galactose 1.16±0.04 %, mannose 0.63±0.04 %, AX 22.8±0.6 % with A/X ratio of 0.60±0.03) was not affected by any of the treatments, except for the glucose content, which decreased to 21.3±1.3 % after 24-h treatment at 90 % both with and without added xylanase (data not shown).

Extractability and Electrophoretic Patterns of Proteins

The physicochemical characteristics of bran proteins are not yet well established, although the protein content of bran is relatively high. In the current study, the impact of bran treatments on the solubility and molecular weight of bran proteins was analyzed by sequential extraction and electrophoretic analysis of salt-extractable and SDS+DTT-extractable (residual) proteins (Fig. 2) and by the spectrophotometrical quantification of protein in the extracted fractions (Fig. 3). The concentration of salt-extractable proteins decreased during the bran treatments, especially during the treatments at 40 % water content. This was detectable by both SDS–PAGE and protein quantification analysis. In the electrophoretic patterns

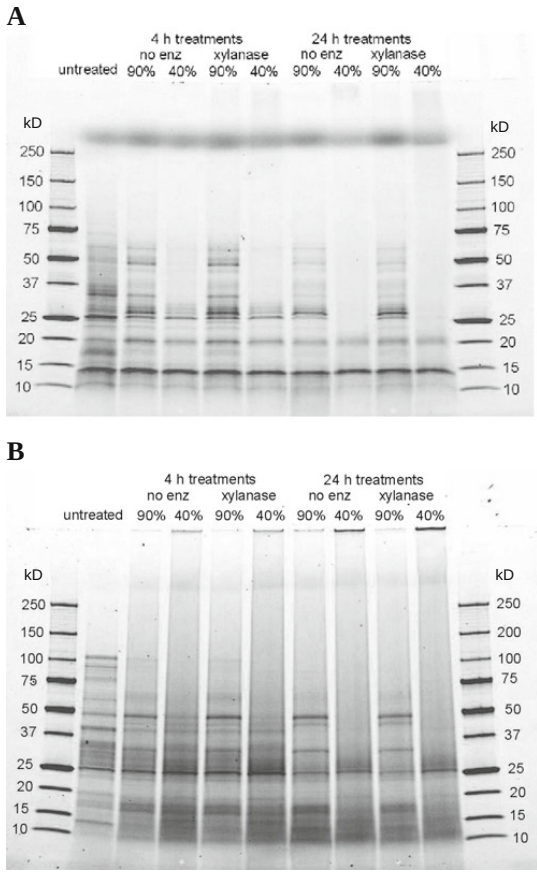

Fig. 2 SDS–PAGE patterns of salt extractable (**a**) and SDS+DTT-extractable (**b**) (residual) proteins of untreated bran and bran samples treated at 40 and 90 % water content

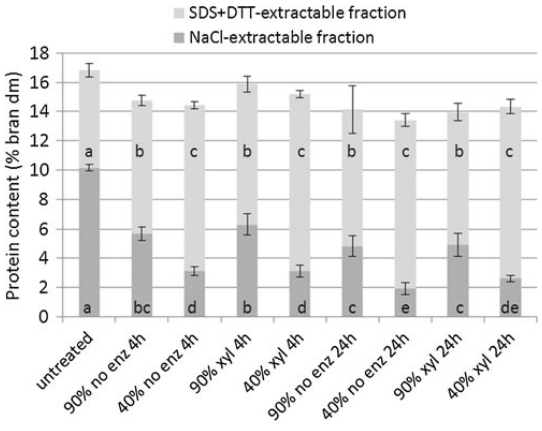

Fig. 3 Protein content (percentage bran dm) of the NaCl and SDS+DTT extracts of untreated bran and bran samples treated at 40 and 90 % water content. The results are expressed as mean (n=4), and the standard deviations are indicated by *error bars*. Columns marked with *different letters* within the same fraction are significantly different (P<0.05)

of the salt-soluble fraction, the proteins above 25 kDa had disappeared from the bran treated at 40 % water content (Fig. 2a). These protein bands also appeared lighter in the sample treated at 90 % for 24 h than in the untreated bran. The initial content of salt-extractable protein in the untreated bran was 10.2 % of bran dm, while after 4-h treatments without added enzyme at 90 and 40 % water, the content of salt-extractable proteins dropped to 5.7 and 3.1 % of bran dm, respectively (Fig. 3). After 24-h treatment without added enzyme, the content of salt-extractable protein decreased further at 40 % (to 1.9 % of bran dm).

Contrary to the decrease in the salt-extractable protein content, the concentration of SDS+DTT-extractable, "residual" protein fraction increased during the bran treatments. Protein aggregation was also detected in this residual fraction. This is evident from the electrophoretic patterns of SDS+DTT-extractable fraction, where no protein above 100 kDa appeared in the untreated bran, while in the treated bran samples, protein "dust" was observed in this region (Fig. 2b). Furthermore, especially in the case of samples treated at 40 %, a lot of protein had remained in the wells of the electrophoresis gel, which also indicates the formation of large protein-containing aggregates. The SDS+DTT-extractable fractions also contained small proteins of molecular weight of 10–20 kDa not detectable in the initial bran (Fig. 2b). The increase in the content of SDS+DTT-extractable proteins was also confirmed by the spectrophotometrical quantification of protein in the fractions. The initial content of SDS+DTT extractable protein in the untreated bran was 6.7 % of bran dm, and after 4-h treatments without added enzyme at 90 and 40 %, the content of SDS+DTT-extractable proteins increased to 9.1 and 11.3 % of bran dm, respectively. The use of xylanase had no visible effect on the electrophoretic patterns of proteins at either water contents. According to the spectrophotometrical quantification, the use of xylanase slightly increased the content of SDS+DTT-extractable protein after 4-h treatment (9.6 % at 90 % and 12.1 % at 40 %), but the increase was not statistically significant.

The formation of the high molecular weight protein aggregates in the SDS+DTT-extractable fraction was most probably due to the formation of covalent bonds during the bran treatments. Because electrophoresis was performed in denaturing conditions, which leads to the reduction of disulfide bonds during preparation of samples for SDS–PAGE, the protein aggregation could not be only due to the formation of disulfide bonds between the proteins. Protein aggregation is probably due to the high shear forces especially during the treatment at water content of 40 %. It is known that heating and shearing may cause degradation or aggregation of proteins by disulfide or covalent bond formation, for example in extrusion processing, and several authors have reported a decrease in protein extractabilities after extrusion, as reviewed by Anderson and Ng (2000). Then again, the small proteins in the SDS+DTT-extractable fractions which were not detectable in the initial bran might have formed by nonenzymatic depolymerization of the larger proteins, which has also been observed to occur in extrusion processing of wheat flour (Anderson and Ng 2000). Another possibility is the action of endogenous proteases known to be present in bran (Galleschi and Felicioli 1994; Umetsu et al. 1981).

The spectrophotometrical quantification of salt-soluble and SDS+DTT-extractable proteins showed that the sum of proteins in these two extracts of processed brans was somewhat lower than the sum of protein in the extracts of untreated bran. It may be that some protein remained in the bran residue (from which the proteins were extracted) due to the possible formation of covalent bonds between proteins and insoluble polysaccharides, thus preventing the extraction of residual protein even with SDS+DTT. It is also possible that strong aggregation and conformational changes in the protein structure had inhibited the access of the colorimetric reagent to the proteins, thus leading to lower color development and lower result.

Impact of Processing on the Hydration Properties of Bran

Treatments at water content of 40 % resulted in lower WBC of the freeze dried sample than the corresponding treatments at 90 % water content (Table 1). With no added enzymes, the initial WBC of bran (2.8±0.2 g/g) was not significantly affected by the 4 or 24 h treatments at 90 % water content (WBC 3.0 g/g after both 4 and 24 h treatments), while after

the treatments at 40 % water content, WBC reduced to 2.5 g/g and 2.3 g/g after 4 h and 24 h, respectively. The use of xylanase decreased WBC at both water contents as compared to the corresponding treatments with no added enzymes (Table 1). The lowest WBC was measured for the sample treated at 40 % with xylanase for 24 h (1.3 g/g).

WHC curves of bran samples, determined by the Baumann apparatus, are shown in Fig. 4. The WHC values (the max value of the curve, Table 1) of the bran changed during the treatments in a similar manner as the WBC (with minor exceptions), but WHC was always 0.6–0.9 units higher than the WBC of the same sample (Table 1). The highest WHC was measured for the sample treated at 90 % water content with no added enzymes for 4 h (3.9 g/g) and the lowest for the sample treated at 40 % water content with xylanase for 24 h (2.0 g/g). The samples did not significantly differ in the kinetics of water holding, as observed from the estimated slopes of the WHC curves (Fig. 4).

Hydration characteristics are one of the most important physicochemical properties concerning the technological applicability of DF preparations (Thebaudin et al. 1997). Water binding is also particularly relevant for the physiological actions of DF (Chaplin 2003). In a simplified way, water bound to insoluble polysaccharides can be presented in two forms: (1) water bound by surface tension in the pores of the matrix and (2) water bound by hydrogen bonds, ionic bonds, and/or hydrophobic interactions. The distribution of water between these two states depends on the chemical structure of the components, the associations between molecules, the size of the particles, the porosity of the material, and the effects of solvents and temperature (Thebaudin et al. 1997). In this study, the use of xylanase decreased WBC and WHC at both water contents, which was expected as water unextractable AX is known to bind more water than WEAX (Courtin and Delcour 2002). Also, the degradation and solubilization of other fiber components, such as β-glucan, have probably affected the WBC in the same way as the solubilization of AX by binding less water. The impact of time on the reduction of WBC and WHC during the treatments was more obvious at 40 than at 90 %. This may be due to the significant decrease of the bran particle size during the treatment at 40 %. In general, particle size reduction of bran and other DF preparations decreases water binding capacity (Noort et al. 2010; Auffret et al. 1994; Zhu et al. 2010). When particle size increases, so does the trapped volume due to imperfect packing and consequential apparent water binding (Thebaudin et al. 1997). The effect of grinding on WBC is attributable not only to particle size reduction but also to the altering of the physical structure of the fiber matrix (Auffret et al. 1994).

Hydration properties were measured using two different methods, WBC by a centrifugation method and WHC by the Baumann method. The WBC can be defined as the quantity of water that remains bound to the hydrated fibers following the application of an external force (pressure, or most commonly, centrifugation), while the WHC is defined by the quantity of water that is bound to the fibers without the application of an external force (except for gravity and atmospheric pressure) (Thebaudin et al. 1997). In the Baumann method, the measurement is based on the principle of the diffusion of a liquid by capillary action. Both methods gave similar results about the impact of the different treatments, but the WBC was always lower than the WHC of the same sample measured by the Baumann apparatus. This is probably due to the fact that in the centrifugation method, the water-soluble components of the sample are lost in the supernatant, thus reducing the mass of the sample that is holding water (Rasper and DeMan 1980). Furthermore, the water retained by centrifugation depends on the g-force used (Chaplin 2003), while in the Baumann method, no external force is used.

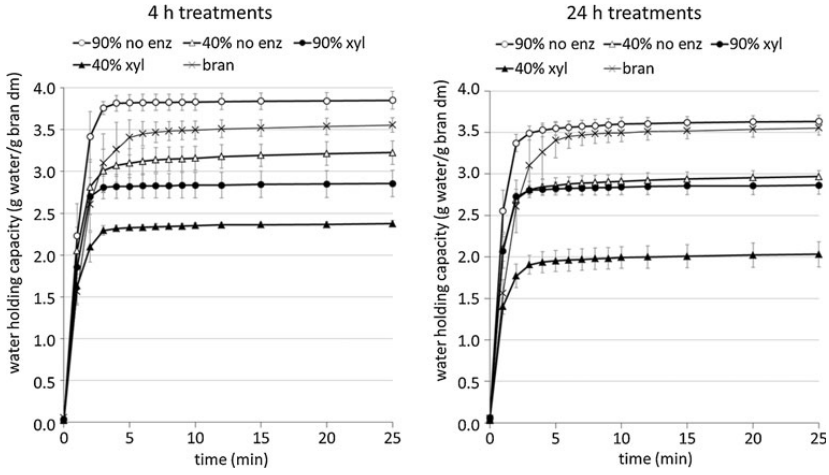

Fig. 4 Water holding capacity as a function of measurement time of untreated bran and bran samples treated at 40 and 90 % water content (determined from freeze dried and ground samples). The results are expressed as mean (n=4) and the standard deviations are indicated by *error bars*

Conclusions

The processing at 40 % water content brought about larger changes to bran properties than processing at 90 % water content. The physical structure of the bran changed more by the treatments at 40 % water content, also without added enzymes. The results indicated the strong impact of the shear forces at the 40 % treatment, which also enhanced the solubilization of polysaccharides. However, the A/X ratio results suggest that the solubilization of AX was caused by enzymatic action, and not by mechanical degradation, probably due to improved availability of the substrates. The observed changes in bran proteins were more obvious at 40 % treatment, and caused either by themomechanical mechanisms or by the action of bran-associated enzymes. Water binding capacity of bran was lower after the treatment at 40 than at 90 %, probably due to the smaller particle size of bran treated at 40 %, and higher content of water-extractable carbohydrates. The study showed that both structural and physicochemical properties of bran were affected by the water content and the way of processing, probably mostly through mechanisms related to the consistency of the bran–water mixture. This opens up new possibilities for hybrid processing aiming at improved bran applicability.

