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Hydrolysis of konjac glucomannan by *Trichoderma reesei* mannanase and endoglucanases Cel7B and Cel5A for the production of glucomannooligosaccharides

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In this paper we describe the enzymatic hydrolysis of konjac glucomannan for the production of glucomannooligosaccharides using purified *Trichoderma reesei* mannanase, endoglucanases EGI (*Tr* Cel7b) and EGII (*Tr* Cel5a). Hydrolysis with each of the three enzymes produced a different pattern of oligosaccharides. Mannanase was the most selective of the three enzymes in the hydrolysis of konjac mannan and over 99% of the formed oligosaccharides had mannose as their reducing end pyranosyl unit. *Tr* Cel5A hydrolysate shared similarities with mannanase and *Tr* Cel7B hydrolysates and the enzyme had the lowest substrate specificity of the studied enzymes. The hydrolysate of *Tr* Cel7B contained a series of oligosaccharides with non-reducing end mannose (M) and reducing end glucose (G) (MG, MMG, MMMG, and MMMMG). These oligosaccharides were isolated from the hydrolysate by size exclusion chromatography in relatively high purity (86-95%) and total yield (23% of substrate). The isolated oligosaccharides were characterized using acid hydrolysis and HPAEC-PAD (carbohydrate composition), HPLC-RI and HPAEC-MS (to determine the DP of purified oligosaccharides), enzymatic hydrolysis (determination of non-reducing end carbohydrate) and NMR (both 1D and 2D, to verify structure and purity of purified compounds). Hydrolysis of konjac mannan with a specific enzyme, such as *T. reesei* Cel7B or mannanase, followed by fractionation with SEC offers the possibility to produce glucomannooligosaccharides with defined structure. The isolated oligosaccharides can be utilised as analytical standards, for determination of bioactivity of oligosaccharides with defined structure or as substrates for defining substrate specificity of novel carbohydrate hydrolyzing enzymes.

Key words: oligosaccharide, Konjac mannan, glucomannan, enzymatic hydrolysis

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Abbreviations: GM, β-D-Glc*p*-(1→4)-D-Man*p*; MM, β-D-Man*p*-(1→4)-D-Man*p*; MG, β-D-Man*p*-(1→4)-D-Glc*p*; MMM, β-D-Man*p*-(1→4)- β-D-Man*p*-(1→4)-D-Man*p*; MMG, β-D-Man*p*-(1→4)- β-D-Man*p*-(1→4)-D-Glc*p*; GGM, β-D-Glc*p*-(1→4)- β-DGlc*p*-(1→4)-D-Man*p*; GMM, β-D-Glc*p*-(1→4)- β-D-Man*p*-(1→4)-D-Man*p*; MGG, β-D-Man*p*-(1→4)- β-D-Glc*p*-(1→4)-D-Glc*p*; MMMG, β-D-Man*p*-[(1→4)- β-D-Man*p*]2-(1→4)-D-Glc*p*; MGGM, β-D-Man*p*-(1→4)-[β-D-Glc*p*-(1→4)]2-D-Man*p*; GGGM, β-DGlc*p*-(1→4)-[β-D-Glc*p*-(1→4)]2-D-Man*p*; MMMMG, β-D-Man*p*-[(1→4) - β-DMan*p*]3-(1→4)-D-Glc*p*.

1. Introduction

Mannans are hemicelluloses that have a role as structural polymers in plant cell walls and as storage carbohydrate in plant seeds. Mannans can be classified in four subfamilies; linear mannan, glucomannan, galactomannan and galactoglucomannan. The main structure of linear mannan consists of mannopyranosyl units bound together with β-D-1,4 linkages.[1] The structure of glucomannan consists of D-mannopyranosyl and D-glucopyranosyl units linked together by β-D-1,4 –linkages.[2] Galactomannans, mainly found in seeds, are substituted in different degrees with 1,6-linked α-galactopyranosyl units.[1] The main hemicellulose present in softwood cell walls, galactoglucomannan, has a backbone of mannopyranosyl and glucopyranosyl units and is substituted with α-galactopyranosyl units in varying ratios.[3]