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PUBLICATION III

Use of an extruder for pre-mixing enhances xylanase action on wheat bran at low water content

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Use of an extruder for pre-mixing enhances xylanase action on wheat bran at low water content

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HIGHLIGHTS

- Xylanase action (AX solubilisation and hydrolysis) on wheat bran was studied.
- Impacts of mixing method, water content and bran particle size were studied.
- With xylanase, MW of WEAX was not significantly affected by these variables.
- Extruder enhanced AX solubilisation as compared to blade mixing at low water content.
- Plasticization in extruder probably enhanced xylanase action via improved diffusion.

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ABSTRACT

The aim of the work was to test the hypothesis that at low water content enzyme action on biomass is enhanced when the raw material is in the form of a continuous mass instead of powder/granular form. Effects of two pre-mixing methods, blade-mixing and extrusion, on xylanase action were studied during stationary incubation of wheat bran of different particle sizes, also in comparison with incubation at high water content with continuous stirring. The use of an extruder enhanced arabinoxylan (AX) solubilisation at low water content (<54%), as compared to blade-mixing. AX solubilisation was highest in the high-water stirring treatment, but based on molecular weight, xylanase action on solubilised AX was similar as in the extrusion-aided process. Pre-mixing by extrusion enabled efficient enzyme action at low water content without the requirement for continuous mixing, probably due to the enhanced diffusion by the formation of a continuous mass in the extruder.

1. Introduction

In the extrusion process food ingredients are forced to flow, under mixing, heating and shear, through a die that forms and/or puff-dries the ingredients (Rossen and Miller, 1973). Extrusion technology can be used either to form ready-to-eat foods (snacks, cereals, pasta, confectionery) or to modify food ingredients. A less studied approach is to use extruders as bioreactors for enzymatic processes under elevated temperature, pressure and shear, at moisture levels as high as 70% or more. "Wet extrusion" with feed moisture content above 40% has been possible only since the late 1980s due to developments with twin screw extruders including sophisticated barrel designs, screws and dies (Akdogan, 1999). Compared to traditional stirred-tank reactors, extruders and screw reactors present a cost competitive alternative reactor type for enzymatic modification of biomaterials, especially when targeting dry end products, since the extruders can operate at higher solids content, thus reducing the need for addition and removal of large amounts of water.

The use of an extruder for enzymatic modification has previously been studied mainly for liquefaction of starch by thermostable α-amylase (Linko, 1989; Tomás et al., 1997; de Mesa-Stonestreet et al., 2012). The impact of water content has also been studied, and in most cases the conversion has been highest at the highest water content studied, typically at 55–70% water content, as reviewed by Linko (1989) and Akdogan (1999), although Tomás et al. (1997) reported maximum starch hydrolysis at an intermediate water content of 60% when studying in the range of 55–65%. However, as starch needs to be gelatinized for efficient liquefaction, these extrusion treatments were typically performed above 70 °C or even above 100 °C. Studies concerning the use of extrusion for enzymatic modification of other cereal materials or for other targets are rare. However, two recent papers reported the use of cell wall degrading enzymes for enzymatic hydrolysis of oat bran β-glucan at a water content of 50% (Sibakov et al., 2013a) and for modification of brewer's spent grain at a water content of 65% (Steinmacher et al., 2012).

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Several biocatalytic and thermomechanical methods and processing conditions have been studied as potential means to modify the properties of wheat bran and its components, since wheat bran is a nutritionally appealing (high in dietary fiber, protein and phytochemicals) and widely available raw material which is currently under-utilised as a food ingredient due to its technological and sensory challenges (Sibakov et al., 2013b). Particle size is an important parameter for the use of bran, affecting both its physiological effects and technological functionality (Hemery et al., 2011; Robin et al., 2012; Zhang and Moore, 1999). Decreasing the particle size by grinding increases the surface area available for reactions. It has been shown that decreasing the particle size of plant materials may enhance their enzymatic hydrolysis (Silva et al., 2012; Niemi et al., 2012; Mahasukhonthachat et al., 2010; Dasari and Berson, 2007). Reduction of particle size can also affect the physicochemical properties of bran, such as water uptake and solubility (Mahasukhonthachat et al., 2010; Zhu et al., 2010), as well as the rheological behaviour of biomass slurries (Dasari and Berson, 2007; Viamajala et al., 2009), which may play an important role in enzymatic processes especially at low water content, when only a limited amount of free water is available.

Solubilisation of the arabinoxylan (AX) of cereal bran by endoxylanases has been shown to modify the technological properties of the bran (Katina et al., 2012; Lebesi and Tzia, 2012). We have previously shown that enzymatic solubilisation of bran AX can be efficiently performed even at low water content (40%) using continuous mixing (Santala et al., 2011, 2012). However, when processing at low water content, i.e. at high consistency, continuous mixing requires a high amount of energy, which may not be feasible in industrial processes. In the previous study (Santala et al., 2011), the enhanced AX solubilisation was related to the formation of a compact plastic mass during the treatment at the water content of 40%, because it resulted in the reduction of bran particle size due to the high shear during the treatment with continuous mixing (Santala et al., 2012). However, it is also possible that the formation of a continuous mass from the granular/powdery material can be used as a means to facilitate enzyme action even without continuous mixing. In the current study the aim was to test this hypothesis by studying the effects of two different pre-mixing and forming methods, blade-mixing and extrusion, on xylanase action during stationary (i.e. without stirring) incubation of wheat bran. The impact of treatment water content and bran particle size on the solubilisation and hydrolysis of AX during the different processes was investigated. Further, the aim was to compare the stationary enzyme incubation at low water content to incubation with continuous stirring at high water content.

2. Methods

2.1. Bran

Commercial wheat bran (Fazer Mill & Mixes, Lahti, Finland) was used as raw material and ground by TurboRotor technology (Mahltechnik Görgens GmbH, Dormagen, Germany) to three different levels of fineness. The particle size distributions of the bran raw materials (Table 1) were determined in triplicate from dry bran dispersions by laser light diffraction (Mastersizer 3000, Malvern, Worcestershire, UK) and calculated from the volumetric distribution of the particles using the Fraunhofer optical model. The heat damage possibly associated with intensive milling treatments could be avoided, since in the grinding technology used the high air throughput and short residence times ensured that the product temperature remained below 45 °C. The dietary fibre (DF) content of the brans (Table 1), was analysed by AOAC method No. 991.43 (Prosky et al., 1988). For the quantification of total AX (Table 1), 0.1 g of bran was mixed with 5 ml of 0.5 M H_2SO_4 and boiled for 30 min and centrifuged, followed by a colorimetric determination (Douglas, 1981).

2.2. Hydration properties

Water holding capacity was determined by the Baumann apparatus as described previously (Santala et al., 2012) using a sample size of 50 mg and measurement time of 30 min.

2.3. Xylanase enzyme preparation

A commercial *Bacillus subtilis* xylanase preparation, Depol 761P (Biocatalysts Ltd., Cardiff, UK), was used for the bran treatments. The activity profile (xylanase 28,660 nkat/g, polygalacturonase 1317 nkat/g, β-glucanase 1625 nkat/g, α-amylase 44 nkat/g, and β-xylosidase 2 nkat/g) of the preparation was previously reported by Santala et al. (2012). According to the manufacturer, the optimum temperature range of the enzyme preparation is 45–55 °C.

2.4. Extrusion-aided and blade-mixed treatments at water contents of 37–60%

The process scheme of the treatments is presented in Fig. 1. For the enzymatic treatments at water contents of 37–60%, the xylanase preparation (in powder form, dosed according to its xylanase activity at 200 nkat/g bran dm) was first mixed carefully with 450 g of dry bran, after which the mixture was pre-conditioned to a moisture content of 20% by adding water slowly while mixing (speed setting 2) with a Kenwood KM300 mixer (Kenwood Ltd., Havant, United Kingdom) with a K-shaped blade for 2 min. Pre-conditioning was also performed for the blank extruder treatments (i.e. without enzyme addition). For the extrusion-aided treatments, the pre-conditioned bran mixture was transferred to the feeding bowl of a co-rotating twin screw extruder (APV MPF 19/25, Baker Perkins Group Ltd, Peterborough, UK) within 20 min and fed to the extruder at a rate of 26 g/min. The barrel temperature was set at 50 °C and it was monitored that the temperature in the barrel remained at 50 °C during all the treatments. The screw speed was 65 rpm. Water was pumped to the barrel at an appropriate rate in order to obtain moisture contents of 37 ± 0.5%, 42 ± 1%, 48 ± 1%, 54 ± 1%, or 60 ± 1% in the

Table 1
Properties of the bran raw materials.

	Unground	Coarse	Fine	Ultrafine
Median particle size (μm)	1001 ± 9	702 ± 59	327 ± 9	81 ± 2
90% of particles < (μm)	2257 ± 16	1873 ± 163	895 ± 57	401 ± 28
10% of particles < (μm)	283 ± 5	127 ± 13	29 ± 1	10 ± 1
Total DF (% bran dm)	48.0	48.9	47.9	48.4
Soluble DF (% bran dm)	3.1	3.5	4.1	4.6
Total AX (% bran dm)	20.6 ± 0.4	20.5 ± 0.3	20.3 ± 0.6	20.6 ± 0.2
WEAX (% bran dm)	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
Water holding capacity(g/g bran dm)	3.7 ± 0.2	3.7 ± 0.2	3.7 ± 0.1	3.3 ± 0.1

The results are expressed as means ($n = 4$) ± standard deviation.

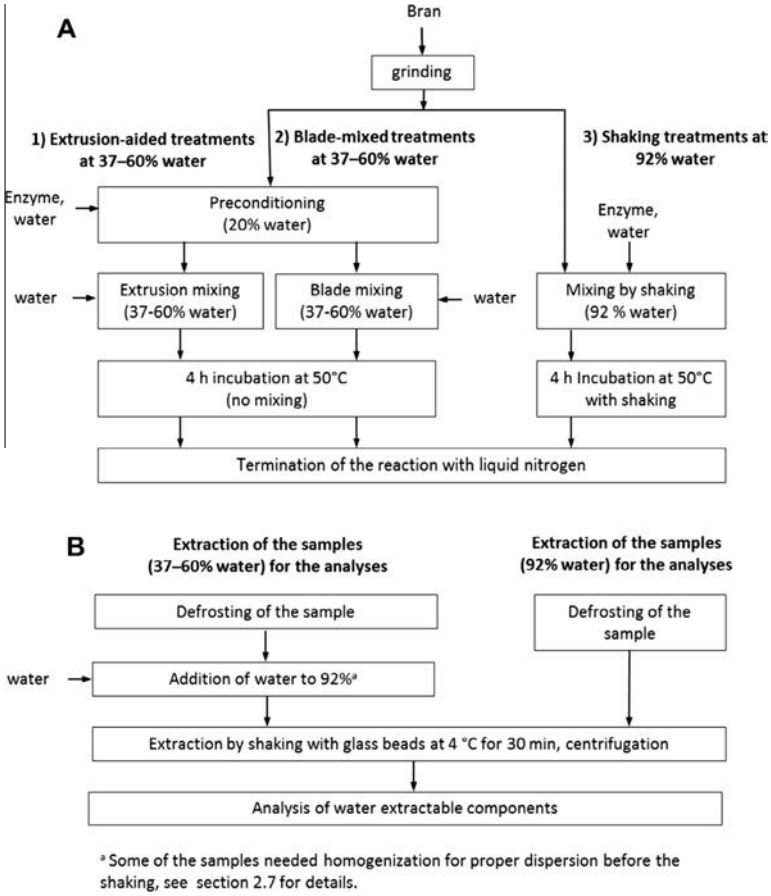

Fig. 1. Process scheme of the bran treatments (a) and extraction procedure (b) for the subsequent analyses.

extrudate. The residence time inside the barrel was about 3 min. Samples were collected from the die exit (diameter 3 mm, each sample was collected for 2 min, corresponding to approx. 20.8 g bran dry matter per sample), after which they were either frozen immediately using liquid nitrogen, or incubated further in sealed containers at 50 °C for 4 h and then frozen using liquid nitrogen.

For the treatments without extruder treatment (hereafter referred to as 'blade-mixed treatments') at water contents of 37–60%, the pre-conditioned bran mixture was pre-heated in a heat chamber at 55 °C for 12 min, after which it was brought to the intended moisture content by adding pre-heated (55 °C) water by spraying while mixing with a Kenwood mixer (K-blades, speed setting 2) for 3 min at 55 °C. The mixture was divided into samples which were either frozen immediately using liquid nitrogen, or incubated further in sealed containers at 50 °C for 4 h and then frozen using liquid nitrogen. Extrusion-aided and blade-mixed treatments were performed in duplicate.

2.5. Incubations at water content of 92%

Bran (3.00 g of bran dm) and the xylanase preparation (in powder form, 200 nkat/g bran dm) were weighed in tared 50 ml centrifuge tubes. Pre-heated water (50 °C) was added to reach a water content of 92%. The sample was then mixed by vortexing and incubated for 4 h with continuous shaking (120 rpm) at 50 °C, after which it was frozen using liquid nitrogen. For the treatments with the lower xylanase dose (20 nkat/g bran), the enzyme was added in the form of a solution (600 nkat/ml, prepared by dissolving the powdered xylanase preparation in distilled water at room temperature by magnetic stirring for 30 min, followed by centrifugation) for more precise dosing. Treatments were performed in duplicate.

2.6. Moisture content analysis

The moisture content in the untreated and ground bran was analysed by oven drying a sample of 1–2 g at 130 °C for 1 h. For the treated samples (with moisture content up to 60%), the sample size was 4–7 g and it was freeze dried (in the tared moisture dish) prior to the oven drying (130 °C, 1 h) in order to ensure complete removal of moisture. The analysis was made in triplicate.

2.7. Extraction of bran samples for the AX analyses

The frozen bran samples were extracted in cold water after rapid defrosting in order to avoid additional enzymatic action during the extraction process. The required quantity of frozen or zuntreated sample (except for the samples incubated at 92% water content), corresponding to 3 g of bran dm, was weighed into a

tared 50 ml centrifuge tube and stored at 4 °C (max. 1 h) until the extraction. The sample was diluted by a factor of 12 (resulting in a total volume of approx. 36 ml and a sample concentration of 8%) using ice-cold distilled water. The actual bran concentration in the extraction mixture was calculated by weighing the tube with diluted sample after water addition. Approximately 24 glass beads (size 4.5–5.5 mm) were added and the sealed tube was shaken in a reciprocating shaker at 4 °C for 30 min. If the sample was not properly suspended with this procedure (due to compact consistency or unhydrated clumps in fine bran treated at a water content of 37% and ultrafine bran treated at water contents of 37% and 42%), the sample was homogenised (10 s, 10,000 rpm with a SilentCrusher M, Heidolph Instruments, Schwabach, Germany) before the extraction. It was confirmed with suitable pre-tests that the use of homogenisation did not increase AX solubilisation of those samples that were completely suspended by the basic procedure. After centrifugation, the supernatant was boiled for 20 min and recentrifuged. The supernatant was stored at −20 °C until analysis. The samples incubated at 92% water content were defrosted in a water bath for about 30 min ensuring that the sample temperature did not exceed 4 °C, after which glass beads were added and the extraction was continued as described above.

2.8. Quantification and molecular weight (MW) distribution of AX

The contents of AX in the water extracts (hereafter referred to as water extractable AX, WEAX) were determined by a colorimetric phloroglucinol method (Douglas, 1981) using xylose as a standard. Free pentose sugars were corrected by a factor of 0.88 to anhydro sugars. For the analysis of the apparent MW distribution of WEAX, other poly- and oligomeric compounds were removed from the water extract by an enzymatic procedure, followed by EtOH precipitation, using a method modified from that of Andersson et al. (2009). All enzymes were obtained from Megazyme International Ireland. First 1.5 ml of the sample water extract was diluted with 1.5 ml of 0.16 M phosphate buffer (pH 6.5). 12.5 μl of α-amylase (from *Bacillus licheniformis*, 3000 Units/ml on Ceralpha reagent, 40 °C, pH 6.0), 50 μl of lichenase (*B. subtilis*, 1000 U/ml on barley β-glucan, 40 °C, pH 6.5) and 12.5 μl of protease (Subtilisin A from *B. licheniformis*, 300 Units/ml on casein, 40 °C, pH 7.0) were then added. The sample was incubated at 60 °C in a shaking water bath for 70 min and cooled down in an ice-water bath. The complete hydrolysis of interfering oligosaccharides was ensured by a further incubation with β-glucosidase, amyloglucosidase and fructanase. The pH of the supernatant was first adjusted to 4.3 ± 0.1 with 0.325 N HCl, after which 75 μl of β-glucosidase (*Aspergillus niger*, 40 U/ml on p-nitrophenyl β-glucoside, 40 °C, pH 4.0), 7.5 μl of amyloglucosidase (3300 U/ml on soluble starch, 40 °C, pH 4.5) and 75 μl of Fructanase Mixture (exo-inulinase 1100 U/ml on kestose, 40 °C, endo-inulinase 95 U/ml on fructan, 40 °C) were added and the sample was incubated at 50 °C in a shaking water bath for 35 min. After boiling for 20 min and centrifugation, WEAX was separated from smaller proteins and hydrolysed contaminating sugars by EtOH precipitation, effected by carefully mixing 1.0 ml of the supernatant with 2 volumes of 95% ethanol. After 75 min in an ice-water bath, the precipitate was collected by centrifugation and dissolved in 2 ml of distilled water by heating in a boiling water bath for 20 min with occasional mixing. The dissolved sample was filtered through a 0.45 μm filter and analysed by HP-SEC. The liquid chromatograph with Alliance 2690 separation module and M-2414 refractive index detector consisted of three columns (7.8 × 300 mm) of μHydrogel 500, μHydrogel 250 and μHydrogel 120. All the equipment was purchased from Waters Inc. (Milford, MA, USA). The eluent was 0.2% H_3PO_4 at a flow-rate of 0.5 ml/min. The columns were at 60 °C and the injection volume was 100 μl. Pullulan standards (Waters Inc., Milford, MA, USA) ranging from 1,660,000 to 5900 Da and maltopentaose were used for calibration. Average MW of the sample was calculated using a 3rd order calibration curve between 32.7 min (the elution point of the largest standard) and 53.0 min (corresponding to a MW of approx. 2 kDa). The amount of WEAX in the HP-SEC sample of treated brans was also analysed by the colorimetric phloroclucinol method (Douglas, 1981). The efficiency of the sample pretreatment was confirmed by HP-SEC, which showed that with the enzymatic procedure used, commercial wheat starch (Fluka Chemie, Buchs, Switzerland, dissolved in 0.08 M phosphate buffer, pH 6.5, at a concentration of 3%), barley β-glucan (Megazyme International Ireland, dissolved at a concentration of 0.5%), and fructan/cellulose mixture (Megazyme International Ireland, dissolved at a fructan concentration of 0.5%), were hydrolysed to oligosaccharides smaller than 2 kDa, whereas the MW of commercial wheat AX (Megazyme International Ireland, dissolved at a concentration of 0.5%) remained unchanged as compared to the AX solution before the enzymatic treatment.

2.9. Particle size determination of treated bran samples

For the analysis of particle size distribution, the treated bran samples were defrosted and dispersed in water as for the extraction of samples for AX analyses (diluting bran with water by a factor of 12 and shaking with glass beads for 30 min), with the exception that the procedure was performed at room temperature. The particle size distributions of the bran–water suspensions were measured by laser light diffraction (Mastersizer 3000, Malvern, Worcestershire, UK) and calculated from the volumetric distribution of the particles using the Fraunhofer optical model.

2.10. Statistical analysis

All bran treatments were made in duplicate, and each sample was analysed at least in duplicate. Thus all the results were calculated as means of at least four analysis results. Data were subjected to analysis of variance using IBM SPSS Statistics 19 (IBM Corporation, Somers, NY, USA), and significant differences ($P < 0.05$) between individual means were identified by the Tukey's test.

3. Results and discussion

3.1. Impact of pre-mixing method on the formation of bran–water mixture and on AX solubilisation at low water content

The impact of two pre-mixing methods, blade-mixing and extrusion at ambient temperature, on xylanase action during 4 h stationary incubation of coarsely (Dv50 = 700 μm) and ultrafinely (Dv50 = 80 μm) ground wheat bran at different water contents was evaluated by analysing the amount of WEAX formed (Fig. 2). The level of WEAX after the treatments varied between 2.6 and 6.3% of bran dm, corresponding to a degree of solubilisation of 13–30% of the total AX content of the bran. The solubilisation of AX was in both brans higher after the extrusion-aided treatments than after blade-mixed treatments at the same water content (Fig. 2a). The greatest difference between the treatments was observed at water contents below 54%: for example, after the blade-mixed treatment of coarsely ground bran (Dv50 = 700 μm) at the lowest water content studied (37%), the amount of WEAX was 2.6% of bran dm, whereas after the extrusion-aided treatment the corresponding value was 4.5% (Fig. 2a).

With the coarsely ground bran, the solubilisation of AX increased rather linearly with the increase of water content in the blade-mixed treatments, and no significant differences in the physical form (moist granular material) Fig. S1, Supplementary information)

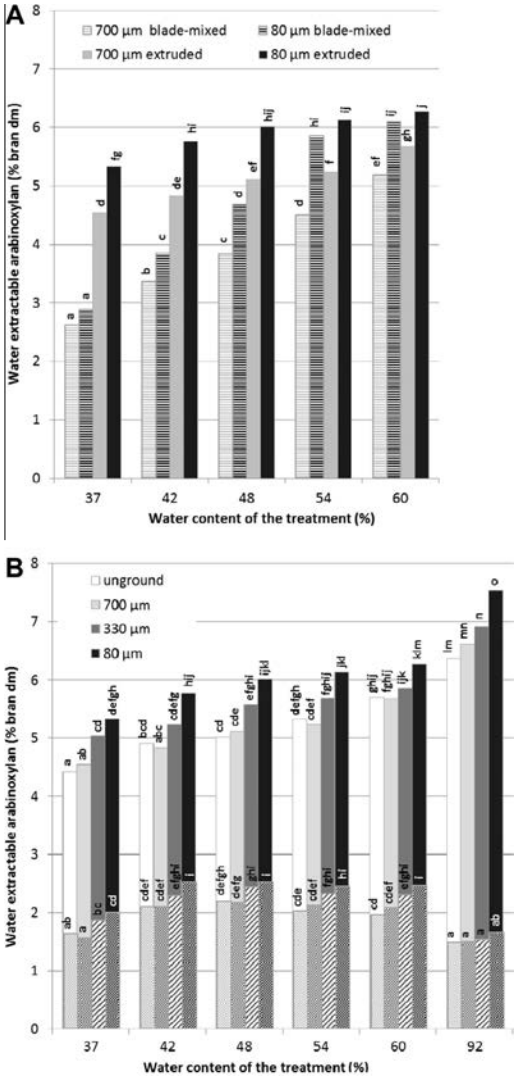

Fig. 2. The amount of water extractable arabinoxylan after blade-mixed and extrusion-aided xylanase treatments (including 4 h stationary incubation) at water contents of 37–60% with coarse and ultrafine bran (a), and after the extrusion-aided and shaking treatments with brans of different mean particle sizes (b). In b the long columns represent samples treated with xylanase and the small columns represent samples without added enzymes. The results are expressed as means (n = 4–8). Values marked with different letters within a set of corresponding samples (in a all samples were analysed in one set; in b the samples with xylanase were analysed separately from the samples with no added enzymes) are significantly different ($P < 0.05$).

of the bran–water mixtures were observed. At a water content of 60%, the level of WEAX after the blade-mixed treatment (5.2% of bran dm) was close to that of the extrusion-treated bran (5.7%). However, in the case of the fine bran, there was a notable increase in the AX solubilisation between 48% (WEAX 4.7% of bran dm) and 54% (WEAX 5.9%) water content, when the AX solubilisation was as high as that of the extrusion-processed bran (6.1%). At a water content of 54%, the bran–water mixture of the ultrafine bran formed a continuous, plastic mass during the 3 min blade mixing, whereas at 48% the mixture remained in the form of a moist granular powder (Fig. S1). This suggests that the formation of a continuous mass (plasticization) might have enhanced the action of the enzyme. The enhanced enzyme activity in the extrusion-aided treatments below a water content of 54%, as compared to the blade-mixed treatments at the same water content, might similarly be due to the formation of plastic mass by the pressure and mechanical shaping in the extruder. In the extrusion-aided treatments the bran mixture was forced through a die after intensive mixing in the extruder barrel, causing the formation of uniform 'sticks' of moist bran mass (Fig. S1). It has also previously been suggested that the reduction of enzyme action at low water content is related to the rheological properties and physical form of the biomass and its polymers, which in turn is related to the state of water in the mixture (Viamajala et al., 2009; Roberts et al., 2011). Viamajala et al. (2009) pointed out that at high solids concentrations the absorption of water within the biomass particles may result in the absence of continuous free water phase, causing the bulk to behave as a wet granular material as portions of the "void" volume contain air rather than liquid. Based on their visual observations, this occurred at insoluble solid concentrations of 30–40% (w/w), corresponding to water contents of 60–70%. Once there is no free water in the system, the material becomes difficult to shear and mix uniformly (Viamajala et al., 2009). The results of the current study suggest that it might be possible to change the granular structure of the material to a continuous mass by using extrusion, without increasing the water content. The continuous form of the material probably enhanced enzyme action by improving diffusion, which is considered a major factor affecting enzymatic reaction rates especially at high solids concentration (Lavenson et al., 2012).

3.2. Impact of high water content and continuous shaking on AX solubilisation

Solubilisation of AX in a conventional high-water (92% in the current study) system with shaking was also investigated and compared to the low water content (⩽60% in the current study) treatments (Fig. 2b), since hydrolytic enzymatic reactions are typically performed at high water content due to the expected reduction of enzymatic hydrolysis at higher solid concentrations. High-solids (i.e. low-water) enzymatic hydrolysis have been roughly defined to take place at the solids content where significant amounts of free liquid are not present (Hodge et al., 2009). The water holding capacity of the bran used in the current study was 3.3–3.7 g water/g bran (Table 1), which corresponds to water contents of 77–79%. Thus, the extrusion-aided treatments performed at water content of 60% and below can be defined as "high-solids" or correspondingly "low-water" treatments.