Glucomannan structure consists of four different β-D-1,4 –linkages: mannose to mannose, mannose to glucose, glucose to mannose and glucose to glucose. These linkages can be hydrolysed by endo-1,4- β-mannanases (mannanase, EC 7.2.1.78) and endo- β-glucanases (EG, endoglucanase, EC 3.2.1.4). Mannanases catalyse the random hydrolysis of β-D-1,4-mannopyranosyl linkages in the main chain of mannans, glucomannans, and galactomannans.[4,5] Endoglucanases belong to the cellulose degrading enzymes and hydrolyse the β-D-1,4 –linkages in cellulose chain. In addition to cellulose, at least the glucose to mannose and glucose to glucose linkages in glucomannan are hydrolysable to endoglucanases. Substrate specificity of enzymes vary depending on their structural features and endoglucanases from certain glycoside hydrolase (GH) families are able to hydrolyse hemicelluloses such as mannans and xylans as well.[6] Enzymes hydrolysing the non-reducing end mannopyranosyl and glucopyranosyl units from oligosaccharides released from glucomannan are β-mannosidase (EC 3.2.1.25) and β-glucosidase (EC 3.2.1.21), respectively. The α-galactopyranosyl units can be hydrolysed by α-galactosidase (EC 3.2.1.22).[4]

Linear glucomannans from plants are readily available in commercial sources, for example konjac glucomannan from the tuber of plant *Amorphophallus konjac* has been studied in detail and is used as gelling and thickening agent in food and beverages.[5] Additionally, oligosaccharides prepared by enzymatic hydrolysis of konjac glucomannan can be used as prebiotics as the hydrolysate efficiently enhances the growth of lactobacilli and bifidobacteria.[7] The specific nature of enzyme hydrolysis makes enzymes excellent tools for targeted and controlled hydrolysis of polysaccharides. The advantage of enzymatic hydrolysis over acid hydrolysis is specificity and thus, although the process is slower, oligosaccharides with desired degree of polymerization (DP) are obtained without the formation of monosaccharides or furfural.[8] The choice of enzyme will have an effect on the oligosaccharide profile to be obtained in the hydrolysis of glucomannan. For example hydrolysis of konjac mannan by *P. funiculosum* endoglucanase leads to formation of MG, MM, MMG and MMM whereas GM, MM, GGM and GMM have been detected after hydrolysis by *A. niger* and *Streptomyces* sp. mannanase.[9] The substrate specificity can also be exploited in analytical purposes. For example, glucomannan

content of e.g. konjac flour can be determined by enzymatic hydrolysis with mannanase followed by the alkali treatment to remove acetyl groups and further hydrolysis by β-mannosidase and β-glucosidase.[10] Based on the quantities of different oligosaccharides formed by the enzymatic hydrolysis it has been concluded that the glucose and mannose units are distributed randomly along the polysaccharide chain and konjac mannan thus represents a Bernoulli-type chain,[9] thus even features of the polymer structure can be revealed by enzyme hydrolysis.

Trichoderma reesei (syn. *Hypocrea jecorina*) is an efficient plant biomass degrading fungus, which is currently one of the main sources of industrial glycoside hydrolases and a commonly utilized production host of industrial enzymes. The genome of *T. reesei* was recently sequenced and the genes encoding carbohydrate-active enzymes were thoroughly investigated. The genome of *T.reesei* contains 200 glycoside hydrolase encoding genes, seven and 6 of which encoding known cellulases and hemicellulases, respectively.[11] The aim of the present work was to produce glucomannooligosaccharides from konjac mannan by enzymatic hydrolysis using selected purified *T. reesei* glycoside hydrolases. Although the cellulolytic enzymes of *T. reesei* have been widely studied, details on the substrate specificity of the enzymes from this industrially relevant organism are still needed. In this paper we present detailed analysis of the hydrolysis products of konjac glucomannan obtained by hydrolysis with two *T. reesei* endoglucanases belonging to glycosyl hydrolase (GH) families 5 and 7 (*Tr*Cel5A and *Tr*Cel7B, formerly known as EGII and EGI, respectively) and one mannanase belonging to GH family 5.[12,13]