The solubilisation of AX with xylanase was higher in the high water content treatment (WEAX 6.4% of dm for coarsely ground bran) than in the corresponding low water treatments in the extruder (4.4–5.7% at water contents of 37–60%). On the other hand, efficient mixing and mass transfer are generally considered to be essential for the efficient performance of enzymatic reactions (recently reviewed by Lavenson et al. (2012)), and in that respect even higher differences would be expected because of the lack of stirring during the low water content incubations. Probably the efficient initial mixing was sufficient to facilitate the enzyme action during the extrusion-aided treatment. The impact of mixing on biomass hydrolysis at high solids concentration (15–30%) was studied by Roche et al. (2009), who compared small-scale enzymatic saccharification vessels with three different mixing mechanisms: shaking, gravitational tumbling and hand stirring. They reported that effective initial mixing to promote good enzyme distribution and continued, but not necessarily continuous mixing is necessary in order to facilitate high biomass conversion

rates. On the other hand, very high shear rates might induce inactivation of enzymes (as reviewed by van der Veen et al., 2004), and it is possible that the lower AX solubilisation in the extrusion-aided process as compared to the high-water treatment was partly caused by the enzyme inactivation.

Contrary to the treatments with added xylanase, in the blank treatments with no added enzymes the solubilisation of AX was notably higher in the extrusion-aided processes at the water contents of 42–60% than in the high water content (92%) treatments (Fig. 2b). For example, in case of the ultrafine bran, the level of WEAX after these treatments was 2.5% bran dm, whereas at high water content the level was only 1.7%. The solubilisation of AX was most probably caused by the action of endogenous hydrolytic enzymes of the bran material (Dornez et al., 2009), and the results suggest that low moisture content could be favourable for the solubilisation of AX by endogenous bran enzymes. This would be logical, as the natural environment of the endogenous enzymes is not necessarily highly aqueous. It could also be speculated whether it is possible that the activity of enzyme inhibitors, known to be present in bran material (Jerkovic et al., 2010), could have been reduced by the low water content.

3.3. Impact of bran particle size on bran processing with xylanase

The impact of bran particle size on the solubilisation of AX was studied using brans ground to four different mean particle sizes (1000, 700, 330 and 80 μm). The analysis of soluble and insoluble DF content showed that the total DF content was similar in all the brans (48–49% of bran dm), whereas the level of soluble DF increased with decreasing particle size (from 3.1% to 4.6%), which is often observed after intensive milling (Hemery et al., 2011; Zhu et al., 2010). Similarly, the total AX content was similar in all the brans (20.3–20.6% of bran dm), whereas the content of WEAX increased with decreasing particle size (from 0.6% to 0.9%). The reduction of bran particle size also enhanced the solubilisation of bran AX during all treatments, but the impact was rather small (Fig. 2). For example, at a water content of 48% after the extrusion-aided treatments with xylanase, the WEAX content varied from 5.0% (of unground bran dm) to 6.0% (ultrafine bran). In most points studied, the differences between different brans were statistically significant only when comparing the ultrafine bran to the coarse and unground bran. It was also tested whether the high xylanase dose or the use of glass beads in the extraction method used in the AX assay could have hidden the impact of the particle size on AX solubilisation, but the trend (only a rather small increase in AX solubilisation with reduction of bran particle size) was also the same when a 10% enzyme dose was used in the shaking treatment and the extraction was performed without addition of the glass beads (data not shown).

Several studies have reported that the reduction of particle size of plant-based substrates enhances the efficiency of enzyme action (Silva et al., 2012; Niemi et al., 2012; Mahasukhonthachat et al., 2010; Dasari and Berson, 2007). However, to our best knowledge, the impact of particle size on enzyme function over a wide range of water contents, or together with different processing or mixing methods, had not previously been studied. The present results showed that the impact of bran particle size on enzyme function was similar in all the processing methods and water contents studied, and it was also similar when no added enzymes were used. The only notable exception was in case of the blade-mixed treatments at a water content of 54%, when the difference between the ultrafine and coarsely ground bran was higher than in the other water contents. The enhanced AX solubilisation of the ultrafine bran was most probably caused by plasticization of the ultrafine bran mixture in these conditions (Fig. S1), as already discussed. The higher susceptibility of the ultrafine bran to form continuous mass might be explained by its lower water holding capacity (Table 1). When material binds less water, more water remains to act as a plasticizer. It was also noted that reduction of particle size caused a reduction in the torque values of the extruder, especially at water contents of 54–60% (data not shown), indicating a reduction of viscosity with decreasing bran particle size. Dasari and Berson (2007) and Viamajala et al. (2009) also reported a reduction in viscosity as a result of decreasing particle size. Dasari and Berson (2007) suggested that the reduction in viscosity due to smaller particle size may allow for higher solids loading (i.e. lower water content) and thus reduced reactor sizes in large-scale processing. From the results of the current study it can also be concluded that reduction of bran particle size can be used as a means to maintain AX solubilisation when decreasing the water content. For example, when using the coarse bran (Dv50 = 700 μm), the level of 5.7% of WEAX was reached at a water content of 60%, whereas in case of the ultrafine bran this level was already reached at a water content of 42% (Fig. 2b).

In our previous study in which we studied the solubilisation of AX with xylanase at water contents of 20–90% using continuous mixing, the solubilisation was highest at 40% and 90% water contents (Santala et al., 2011). Reduction of particle size due to the high shear during the treatment with continuous mixing was considered to be one explanation for the enhanced solubilisation at 40% water content (Santala et al., 2012). In the current study, particle size measurement of the extruded sample before incubation showed that the particle size of the coarse bran had decreased during the extrusion, especially at the low water content (Table 2). In the vessel treated samples the particle size was 892–938 μm after the blade mixing (before incubation) at all water contents analysed, whereas in the extruded sample the particle size was 348 μm at a water content of 37%, and 770 μm at a water content of 60%. Reduction in particle size was presumably due to the higher torque (data not shown) exerted on the bran mixture at low water content. Particle size was reduced similarly or even slightly more in the samples with no added enzyme (data not shown), probably due to the lack of lubricating effect of the AX solubilisation caused by the added enzymes. The mechanical particle size reduction in the extruder could have enhanced the AX solubilisation. However, the reduced particle size of the coarse bran was still much higher than that of the blade-mixed ultrafine bran (84–85 μm), which showed lower AX solubilisation after blade-mixed treatment than the coarse bran in the extruder. This confirms that the enhanced AX solubilisation in the extruder-aided treatment was not merely caused by the particle size reduction in the extruder. In addition, the particle size of the blade-mixed ultrafine bran was similar at all water contents studied, confirming that the use of homogenisation in the extraction procedure of the 37% water content samples did not affect the particle size of the bran. The particle sizes of the blade-mixed samples (892–938 μm for coarse and 84–85 μm for ultrafine bran) were slightly higher than those of the untreated brans measured with the dry method (702 and 81 μm), which is probably due to the swelling of the blade-mixed brans by the water of the wet method used for the particle size analysis.

3.4. Impact of different processing methods on the molecular weight of water extractable AX

The apparent weight average molecular weights (MW) of WEAX were analysed from enzymatically purified and EtOH-precipitated AX fraction of selected bran samples (Table 3 and Fig. 3). EtOH precipitation is a generally accepted method for separating WEAX from contaminating protein and mono- and oligosaccharides for the analysis of WEAX MW, due to the lack of a specific detection method for AX. The concentration of EtOH should be low enough to avoid the precipitation of oligosaccharides that would overlap

Table 2
Impact of different pre-mixing methods on the bran particle size at different water contents measured before incubation of the extruded or blade mixed samples with xylanase.

Treatment water content (%)	Coarse bran (d50 = 700 μm) Median particle size (μm)		Ultrafine bran (d50 = 80 μm) Median particle size (μm)	
	Extruded	Blade-mixed	Extruded	Blade-mixed
37	348 ± 13a	902 ± 35d	55 ± 2a	84 ± 5c
48	603 ± 21b	938 ± 31d	76 ± 2b	84 ± 5c
60	770 ± 32c	892 ± 41d	85 ± 3c	85 ± 5c

The results are expressed as means ($n = 6$) ± standard deviation. Values marked with different letters within the same bran type (coarse/ultrafine) are significantly different ($P < 0.05$).

Table 3
Average apparent molecular weight and content of water extractable AX (WEAX) precipitated at 65% EtOH in the brans after different treatments.

	Treatment water content (%)	Coarse bran (d50 = 700 μm) EtOH (65%) precipitated WEAX			Ultrafine bran (d50 = 80 μm) EtOH (65%) precipitated WEAX		
		Average MW (kDa)	Content in bran (% of dm)	% of total WEAX	Average MW (kDa)	Content in bran (% of dm)	% of total WEAX
Untreated bran		158 g	0.4 a	75	143 f	0.6 ab	83
Extrusion-aided treatment without added enzymes	37	131 e	1.3 de	85	133 e	1.7 gh	86
	48	79 d	1.3 d	60	76 cd	1.7 fgh	68
	60	68 b	1.2 cd	57	68 b	1.4 defg	58
Shaking treatment without added enzymes	92	66 b	0.9 bc	57	68 bc	1.1 cd	67
Extrusion-aided treatment with xylanase	37	51 a	1.1 cd	25	52 a	1.7 efgh	32
	48	52 a	1.4 def	27	51 a	1.9 h	31
	60	56 a	1.4 def	24	52 a	1.9 h	31
Shaking treatment with xylanase	92	51 a	1.9 h	29	48 a	2.7 i	35
Blade-mixed treatment with xylanase	37	69 bc	1.3 d	50	73 bcd	1.8 gh	62
	48	55 a	1.3 def	35	52 a	1.8 h	39
	60	53 a	1.3 d	25	51 a	2.0 h	33

The results are expressed as means ($n = 4$). Values marked with different letters within the same analysis (MW/AX content) are significantly different ($P < 0.05$).

with the unwanted residues at the low molecular size end of the chromatogram. However, with decreasing EtOH concentrations, more of the original WEAX is lost as only WEAX with high MW is precipitated (Swennen et al., 2005). An EtOH concentration of 50–65% has generally been used for separation of WEAX (Andersson et al., 2009; Ganguli and Turner, 2008) and 65% was selected for the current study.

When enzyme was used, the MW of EtOH-precipitated WEAX of the extrusion-processed brans (51–56 kDa) was similar to that of the bran treated at high water content with shaking (48–51 kDa), as well as that of the brans treated by the blade-mixed method at ⩾48% water content (51–55 kDa). In the blade-mixed treatment at 37% water content the MW was higher (69 and 73 kDa with coarse and ultrafine brans), indicating that AX was hydrolysed less efficiently. This is in accordance with the lower level of AX solubilisation during the vessel treatment at 37% water as compared to all other xylanase treatments.

Without added enzyme, the MW of EtOH-precipitated WEAX varied when bran was processed at different water contents by the extrusion-aided process. The MWs of AX in the untreated brans (coarse bran 158 kDa and ultrafine bran 143 kDa) were very close to the values obtained by Zhang et al. (2011) for wheat bran WEAX using a different HP-SEC method (152 kDa). At the lowest water content (37%), the MW of AX of the processed brans (coarse bran 131 kDa and ultrafine bran 133 kDa) did not markedly change from that of the untreated brans, suggesting that at 37% water content, endogenous enzymes of bran material were not able to hydrolyse WEAX. At a higher water content of 48% the MW was already significantly lower (79 and 76 kDa), and at 60% water content the MW was further decreased to the same level as after the shaking treatment at high water content (66–68 kDa).

The results suggest that with added xylanase, the MW was not dependent on the used water content or processing method (extrusion-aided or shaking treatment). By contrast, without added enzymes the hydrolysis of WEAX increased with increasing water content, evidently due to the action of endogenous bran enzymes, whereas the solubilisation of AX was favoured by the low water content process (extrusion-aided treatment), as already discussed. Similarly, in a previous study in which a process with continuous mixing was used, solubilisation of wheat bran AX was higher and less depolymerisation occurred at low (40%) water content as compared to high (90%) water content (Santala et al., 2011). According to Sibakov et al. (2013a), the use of a water content of 90% for enzymatic hydrolysis of β-glucan resulted in rapid breakdown into short oligosaccharides, whereas low water content (50%) enabled a controllable depolymerisation of high MW β-glucan. In the current study, the difference between the impact of water content on the MW of WEAX with and without added xylanase might be due to the differences in the susceptibility of the exogenous and endogenous bran enzymes to water content.

In order to learn how well the determined MW represents the total WEAX of the bran sample, the amount of WEAX in the HP-SEC sample was also analysed (Table 3), as the method used for the MW analysis applies only to the WEAX fraction precipitating at 65% EtOH. The level of WEAX in the HP-SEC samples was 1.1–1.4% of bran dm for coarse bran after all treatments at water contents of 37–60%. After the shaking treatments, the amount was higher when treated with xylanase (1.9% of bran dm), and lower without added enzyme (0.9%). The trend was the same in the case of the ultrafine bran, but the levels were somewhat higher than in case of the coarse bran (Table 3). When compared to the total level of WEAX in the original sample (Fig. 2), the precipitated WEAX

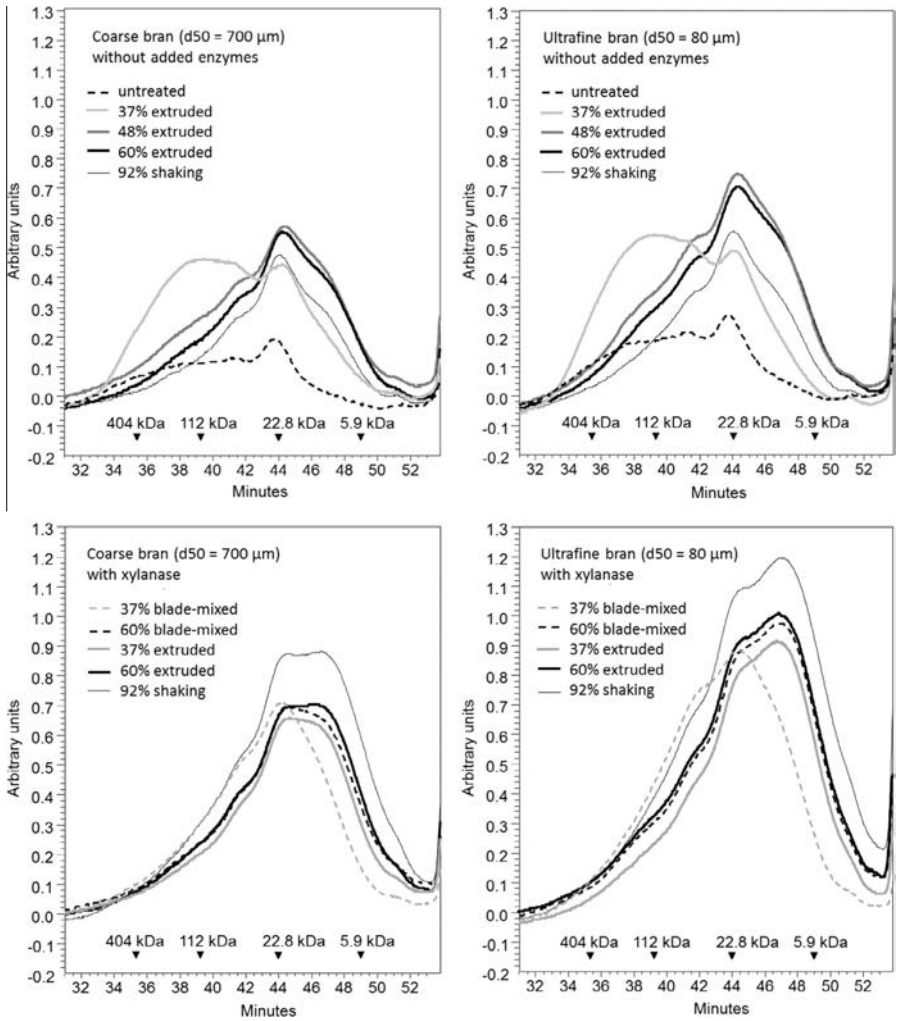

Fig. 3. Molecular weight distribution of water extractable arabinoxylan (precipitated at 65% EtOH) after blade-mixed and extrusion-aided treatments (including 4 h stationary incubation) at water contents of 37–60% and after shaking treatment (water content 92%) with coarse and ultrafine brand.

amounts in the HP-SEC samples corresponded to 24–39% (Table 3) of the total WEAX when bran had been processed with enzyme, regardless of the process used or the water content, except for the vessel treatment at 37% water content, when the precipitation level was higher (50 and 62% for coarse and ultrafine bran, respectively), obviously due to the lower level of total WEAX. The rest of the WEAX were smaller oligosaccharides which did not show in the MW chromatograms. Without added enzymes and in the untreated bran, the analysed MW of WEAX represented as much as 57–86% of the total WEAX (Table 3). The levels of WEAX in HP-SEC samples are not always reported, although they should be taken into account because the level of WEAX in the original sample and the analysed MW are not necessarily comparable as such, especially in the case of enzymatic treatments. It is known that both the level of AX solubilisation (WEAX content as compared to water insoluble AX), as well as the MW of the WEAX are important factors affecting the technological properties of cereal AX (Courtin and Delcour, 2002).

4. Conclusions

An extruder-aided process enabled efficient xylanase action on wheat bran at low water content without the requirement for continuous mixing, probably due to enhanced diffusion by the formation of continuous mass in the extruder. The method may be applicable to other biomass sources as well. AX solubilisation with added xylanase was highest at high-water stirring treatment, but without added xylanase, solubilisation was highest in the extrusion-aided process. Based on molecular weight, xylanase action on solubilised AX was similar in the high-water process and extrusion-aided process. Decreasing bran particle size improved AX solubilisation in rather similar manner in the processes studied.

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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.biortech.2013.09.029.

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PUBLICATION IV

Enzymatic modification and particle size reduction of wheat bran improves the mechanical properties and structure of bran-enriched expanded extrudates

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Enzymatic modification and particle size reduction of wheat bran improves the mechanical properties and structure of bran-enriched expanded extrudates

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KEY WORDS

Extrusion; wheat bran; particle size; enzymatic modification

ABBREVIATIONS

AX, arabinoxylan; C_i, crispiness index; DF, dietary fibre; DM, dry matter; ER, expansion rate; F_{cr}, crushing force; F_{max}, maximum point of the force-deformation curve; IDF, insoluble dietary fibre; SDF, soluble dietary fibre; WEAX, water extractable arabinoxylan; WHC, water holding capacity.

ABSTRACT

The aim of this study was to examine enzymatic modification of wheat bran, performed in a low-moisture process, and the reduction of bran particle size as means of improving the technological performance of wheat bran in expanded extrudates. Modification of bran by hydrolytic enzymes increased the crispiness and decreased the hardness and piece density of extrudates containing wheat bran and endosperm rye flour in 20:80 ratio. These improvements correlated ($P < 0.01$ or 0.05) with an increased content of water extractable arabinoxylan and decreased water holding capacity of the bran, as well as with increased longitudinal expansion of the extrudates. Furthermore, bran with a fine average particle size (84 µm) produced extrudates with improved mechanical properties and higher radial expansion than coarse bran (particle size 702 µm). The impact of bran particle size was also observed in the cellular structure of the extrudates as differences in cell size and homogeneity. The bran drying method, oven or freeze drying after enzymatic modification, did not have a major impact on the properties of the extrudates. The study showed that the functionality of wheat bran in extrusion can be improved by enzymatic modification using a low-water process and by reduction of bran particle size.

1. INTRODUCTION

Extrusion technology is a versatile option for the production of different types of ready-to-eat snacks with puffed structure and cooked characteristics appealing to consumers (Brennan, 2013). During the past 10–15 years, consumers have become more health conscious and are increasingly demanding tasty snack products which satisfy their hunger and yet are low in fat, rich in dietary fibre (DF) and preferably fortified with vitamins and minerals (Brennan, 2013). Wheat bran is a good source of DF and contains a relatively high amount of protein and phytochemicals. However, as recently reviewed by Robin et al. (2012) and Sozer and Poutanen (2013), increasing the amount of DF or bran in extrusion formulations typically causes deterioration of the textural properties of the product, e.g. by increasing the density and hardness and decreasing the expansion volume and crispiness of the product. Extrudate expansion, which is crucial for the formation of the appetizing and crispy textures, is governed by a complex series of events, in which starch plays a major role (Moraru and Kokini, 2003). The impact of DF on the texture of extruded products is generally considered to depend mainly on its interactions with starch and on its effects on the mechanistic steps of expansion, i.e. starch transformation, nucleation of bubbles, extrudate swell, growth of bubbles, and bubble collapse (Moraru and Kokini, 2003; Robin et al., 2012). Particularly insoluble DF (IDF) has been reported to be detrimental to the extrudate characteristics (Robin et al., 2012). The adverse effects of IDF in extrusion have been related to the changes they cause in the rheological properties of the batter melt and in the amount of free water available for starch transformations and expansion, as well as to the physical disruption of the continuous starch matrix and gas cell walls by the fibre particles (Pai et al., 2009; Moraru and Kokini, 2003; Robin et al., 2012).

Different strategies have been studied as means to improve the technological performance of DF ingredients in extruded products. Decreasing DF ingredient particle size has been reported to increase the expansion of extrudates containing DF (Lue et al., 1991; Blake, 2006; Alam et al., 2013). However, particle size reduction has not improved expansion when the size differences or addition levels are low (Robin et al., 2011b; Blake, 2006; Alam et al., 2013). It has also been reported that soluble DF (SDF), such as pectin, inulin or guar gum, generally performs better than fibres that are mostly insoluble (IDF), e.g. wheat bran (Yanniotis et al., 2007; Brennan et al., 2008), and it has been suggested that increasing the solubility of DF prior to extrusion could be a means to improve the functionality of DF in extruded products (Robin et al., 2012). However, only few studies have examined this possibility. Pai et al. (2009) showed that increasing the SDF content of corn bran with concomitant reduction of IDF by a chemical treatment resulted in higher expansion as compared to untreated bran. The improved expansion was related to favourable changes in melt viscosity and better interaction of SDF with starch (Pai et al., 2009).

Modification of bran by enzymatic processing has been shown to facilitate the addition of bran, and thus DF, to food products, and the beneficial effects of these processes have often been related to the transformation of IDF to SDF (Lebesi and Tzia, 2012; Coda et al., 2014). However, to our best knowledge enzymatically modified bran has not previously been studied as an ingredient in extrudates. Enzymatic treatments are typically preformed in high water content, which is not economical especially if the modified ingredient should be dried prior to its subsequent use in a low moisture process such as extrusion. On the other hand, enzymatic processing at low water content, i.e. high consistency, typically causes reduction of enzyme action and requires a substantial amount of energy for mixing. It has been shown that efficient xylanase action on wheat bran at low water content and without continuous mixing can be accomplished by the use of an extruder-aided pre-mixing process (Santala et al., 2013a).

In the current study the enzymatic degradation of wheat bran was investigated as a means of improving the quality of bran-supplemented endosperm flour-based expanded extrudates. The aims were 1) to study the impact of different variations of the extruder-aided low-moisture enzymatic treatment and the subsequent drying step on the physicochemical properties of wheat bran of two different particle sizes, and thereafter 2) to study how the physicochemical properties of the modified bran ingredient and the reduction of bran particle size affect the macro- and microstructure and mechanical properties of bran-supplemented expanded extrudates.

2. MATERIALS AND METHODS

2.1. Cereal raw materials

Commercial wheat bran (Fazer Mill & Mixes, Lahti, Finland) was ground by TurboRotor technology (Mahltechnik Görgens GmbH, Dormagen, Germany) to two different levels of fineness so that the mean particle sizes were 702 µm (hereafter referred as coarse bran) and 84 µm (fine bran). The grinding process did not contain any sieving or fractionation steps, thus the fine and coarse bran were composed of the same bran raw material. The high air throughput and short residence times used in the grinding technology ensured that the product temperature remained below 45 °C, thus avoiding the heat damage often associated with intensive grinding treatments. The total and water soluble DF contents were 49.9 and 6.7% in the coarse and 48.0 and 8.2% in the fine bran, respectively. Arabinoxylan content was 20.5% (coarse) and 20.6% (fine) and starch content was 16.7 (coarse) and 16.5% (fine).

Rye endosperm flour (Helsinki mills ltd. Järvenpää, Finland) was used as a base material for the expanded extrudates. The total and soluble DF contents were 11.8% and 9.6%, respectively, and the starch content was 84.7%.

2.2. Enzyme preparations

Commercial hydrolytic enzymes, Depol 761P (Biocatalysts Ltd, Cardiff, UK), a xylanase preparation derived from *Bacillus subtilis*, and Veron CP (AB Enzymes GmbH, Darmstadt, Germany), a cellulolytic enzyme preparation with hemicellulase side activities from *Trichoderma reesei*, were used either individually or in combination for the bran treatments. The activity profile of Depol 761P, endoxylanase 28,660 nkat/g, polygalacturonase 1,317 nkat/g, β-glucanase 1,625 nkat/g, α-amylase 44 nkat/g, and β-xylosidase 2 nkat/g, was previously reported by Santala et al. (2013b). The activity profile of Veron CP, determined by the methods described by Santala et al. (2013b), was as follows: endoglucanase 18974 nkat/g, cellulase (filter paper as substrate) 53 filter paper units/g, β-glucanase 75760 nkat/g, xylanase 14610 nkat/g, β-xylosidase 257 nkat/g, polygalacturonase 8469 nkat/g, mannanase 3022 nkat/g, α-arabinosidase 530 nkat/g, β-glucosidase 528 nkat/g and α-amylase 94 nkat/g. The preparation was free from ferulic acid esterase and proteinase. Enzyme preparations were dosed according to their xylanase activity at 200 nkat/g bran dry matter (DM) (treatments with Depol 761P) or 100 nkat/g (treatments with Veron CP, corresponding to an endoglucanase activity of 130 nkat/g). For the treatments with a combination of the enzymes the dosages as xylanase activity were 200 nkat Depol 761P and 100 nkat Veron CP /g bran DM.