2. Results

2.1 Enzymatic hydrolysis of konjac mannan

All the three studied enzymes, *Tr* Cel5A, *Tr* Cel7B and mannanase from *Trichoderma reesei*, hydrolysed konjac mannan efficiently and the amount of released reducing sugars was approximately 50% of substrate after the 48 hour hydrolysis (Figure 1), which corresponds on average to total hydrolysis of the substrate into disaccharides. Hydrolysis with mannanase released reducing sugar ends the most of the three studied enzymes and the hydrolysis was the fastest in the beginning of the incubation. One of the two studied endoglucanases, *Tr* Cel7B, hydrolysed konjac mannan faster, although the overall hydrolysis by *Tr* Cel5A was higher. At the end of the 48 h incubation, the hydrolysis by *Tr* Cel7B and mannanase had plateaued suggesting that hydrolysis was close to maximal level. The hydrolysis by *Tr* Cel5A was still slowly progressing after 48 h, although the hydrolysis was relatively slow.

The mono- and oligosaccarides formed by the hydrolysis by *Trichoderma reesei* enzymes were analysed by HPAEC-PAD/MSQ (Figure 2). The yield of each hydrolysis product was calculated as % of substrate after anhydrocorrection. Monosaccharides were released from the substrate by all the three enzymes as both glucose (8.2% of substrate by *Tr* Cel7B and 2.6% by

Tr Cel5A) and mannose (7.7% of substrate by mannanase and 1.5% by *Tr* Cel5A). Thus, the lowest amount of monosaccharides was released by *Tr* Cel5A, but it also was the only enzyme of the three to release significant amount (over 1% of substrate) of both mannose and glucose from the substrate. Linear manno-oligosaccharides (DP 2-6) were detected in all hydrolysates in total of 17%, 18% and 4.7% for mannanase, *Tr* Cel5A and *Tr* Cel7B, respectively.

In addition to mannose and mannobiose, mannanase hydrolysate contained minor amount of mannotriose and oligosaccharides with glucose in their non-reducing end and mannose in the reducing end , namely GM, GGM and GMM (Figure 2B, retention time 52.83, 57.47 and 73.76 min). These oligosaccharides were identified and quantified using in-house standards and represented 62% of the substrate. The main hydrolysis products of *T. reesei* mannanase consisting of only mannose were mannose (7.7% of substrate) and mannobiose (16% of substrate). Two peaks with DP4 in the mannanase hydrolysate eluting at 78.96 min and 92.48 min (Figure 2B) could not be identified with the analytical standards in use, their yields were estimated to present 7.6 and 4.5% of substrate, respectively. The concentrations of the unidentified oligosaccharides in the hydrolysate were estimated based on the standard eluting closest to them. Altogether 89% of the konjac mannan substrate was hydrolysed into identified products by *Trichoderma reesei* mannanase, summing up to 101% yield with the unidentified peaks, although some minor peaks with DP5-6 in the chromatogram were not included in the calculations.

The main oligosaccharide products (unidentified peaks 1-4) in the *Tr* Cel7B hydrolysate could not be identified with the utilised commercial or in-house standards (Figure 2A, retention time 16.42, 20.18, 26.62 and 34.93 min). The concentrations of the unidentified oligosaccharides in the hydrolysate were estimated based on the standard eluting closest to them. The four unidentified peaks were estimated to represent 48% of the substrate in the hydrolysate of *Tr* Cel7B and 32% in the hydrolysate of *Tr* Cel5A. In addition to the unidentified peaks *Tr* Cel5A hydrolysate contained linear manno-oligosaccharides with DP 2-4 (Figure 2C, retention time 24.42, 30.68 and 40.48 min) and oligosaccharides with glucose in their non-reducing end and mannose in the reducing end (GM and GMM, Figure 2C, retention time 53.75 and 57.71 min). Thus, the hydrolysate formed by *T. reesei* Cel5A shared similarities with the *Tr* Cel7B and mannanase hydrolysates indicating that the enzyme has lower substrate specificity than the two other enzymes studied.

2.2 Isolation and identification of glucomannooligosaccharides from *Trichoderma reesei* Cel7B and mannanase hydrolysates

In order to isolate and identify the unidentified oligosaccharides present in the *Tr* Cel7B and mannanase hydrolysate the freeze-dried hydrolysates were fractionated according to size using size exclusion chromatography. The oligosaccharide composition of the collected fractions was

analysed by HPAEC-PAD and fractions containing the unidentified oligosaccharides were combined and freeze-dried. The HPAEC-PAD chromatograms revealed presence of one main oligosaccharide compound in each combined fractions of *Tr* Cel7B hydrolysate (Figure 3, each panel, grey line). The yield of each unidentified oligosaccharide is depicted in Table 1. The overall yield of isolated oligosaccharides was 23% of the konjac mannan used in the hydrolysis. The DP of the oligosaccharides in the combined fractions was determined by HPLC-RI using linear manno-oligosaccharide and cello-oligosaccharide standard mixtures of DP 1-6. In the chromatograms of each isolated unidentified oligosaccharide only one main peak was observed although some impurities with were detected (Table 1). The purity of the isolated oligosaccharides was estimated based on HPLC-RI by comparing the area of the main DP peak to the total peak area. HPAEC-MS was used to confirm the molecular mass of unidentified oligosaccharides.

The carbohydrate composition of the unidentified oligosaccharides 1-4 was determined by acid hydrolysis (Table 1). In order to determine the non-reducing end carbohydrate of the unidentified oligosaccharides, they were hydrolysed with β-mannosidase and β-glucosidase. None of the unidentified oligosaccharides were hydrolysable with β-glucosidase (data not shown), whereas β-mannosidase hydrolysed the four oligosaccharides almost completely into mannose and glucose (Figure 3, each panel, black line). The carbohydrate composition of the combined fractions based on enzymatic hydrolysis by β-mannosidase slightly differed from the composition determined by acid hydrolysis (Table 1). Based on the DP, MSQ detection, carbohydrate composition and secondary enzymatic hydrolysis by β-mannosidase and β-glucosidase, the unidentified oligosaccharides were tentatively identified as MG (unidentified 1), MMG (unidentified 2), MMMG (unidentified 3), and MMMMG (unidentified 4).

The two unidentified peaks in mannanase hydrolysate were eluted into the same fractions by size exclusion chromatography and could not therefore be separated from each other. Secondary hydrolysis with β-mannosidase , β-glucosidase, and *T.reesei* Cel7B was carried out to find out their structure. Unidentified 6 was hydrolysed into glucose and mannose at ratio 2.7:1 by β-glucosidase whereas unidentified 5 was not affected suggesting that it has mannose as the non-reducing sugar unit. Accordingly, unidentified 5 was hydrolysed by β-mannosidase the main hydrolysis products being GGM (at *m/z* 511*)* and mannose (at *m/z* 187). Interestingly the peak identified as GMM (at *m/z* 511) was not completely hydrolysed by β-glucosidase and was not at all affected by β-mannosidase (Table 2) suggesting that the secondary hydrolysis with β-glucosidase was incomplete. Both of the unidentified oligosaccharides were hydrolysable by *T. reesei* Cel7B, which released MG, GM, MGG, cellobiose, Man and Glc (data not shown). Based on the response to secondary enzymatic hydrolysis with β-mannosidase , β-glucosidase, and *T.reesei* Cel7B the unidentified peaks were tentatively identified as MGGM (unidentified 5) and GGGM (unidentified 6) (Table 2). HPAEC-MS was used to confirm that the peak mass (m/z) values were 673 as Li-adducts $[M + Li]^+$ at $[M + 7]^+$ for both unidentified peaks (5 and 6) i.e. for oligohexoses it means that they are tetrasaccharides (DP4). As the two oligosaccharides were

of same size, they could not be separated by size exclusion from each other and their identity was not further confirmed by NMR.