2.3. Production of enzymatically modified bran

Bran modification was performed by extrusion-aided enzyme treatment as described by Santala et al. (2013a). 450 g of bran with an initial moisture content of 10.7% (coarse) or 5.5% (fine) was first mixed with the enzyme preparation(s) (in powder form) and pre-

conditioned to a moisture content of 20% by adding water slowly while mixing (speed setting 2) with a Kenwood KM300 mixer (Kenwood Ltd., Havant, UK) with a K-shaped blade for 2 min. Pre-conditioning was also performed for the blank treatments (i.e. without enzyme addition). The pre-conditioned bran mixture was transferred to the feeding unit (a co-rotating twin screw feeder, K-Tron Soder, Niederlenz, Switzerland) of a twin screw extruder (APV MPF 19/25, Baker Perkins Group Ltd, Peterborough, UK) within 20 min and fed to the extruder at a rate of 26 g/min. The screw configuration is presented in Fig. 1. The barrel temperature was 50 °C and the screw speed was 65 rpm. Water was pumped to the beginning of the barrel at an appropriate rate in order to obtain moisture contents of 48±1% in the bran mixture. Bran mixture was collected continuously from the die exit (diameter 3 mm) and the collected material was transferred every 2 minutes either to incubation (at 50°C for 4 h in sealed containers) or to drying, which was performed either in an oven (samples spread on metal trays and dried with air circulation at 50°C for 18–20 h) or by freezing the sample in liquid nitrogen for subsequent freeze drying. The incubated samples were also immediately dried by oven drying or by freeze drying. The dried samples were ground in a mill (Hosokawa Alpine, 100 UPZ, Retsch GmbH, Germany) with two different settings (coarse bran samples with sieve size 0.5 mm and rotor speed 6000 rpm; fine bran samples with a 0.3 mm sieve and 18 000 rpm) in order to maintain the different particle sizes of the two bran types (coarse and fine).

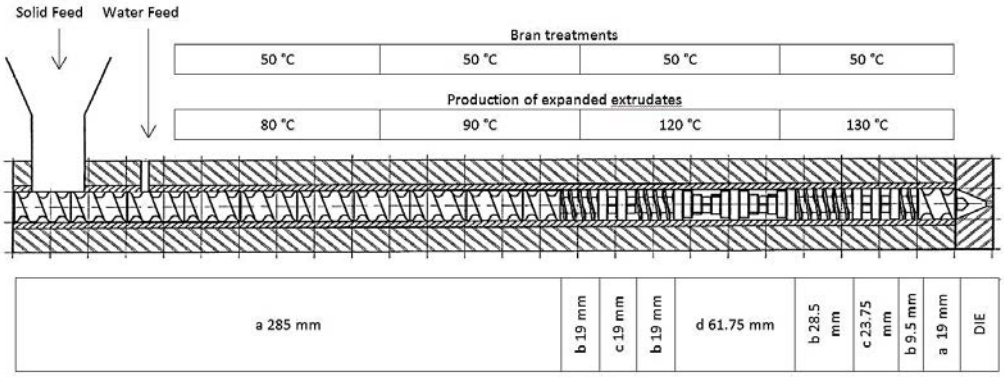

Fig. 1. Screw configuration of the extruder and the temperature profiles used in the experiments. The letters indicate the type of each screw element. Feed screw (a), lead discharge screw (b), mixing paddles at 90º (c), mixing paddles at 30º (d).

2.4. Production of expanded extrudates

Bran samples were mixed with rye endosperm flour in 20:80 ratio on a dry matter basis in a Kenwood KM300 mixer (Kenwood Ltd., Havant, UK) with a K-shaped blade for 4 min. The same extruder, feeder and die were used as for the bran treatments. The feed rate was calibrated separately for each bran ingredient-flour mixture to a level of 60 g/min. Extrusion parameters were selected on the basis of pre-trials performed with the flour base material in order to obtain maximum expansion. Temperatures at the barrel zones 1 to 4 were set at 80, 90, 120 and 130 °C (Fig. 1) and the screw speed was 450 rpm. The water feed rate was adjusted on the basis of the moisture content of the feed material to attain a moisture content of 16%. The extrudates were collected in trays and dried at 50°C for 30 min in an oven dryer with air circulation. The samples were stored at 14°C in sealed pouches. The extrusions were performed in duplicate.

2.5. Analysis of chemical composition and physicochemical properties

The moisture contents of the bran samples were analysed gravimetrically by oven drying samples of 1–2 g at 130 °C for 1 h. Total and soluble DF contents were analysed by AOAC method no. 2009.01 (McCleary et al., 2013) and starch by the Megazyme method (McCleary et al., 1994).

For the analysis of water extractable AX, 3.2 g of bran was mixed with 32 ml of ice-cold distilled water and shaken with glass beads for 15 min at 4°C. After centrifugation, the supernatant was boiled in a water bath for 20 minutes and recentrifuged. The supernatant was stored at -20 °C until analysis. The contents of AX and pentose monosaccharides in the water extracts (hereafter referred to as water extractable AX, WEAX) were determined by a colorimetric phloroglucinol method (Douglas, 1981) using xylose as a standard. For the quantification of total AX, 0.1 g of bran was mixed with 5 ml of 0.5 M H2SO4 and boiled for 30 min and centrifuged, followed by the colorimetric determination (Douglas, 1981).

The median particle size of the modified brans and the untreated fine bran was determined by laser light diffraction in a Beckman Coulter LS230 particle size analyser (Coulter Corporation, Miami, USA) after dispersing the samples in 95% ethanol using magnetic stirring for 1–2 min. Due to the limited analysis range (up to 2000 µm) of the Beckman Coulter analyser, the particle size of the untreated coarse bran was determined from dry bran dispersion in a Mastersizer 3000 apparatus (Malvern, Worcestershire, UK). Median particle sizes were calculated from the volumetric distribution of the particles using the Fraunhofer optical model.

Water holding capacity (WHC) was determined in a Baumann apparatus as described previously (Santala et al., 2013b), using a sample size of 50 mg and measurement time of 30 min.

2.6. Analysis of the expanded extrudates

For the analysis of macrostructural parameters, the extrudates were cut into 20 pieces each of 5 cm length using a band saw (Scheppach, Germany). Expansion rate (ER), specific length and piece density of each sample were calculated as described by Alam et al. (2013).

Mechanical properties of the extrudates were measured by applying uniaxial compression using a texture analyser (TA.XT plus, Stable Micro Systems Ltd., United Kingdom) containing a 30 kg load cell and a 25 mm aluminium probe under 70% strain with a test speed of 1 mm/s. All extrudates were cut into 10 mm pieces (radial section) and equilibrated at 43 % relative humidity (RH) at 21 °C prior to analysis. Measurements were performed for 20 replicates. Exponent software version 6.0.7.0 (Stable Micro Systems Ltd., United Kingdom) was used to obtain values for the calculation of the hardness indicators F_{max} (the maximum point of the force-deformation curve) and crushing force (F_{cr}), i.e. average puncturing force (van Hecke et al., 1998), and crispiness index (C_i), which was calculated by the following equation (Heidenreich et al., 2004):

$$C_i = \frac{L_N}{A \times F_{mean}}$$

where L_N is the normalized curve length (length of actual curve/F_{max}), A is the area under the force-deformation curve and F_{mean} is the sum of the actual force values in the data file divided by the number of data points.

For the stereomicroscope imaging of the radial cross-sections of the extrudates, the samples were cut into 10 mm pieces and examined under a SteREO Discovery.V8 stereomicroscope with an Achromat S 0.5x objective (Carl Zeiss MicroImaging GmbH,

Göttingen, Germany) and imaged using a DP-25 single chip colour CCD camera (Olympus Life Science Europa GmbH, Hamburg, Germany) and the Cell^P imaging software (Olympus).

For the analysis of total and soluble DF contents, the extrudates were ground in a laboratory mill with a 0.5 mm sieve and analysed by AOAC method no. 2009.01 (McCleary et al., 2013).

2.7. Statistical analyses

All bran treatments and extrusions were made in duplicate, and the physicochemical properties of each sample were analysed at least in duplicate. Thus all the results of the physicochemical properties were calculated as means of at least four analysis results. The parameters of macrostructure and mechanical properties were calculated as means of 35–40 results. Data were subjected to analysis of variance using IBM SPSS Statistics 21 (IBM Corporation, Somers, NY, USA), and significant differences ($P < 0.05$) between individual means were identified by the Tukey's test. Correlations between the different variables were determined by subjecting the mean values of each bran sample and the corresponding bran extrudate to the 2-tailed Pearson's bivariate correlation analysis.

3. RESULTS AND DISCUSSION

3.1. Physicochemical properties of the untreated and modified brans

The current study explored extrusion-aided enzymatic degradation and reduction of wheat bran particle size as means of improving the quality of bran-supplemented endosperm flour-based expanded extrudates. Coarse and fine wheat bran were treated either without enzymes followed by direct freeze or oven drying, or with added xylanase enzyme preparation (Depol 761P) followed by 4 h incubation and drying. In order to gain better understanding on the impacts of the use of incubation and different types of enzymes, the fine bran was additionally treated with different combinations of the process parameters and by two different enzyme preparations, Depol 761P and Veron CP, and their combination.

All treatments caused significant reduction in the mean particle size of the coarse bran from 702 to 205–318 µm, whereas the particle size of the fine bran was reduced less, from 84 to 45–69 µm (Table 1). The use of an extruder for pre-mixing of bran and enzymes was studied previously without any drying or regrinding steps (Santala et al., 2013a). It was found that the particle size of the bran decreased during the extrusion mixing, and that the reduction was more severe in the case of coarse bran (from *ca*. 900 to 600 µm) than in case of fine bran (from 84 to 76 µm). In the current study, the particle size reductions were greater, presumably due to the regrinding after drying. The grinding was performed with two different intensities (mild and severe) in order to maintain the different particle sizes of the two bran types (coarse and fine). The reduction of the particle size of the treated brans did not depend significantly on the processing variables used (the use of enzymes, use of incubation vs. direct drying, and the drying method).

Table 1. Properties of the untreated and modified (0 h = treated without incubation, 4 h = treated with 4 h incubation, OD = oven dried, FD = freeze dried) coarse and fine bran ingredients. The results are expressed as means (n=4–6). Values marked with different letters within the results are significantly different ($P < 0.05$). For WEAX content, the statistical analysis was performed separately for samples treated with and without added enzyme preparations (Depol 761P and Veron CP). For median particle size, the statistical analysis was performed separately for fine and coarse bran, and thus the letters indicate significant difference ($P < 0.05$) within each bran type.

			WEAX (% bran DM)		**Median particle size (µm)**		**WHC (g water /g bran DM)**	
			Coarse	Ultrafine	Coarse	Ultrafine	Coarse	Ultrafine
Untreated bran			0.5 a	0.8 b	702 a	84 a	3.7 a	3.3 b
Treated with no added enzymes	0 h	OD	1.4 de	1.6 e	279 b	68 ab	3.0 c	3.1 bc
	0 h	FD	1.1 c	1.3 cd	318 b	61 ab	3.7 a	3.1 bc
	4 h	OD	-	2.7 f	-	69 ab	-	3.0 c
Treated with Depol 761P	0 h	OD	-	4.2 g	-	70 ab	-	2.6 d
	4 h	OD	4.8 h	5.6 i	205 c	52 ab	2.5 d	2.4 d
	4 h	FD	4.9 h	5.7 i	285 b	45 b	3.1 bc	2.4 d
Treated with Veron CP	4 h	OD	-	4.3 g	-	62 ab	-	2.4 d
Treated with Veron CP + Depol 761P	4 h	OD	-	6.2 j	-	57 ab	-	2.4 d

AX is the main DF component of wheat, and solubilisation of AX was assayed by measuring the content of WEAX in bran. Treatment without enzymes with direct drying increased the WEAX content of coarse and fine bran from the initial of 0.5–0.8% to 1.1–1.6% of bran DM (Table 1), indicating that some AX was solubilised already during the extrusion process, probably due to the shear exerted on the bran mixture. It has also previously been reported that mechanical work input may cause the degradation of DF components (Ralet et al., 1990; Hemery et al., 2011; Santala et al., 2013b). When the fine bran sample was further incubated the WEAX content increased to 2.7%, indicating the action of endogenous bran enzymes since there was no shear during the stationary incubation. With Depol 761P enzyme preparation, significant increase in the WEAX content (to 4.2%) occurred already without incubation, indicating that the added enzymes started to act immediately during mixing in the extruder, despite the relatively low water content used (48%). When incubated with Depol, the WEAX content further increased during the incubation, and it was higher in the fine bran (5.6–5.7%) than in the corresponding coarse bran sample (4.8–4.9%). This can be explained by the increase in the surface area of the bran due to the reduction of particle size, which has also previously been reported to make the substrate more accessible to enzymes (Niemi et al., 2012).

The fine bran treated with the combination of Veron CP and Depol 761P enzyme preparations had a slightly higher WEAX content (6.2%) than the brans treated with Depol (5.7%) or Veron (4.3%) alone. This was obviously due to the different doses of endoxylanase and other enzyme activities in the treatments. Bran treatments with Depol 761P contained mainly endoxylanase (200 nkat/g bran), whereas treatment with Veron CP contained only 100 nkat/g endoxylanase and additionally 130 nkat/g endoglucanase and 465 nkat/g β-glucanase, as well as higher levels of other side activities. The enzyme dose was highest in the combination treatment, since it contained both enzyme preparations dosed at the same level as in the

individual enzyme treatments. The use of multiple hydrolytic enzyme activities is generally considered beneficial in the degradation and solubilisation of bran, due to the synergistic action of different enzymes specific for certain cell wall components (Faulds and Williamson, 1995). In the current study, however, instead of studying the synergistic action of the enzymes, the dosages were selected with the aim of obtaining brans with different levels of AX degradation, in order to elucidate the impact of AX solubilisation on the functionality of bran in extrusion.

WHC of the untreated coarse bran (3.7 g water/g bran DM) was higher than that of the untreated fine bran (3.3 g/g). Treatment without enzymes with direct oven drying reduced the WHC of both brans to 3.0–3.1 g/g, and the use of enzymes reduced WHC further (Table 1). Degradation of AX apparently decreased the WHC of WB, since water unextractable AX is capable of binding more water than WEAX (Courtin and Delcour, 2002). Indeed, a significant ($P < 0.01$) negative correlation (-0.864) was found between WEAX content and WHC (Table 2). Particle size is also known to affect the hydration properties of bran (Noort et al., 2010; Santala et al., 2013b), and a significant ($P < 0.01$) positive correlation (0.666) between particle size and WHC was also found in the current study.

Table 2. Pearson's correlation matrix for physicochemical properties of the bran ingredients and the macrostructural and mechanical properties of the bran-supplemented extrudates.

	WHC	**Particle size**	**Expansion rate**	**Specific length**	**Piece density**	**F_{max}**	**Crushing force**	**Crispiness index**
WEAX	-0.864**	-0.454	0.127	0.709**	-0.617*	-0.828**	-0.799**	0.898**
WHC	1	0.666**	-0.355	-0.710**	0.775**	0.844**	0.858**	-0.866**
Particle size		1	-0.682**	-0.355	0.755**	0.604*	0.636*	-0.608*
Expansion rate			1	-0.045	-0.655*	-0.250	-0.319	0.285
Specific length				1	-0.716**	-0.824**	-0.808**	0.747**
Piece density					1	0.807**	0.852**	-0.764**
F_{max}						1	0.958**	-0.935**
Crushing force							1	-0.927**
Crispiness index								1

** Correlation is significant at the 0.01 level.
* Correlation is significant at the 0.05 level.

3.2. Impact of bran properties on the structure of bran-supplemented extrudates

The addition of untreated brans caused a significant decrease in the volumetric expansion of the extrudates, indicated by an increase in the piece density when the untreated coarse (169 kg/m^3) or fine bran (155 kg/m^3) was used as compared to the control extrudate without bran (130 kg/m^3) (Table 3). Expansion of extrudates occurs in both radial and longitudinal directions, and thus overall expansion is governed by both phenomena. As expected, the addition of bran caused a

reduction in the radial expansion of the extrudates, indicated by a reduction in the ER of the extrudates with untreated bran (ER 354–390%) as compared to that of the control extrudate without bran (452%). The specific length of the extrudates increased from that of the control (54 m/kg) when untreated coarse (68 m/kg) or fine (62 m/kg) bran was added (Table 3). In agreement, many sources have reported that radial expansion of cereal extrudates decreases and longitudinal expansion increases in the presence of IDF (Lue et al., 1991; Jin et al., 1995; Robin et al., 2011; Brennan et al., 2008). This has been attributed to the alignment of fibres in the direction of flow (Karkle et al., 2012; Moraru and Kokini, 2003).

Table 3. Macrostructural parameters of the extrudates with and without modified (0 h = treated without incubation, 4 h = treated with 4 h incubation, OD = oven dried, FD = freeze dried) coarse and fine bran ingredients. The results are expressed as means (n = 35–40). Values marked with different letters within the same parameter are significantly different ($P < 0.05$).

			Expansion rate (%)		**Specific length (m/kg)**		**Piece density (kg/m^3)**	
Control (no bran)			452 a		54 a		130 c	
			Coarse	Fine	Coarse	Fine	Coarse	Fine
Untreated bran			354 gh	390 bcd	68 c	62 b	169 a	155 b
Treated with no added enzymes	0 h	OD	371 ef	401 b	78 efg	79 efgh	132 c	113 e
	0 h	FD	351 h	404 b	74 de	69 cd	155 b	126 cd
	4 h	OD	-	371ef	-	82 ghi	-	128 cd
Treated with Depol 761P	0 h	OD	-	399 bc	-	83 ghi	-	108 e
	4 h	OD	355 gh	404 b	84 hij	83 ghi	136 c	106 e
	4 h	FD	367 fg	384 cde	80 fgh	74 ef	133 c	129 c
Treated with Veron CP	4 h	OD	-	372 ef	-	89 j	-	116 de
Treated with Veron CP +Depol 761P	4 h	OD	-	379 def	-	86 ij	-	116 de

The ER decreased less when untreated bran of fine particle size was used (ER 390%) as compared to the use of coarse bran (354%). Similarly, when modified brans were used the ER was always higher in the fine bran extrudates (371–404%) as compared to those of the corresponding coarse bran extrudates (351–371%) (Table 3). Indeed, there was a significant ($P < 0.01$) negative correlation between ER and bran particle size (-0.682) and a significant positive correlation between piece density and particle size (0.755) (Table 2). Smaller particle size has also previously been reported to favour radial expansion of rye bran extrudates (Alam et al. 2013) and corn meal extrudates containing sugar beet fibre (Lue et al., 1991) or corn bran (Blake, 2006). It has been suggested that coarse particles may cause early rupture of gas cells before their optimal expansion, or that reduction of particle size may improve expansion by providing more nucleation sites, and thus more air cells, than coarse fibre particles (Lue et al., 1991). Alam et al. (2013) reported that reduction of rye bran particle size from 440 µm to 28 µm improved expansion, and increased extrudate porosity and the average cell size. In the current study, bran particle size also affected the cellular structure as observed from the radial cross-section images obtained by stereomicroscopy (Fig. 2). In the samples with the coarse bran, the cells were small and the large bran particles were clearly visible, whereas in the samples with the fine bran, the cell size distribution was less homogeneous due to the

presence of some large cells, and the bran particles were less visible. It was also noted that particle size and WHC of the bran were significantly correlated (Table 2), and it is possible that the effect of fibre particle size on expansion and cellular structure was not only related to their physical dimensions, but also to their different hydration properties and their impact on melt rheology, as previously suggested by Sozer and Poutanen (2013).

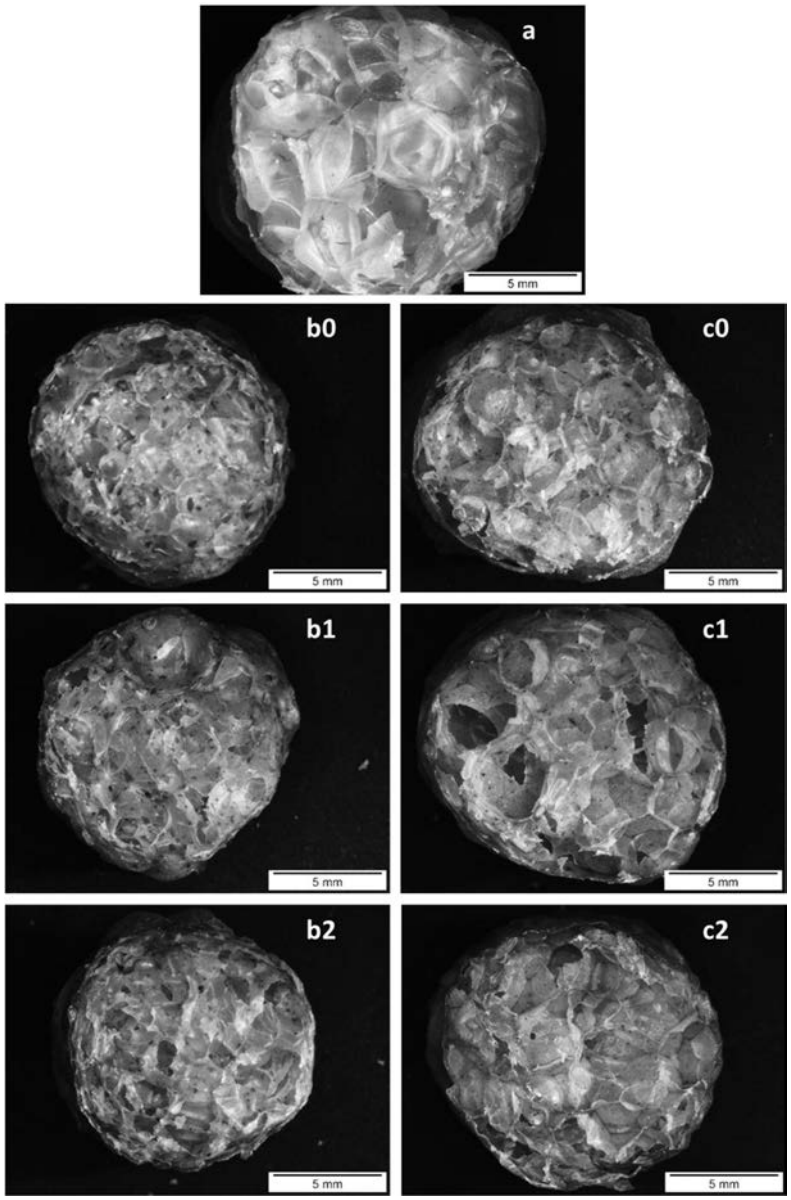

Fig. 2. Stereomicroscope images of radial sections of the extrudates. Control extrudate with no added bran (a), extrudates supplemented with 20% of untreated coarse bran (b0), coarse bran treated with no added enzymes and direct oven drying (b1), coarse bran incubated 4 h with Depol 716P enzyme preparation (b2), untreated fine bran (c0), fine bran treated with no added enzymes and direct oven drying (c1), fine bran incubated 4 h with Depol 716P enzyme preparation (c2).

The use of enzymatically modified brans produced extrudates with equal or lower piece density than that of the control (Table 3). The lowest piece densities were obtained with the enzyme-treated (Depol 761P, Veron CP or their combination) oven dried fine bran (106–116 kg/m^3). Radial expansion remained generally almost unchanged when modified brans were used as compared to the use of untreated brans (Table 3), whereas the specific length increased with modified brans, especially when treated with enzymes (74–89 m/kg), as compared to the specific length of the untreated bran extrudates (62–68 m/kg). A significant negative correlation was observed between piece density and specific length (-0.716) (Table 4). The results indicate that the higher volumetric expansion (lower piece density) of the extrudates with enzymatically modified brans was caused at least partly by increased longitudinal expansion.

Table 4. Mechanical properties of the extrudates with and without modified (0 h = treated without incubation, 4 h = treated with 4 h incubation, OD = oven dried, FD = freeze dried) coarse and fine bran ingredients. The results are expressed as means (n=35–40). Values marked with different letters within the same parameter are significantly different ($P < 0.05$).

			Crushing force (N)		**Hardness (N)**		**Crispiness index (×10^3)**	
Control (no bran)			18.3c		36.4ab		5.8bc	
			Coarse	Fine	Coarse	Fine	Coarse	Fine
Untreated bran			22.5a	21.8 ab	39.4 a	38.4 a	3.4 a	3.9 a
Treated with no added enzymes	0 h	OD	16.6 cd	15.3 def	30.2 cd	30.2 cd	6.3 cd	7.8 de
	0 h	FD	20.6 b	18.3 c	35.8 ab	32.8 bc	4.2 ab	5.8 bc
	4 h	OD	-	15.1 def	-	23.8 fg	-	10.8 gh
Treated with Depol 761P	0 h	OD	-	16.3 de	-	26.8 def	-	9.7 fg
	4 h	OD	15.4 def	13.2 g	26.1 ef	22.5 g	8.4 ef	12.1 hi
	4 h	FD	15.9 de	13.8 fg	28.1 de	25.6 efg	9.6 fg	12.9 i
Treated with Veron CP	4 h	OD	-	14.6 efg	-	27.4 def	-	10.7 gh
Treated with Veron CP +Depol 761P	4 h	OD	-	14.8 defg	-	25.6 efg	-	11.7 hi

Melt viscosity and the level of available water are considered important factors affecting expansion, and the effects of IDF and SDF on expansion have also been related to their effects on these properties (Robin et al., 2011; Moraru and Kokini, 2003). Pai et al. (2009) related the superior performance of alkali-solubilised corn bran on the degree of expansion to the reduction in melt viscosity. They concluded that in order to obtain good extrudate expansion, viscosity should be low enough to allow easy melt stretching, bubble growth and expansion, but high enough to withstand the stretching forces and prevent bubble collapse (Pai et al., 2009). Lobato et al. (2011) reported that addition of inulin to oat bran-supplemented soy flour resulted in improved expansion due to better melt flow properties. In the current study, the correlation analysis showed that specific length was significantly ($P < 0.01$) correlated with both WEAX content (0.709) and WHC (-0.710) (Table 2). It seems likely that the increase in longitudinal expansion was caused by altered rheological properties of the melt due to increase in WEAX content and/or by an increasing level of available water in the system due to reduced WHC. WEAX is known to affect viscosity depending on its molecular weight (Courtin and Delcour, 2002). Furthermore, Robin et al. (2011) reported that addition

of wheat bran resulted in increase in water activity and decrease in the glass transition temperature (T_g) of the melt, which would decrease the starch viscosity at constant temperature (Robin et al. 2011). Thus, it is also possible that in the current study, the increase in the level of available water (decrease in bran WHC) might also have promoted longitudinal expansion due to decreased melt viscosity by reducing melt T_g. However, the above-mentioned possible mechanisms behind the observed effects of bran WHC and WEAX content on expansion remain to be tested experimentally.

It has previously been reported that SDF generally produces higher radial expansion than IDF (Pai et al., 2009; Yanniotis et al., 2007; Brennan et al., 2008), but in the current study no correlation was observed between radial expansion and WEAX content of the bran (Table 2). In accordance, clear differences were not observed when comparing the radial cross-section images of the samples with treated brans to those of the corresponding untreated brans (Fig. 2). This could be due to the relatively low differences in the chemical composition of the flour-bran mixtures in the current study as compared to those of the previous studies. As analysed from selected extrudates (untreated fine and untreated coarse bran extrudates, and extrudates with oven dried fine bran treated with all different process variations), the content of IDF in the extrudates varied between 8.3 and 9.7% and the content of SDF (including oligosaccharides) between 6.5 and 7.9% (data not shown). Thus, the differences in the contents of IDF and SDF were relatively small in the flour-bran mixtures. For example, in the study of Pai et al. (2009), who studied the impact of alkali-solubilized corn bran on extrusion, the differences in SDF content ranged from 1.6 to 64%, whereas other studies have mainly compared the impacts of addition of bran to those of oligosaccharides or gums with no IDF and essentially different chemical composition from that of the bran (Brennan et al., 2008; Yanniotis et al., 2007). The total DF content in all extrudates was between 15.8 and 16.4% of the bran DM (data not shown), indicating that the bran treatments had only minor or no impact on the total DF content of the bran.

3.3. Impact of bran properties on the mechanical parameters of bran-supplemented extrudates

In the current study, the maximum point of the force-deformation curve (F_{max}) and crushing force (F_{cr}) were selected to indicate the hardness of the extrudates. The addition of untreated brans did not significantly change the F_{max} of the extrudates (39.4 N with coarse and 38.4 N with fine bran) as compared to the control extrudate without bran (36.4 N), whereas the F_{cr} of the extrudates increased when untreated coarse (22.5 N) or fine (21.8 N) bran was added, as compared to the control extrudate (18.3 N) (Table 4). This indicates that although the bran addition did not change the force needed to create the initial crack (F_{max}), it made the products more difficult to break down (F_{cr}). However, when the treated brans were used, the hardness generally decreased below that of the control as shown by both indicators (Table 4). Treatment of bran without enzymes with direct oven drying already reduced F_{max} (30.2 N with both bran types) and F_{cr} (16.6 N coarse and 15.3 N fine). F_{max} was further reduced when the fine bran was incubated for 4 h (23.8 N) and when the brans were incubated with Depol 761P (26.1–28.2 N coarse and 22.5–25.6 N fine). The use of Veron CP enzyme preparation produced slightly harder extrudates (F_{max} 27.4 N) than the use of Depol 761P (22.5 N) or the combination of the two enzyme preparations (25.6 N). The extrudates with fine bran were generally less hard than the corresponding coarse bran extrudates (Table 4), as also reported previously for rye bran (Alam et al., 2013).

Crispiness has been defined as a combination of auditory and vibratory sensations occurring in the mouth, but it can also be indicated by instrumental measurements (Heidenreich et al., 2004). The crispiness index (C_i) decreased from that of the control (5.8×10^3) when untreated coarse (3.4×10^3) or fine bran (3.9×10^3) was added (Table 4). Treatment

without enzymes with direct oven drying increased C_i with both brans (6.3×10^3 coarse and 7.8 fi$\times10^3$ fine). C_i was further increased when the fine bran was incubated (10.8×10^3), or when Depol 761P enzyme preparation was used, even without incubation (9.7×10^3). The results showed that bran treatment with enzymes produced more crispy extrudates than untreated bran or bran treated without enzymes with direct drying. C_i values obtained with the use of Veron CP alone (10.7×10^3) or in combination with Depol 761P (11.7×10^3) did not differ significantly from the values obtained with the use of Depol 761P alone (12.1×10^3) (Table 4).

Hardness and crispiness of cereal extrudates are mainly determined by their cellular structure, formed during the expansion of the extrudate, and by the phase properties and composition of the solid matrix (Moraru and Kokini, 2003). In the current study, a significant ($P < 0.01$) positive correlation was observed between improved mechanical properties (decreased hardness and increased crispiness) and decreased piece density and increased specific length (Table 4). Decreased hardness and increased crispiness also correlated ($P < 0.01$) with increased WEAX content and decreased WHC of bran, as well as with fine bran particle size ($P < 0.05$) (Table 4). Particle size also decreased during the bran treatments, but the properties of the extrudates prepared with untreated fine bran with mean particle size of 84 µm were significantly inferior to those of the extrudates made with modified coarse brans with particle size of 205–318 µm. Thus it can be concluded that the reduction of bran particle size after the bran treatments was not the primary reason for improved mechanical properties of the extrudates with modified bran. Rather, the extrudates with modified brans had improved mechanical properties probably due to the effects of the increased WEAX content and decreased WHC of the brans on the extrudate expansion, observed as decreased piece density and increased longitudinal expansion. The mechanical properties might also have improved due to possible changes in the strength of the solid matrix by the altered matrix composition (solubilisation of bran AX). It has been reported that addition of DF increases the hardness of expanded extrudates due to higher cell density and lower cell diameter (Karkle et al., 2012; Yanniotis et al., 2007; Jin et al., 1995; Robin et al., 2011). In the current study, the samples with coarse bran had more homogenous and smaller cell size than the samples with fine bran, as visually observed from the stereomicroscopy images (Fig. 2). However, the reduced hardness and increased crispiness of the extrudates with enzymatically modified brans was not clearly reflected in the cellular structure.

3.4. Impact of bran drying method on the properties of the modified brans and on the bran-supplemented expanded extrudates

In the treatment process brans were dried either by freeze drying or by oven drying. Freeze drying is known to cause minimal structural damage to the products, whereas oven drying is significantly less expensive. When bran was treated without enzymes, the content of WEAX was higher in the oven dried brans (1.4% WEAX in coarse and 1.6% in fine) than in the freeze dried brans (1.1% coarse and 1.3% fine). This was probably due to the action of the bran endogenous enzymes in the beginning of the oven drying. The oven drying was performed at relatively mild temperature (50 °C), enabling the continuation of the enzymatic action until the point where the moisture content was too low to support enzymatic action, whereas the use of liquid nitrogen for the samples that were freeze dried obviously stopped the enzyme reactions immediately. However, when the brans were treated with Depol 761P, the drying method did not have a significant impact on the level of WEAX, probably due to the high enzyme action already during the extruder-mixing, which probably obscured the possible impact of the short continuation of the enzyme action in the oven.

The WHC of the coarse bran was significantly lower after oven drying (3.0 without enzymes and 2.5 with Depol 761P) than after freeze drying (3.7 without enzymes and 3.1 with Depol 761P), both with and without the use of enzymes (Table 1). This can be explained by

the fact that forced heated air dehydration (oven drying) can cause collapse of the structure of DF (Massiot and Renard, 1997), whereas freeze drying preserves their structural features, resulting in higher porosity that allows more water entrapment. However, the drying method did not have an impact on the WHC of the fine bran, probably because it already underwent significant structural collapse due to severe grinding conditions both before and after the treatments.

In the case of the bran-supplemented expanded extrudates, it was noted that oven dried brans produced generally slightly lower piece densities and in case of fine bran also higher specific lengths than the freeze dried samples (Tables 3 and 4). When the brans were treated without enzymes, the oven dried brans also produced slightly less hard (F_{cr} 15.3–16.6 N) and crispier (C_i 6.3–7.8) extrudates than freeze dried brans (F_{cr} 18.3–20.6 N and C_i 4.2–5.8). By contrast, in the Depol 761P treatments with incubation, the drying method did not have a significant impact on the mechanical properties of the extrudates. The differences in the extrudates due to the drying method can be attributed to the slightly different impacts of the drying methods (or lack of impact, as in the case of the Depol 761P treatments) on the WEAX content and WHC of the brans. In addition, on the basis of visual observations, the drying method also affected the colour of the bran ingredients and the expanded extrudates. The oven dried brans were darker in colour, probably due to Maillard or other browning reactions occurring during the oven drying.

4. CONCLUSIONS

Modification of bran by hydrolytic enzymes in a low-moisture process increased the crispiness and reduced the hardness and density of the bran-supplemented expanded extrudates. These improvements correlated with increased level of WEAX and decreased WHC of the bran. Bran ingredient with a fine particle size produced extrudates with better texture and higher radial expansion than coarse bran. Particle size was also decreased by the enzymatic bran treatment, but the results showed that the reduction of bran particle size after the bran treatments was not the primary reason for improved mechanical properties of the extrudates with modified bran. Instead, extrudate texture and structure is proposed to have been improved due to the effects of the altered WEAX content and WHC of the enzymatically treated brans on the extrudate expansion and/or on the properties of the solid matrix. The precise mechanism behind the observed positive effects of enzymatic bran modification on the expansion and mechanical properties of the extrudates remains to be further elucidated.

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Title	**Impact of water content on enzymatic modification of wheat bran**
Author(s)	Outi Santala
Abstract	Enzymatic conversions have a fundamental role in several industrial food manufacturing processes and in the upgrading of agro-industrial residues. Enzymatic reactions are typically conducted in excess water because reduction of water content usually decreases enzymatic conversion. Processing at high solids content could, however, offer several economical advantages. Wheat bran is one of the most important by-products of the cereal industry. Bran is a good source of dietary fibre, protein and health-beneficial compounds, but its use in food applications is limited because unprocessed bran is usually detrimental to product quality. The present work aimed to examine and develop techniques to utilize hydrolytic enzymes, especially xylanase, at reduced water content in order to increase the technological functionality of wheat bran in food applications. The applicability of the modified bran was demonstrated in extruded cereal-based snacks. The results showed that technological functionality of bran can be improved by enzymatic modification at a low water content of 40–50%. Consistency of the reaction mixture, mixing method and bran particle size were important factors affecting the intensity of the modification process at reduced water content. It was possible to increase the enzyme action by changing the granular structure of the material to a continuous paste using an extruder, without increasing the water content. Modification of bran by hydrolytic enzymes by a low-moisture process increased the crispiness and reduced the hardness and piece density of bran-enriched puffed snacks. The results can be utilized for improving the technological functionality of bran in food applications and for developing new processes for the enzymatic modification of plant raw materials at reduced water content.
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Nimeke	**Vesipitoisuuden vaikutus vehnäleseen entsymaattisessa muokkauksessa**
Tekijä(t)	Outi Santala
Tiivistelmä	Entsyymejä hyödynnetään laajasti elintarviketeollisuudessa raaka-aineiden ja lopputuotteiden muokkauksessa sekä maatalouden ja metsäteollisuuden sivutuotteiden prosessoinnissa. Entsyymireaktiot tehdään yleensä suuressa vesimäärässä, koska vesipitoisuuden vähentäminen useimmiten heikentää entsyymien toimintaa. Teollisissa prosesseissa vesipitoisuuden vähentäminen toisi kuitenkin taloudellisia hyötyjä. Vehnälese, joka on yksi viljateollisuuden tärkeimmistä sivutuotteista, on hyvä ravintokuidun, proteiinin ja terveyttä edistävien pienyhdisteiden lähde ja siksi kiinnostava raaka-aine elintarviketeollisuudelle. Käsittelemättömän leseen lisääminen kuitenkin heikentää yleensä elintarvikkeen laatua. Työn tarkoituksena oli tutkia ja kehittää menetelmiä hydrolyyttisten entsyymien, erityisesti ksylanaasien, käyttämiseen matalassa vesipitoisuudessa vehnäleseen ominaisuuksien parantamiseksi elintarvikesovelluksissa. Muokatun leseen teknologinen toimivuus testattiin ekstruusiolla valmistetuissa puffatuissa lesenaksuissa. Tutkimus osoitti, että leseen ominaisuuksia voidaan parantaa entsymaattisella muokkauksella matalassa 40–50 %:n vesipitoisuudessa. Reaktioseoksen fysikaalinen koostumus, sekoitusmenetelmä ja leseen partikkelikoko todettiin tärkeiksi tekijöiksi, jotka vaikuttavat muokkausprosessin tehokkuuteen matalassa vesipitoisuudessa. Työssä havaittiin, että entsyymien toimintaa on mahdollista tehostaa nostamatta vesipitoisuutta muuttamalla lese-vesiseos rakeisesta yhtenäiseksi massaksi ruuvisekoittimen avulla. Matalassa vesipitoisuudessa tehty entsymaattinen leseen muokkaus paransi korkeakuituisten lesenaksujen rapeutta ja vähensi niiden kovuutta ja tiheyttä. Tutkimuksen tuloksia voidaan hyödyntää leseen teknologisen toimivuuden parantamiseen elintarvikesovelluksissa. Tulosten perusteella voidaan myös kehittää uusia entsymaattisia prosesseja kasvimateriaalien muokkaamiseksi matalassa vesipitoisuudessa.
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The results showed that technological functionality of bran can be improved by enzymatic modification at a low water content of 40–50%. Consistency of the reaction mixture, mixing method and bran particle size were important factors affecting the intensity of the modification process at reduced water content. It was possible to increase the enzyme action by changing the granular structure of the material to a continuous paste using an extruder, without increasing the water content. Modification of bran by hydrolytic enzymes by a low-moisture process increased the crispiness and reduced the hardness and piece density of bran-enriched puffed snacks. The results can be utilized for improving the technological functionality of bran in food applications and for developing new processes for the enzymatic modification of plant raw materials at reduced water content.

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