2.3 Identification of glucomannooligosaccharides by NMR spectroscopy

Finally the identification of the unknown oligosaccharides 1-4 was confirmed by ^{1}H and ^{13}C NMR spectroscopy. The structural reporter group regions of the ^{1}H NMR spectra of the four samples in D_2O are shown in figure 4. All samples are P2 purified samples and named after main identified compound. In the spectrum of unidentified 1 (Figure 4, A) two doublets were observed at 5.229 and 4.673 ppm. The coupling constants (3.8 Hz and 8.0 Hz, respectively) confirm that these are the α and β H1 signals of the reducing end glucose.[9] In addition, a signal was observed at 4.763 ppm, a typical position for a β1-4 linked mannose,[9] confirming the structure as Manβ1-4Glc. The 1D ^{1}H spectrum also reveals typical H2 signals of Man and β-Glc at 4.074 and 3.856 ppm, respectively.[15] Although these structural reporter group signals already confirm the structure, full assignment of the ^{1}H and ^{13}C NMR signals was carried out from DQFCOSY, TOCSY and HSQC spectra, and are presented in tables 3 and 4, respectively. The ^{13}C NMR data reveals clear glycosylation shifts of the C4 signals of the both anomers of the glucose, further confirming the position of linkage between Man and Glc. The ^{13}C chemical shifts were similar to the ones published earlier for the same disaccharide structure.[16]

Structural reporter group signals similar to the ones observed above for MG were found also in the ^{1}H spectrum of unidentified 2 (Figure 4, B), but now there were two signals at the H1 and H2 regions of mannose, confirming the structure as MMG. The same trend continued in the spectra of unidentified 3 (Figure 4, C) showing a new signal at 4.774 ppm, between the typical anomeric signals of terminal and glucose-bound mannoses already present in the spectrum of MMG.[9,15] This confirms the structure as MMMG. In addition, a small signal (4.760 ppm) and a group of doublets (about 4.51 ppm, probably mid-chain Glc)[9] with small intensity were observed, arising from the small fraction (about 10%) of other oligosaccharide structures present in the sample as was already revealed from the chromatography results. The ^{1}H spectrum of the last sample, unidentified 4 (Figure 4, D), showed H1 and H2 signals of a fourth mannose, resonating at similar chemical shifts as the middle-chain mannose signals in MMMG (4.77 ppm).[15] This confirms the structure as MMMMG. The weak anomeric β -glucose signals[9] from the small fraction of other oligosaccharide structures were observed also in this sample.

Full assignments of the ^{1}H and ^{13}C NMR signals (Tables 3 and 4, respectively) were obtained from the 2D experiments and they confirmed the assignments of the structural reporter groups presented above. Two clearly different ^{1}H spin systems were observed for the mannoses in the TOCSY spectra (spectra not shown, see Table 3), corresponding to the terminal and mid-chain mannoses. Although the ^{1}H chemical shifts of the anomeric protons of glucose and mannose

bound mid-chain mannoses were slightly different, no differences could be observed for the other proton or carbon signals. However, in the HSQC spectra of MMMG and especially MMMMG, the H4-C4 peak of the mid-chain mannoses was exceptionally broad in the ^{1}H dimension and, in the case of MMMMG, also unsymmetrical. This suggests that there is a small difference in the ^{1}H chemical shift of these overlapping signals, but the resolution of the spectra was not high enough to split the peak. In all cases, the ^{13}C chemical shifts of C4s of the mid-chain mannoses and the reducing end glucose showed a clear glycosylation shift of about 10 ppm, as illustrated for MMG in figure 5. This confirms that C4 is the glycosylation site in each linkage. The ^{13}C chemical shifts of MMG were similar to the ones published for the same structure.[16] The 2D experiments also confirmed that the ^{1}H signals at about 4.51 ppm arise from β-Glc moieties having a ^{1}H spin system very similar to the glucoses in the main oligosaccharides. This suggests that these are oligosaccharides with a non-reducing glucose substituted at position 4.[9]

2.4 Analysis of hydrolysis specificity of *Trichoderma reesei* Cel7B, Cel5A and mannanase

In order to make conclusions of the enzyme specificity, the observed oligosaccharides were analysed based on their reducing and non-reducing end pyranosyl units (Table 5). The most selective hydrolysis pattern was observed with *T .reesei* mannanase, which formed over 99% of oligosaccharides with reducing end mannose. As expected, the hydrolysis products of the two endoglucanases differed from those of the mannanase. *T. reesei* Cel7B clearly preferred glucopyranosyl units in its -1 subsite of the active site, as 84% of the identified oligosaccharides possessed glucose in their reducing end (Table 5). As much as 91% of the formed oligosaccharides had mannose in their non-reducing end, which is explained by the preference of glucopyranosyl in the -1 subsite and the ability of the enzyme to degrade the glucose to mannose bond. Unexpectedly some linear manno-oligosaccharides (~5% of substrate) were present in the *T.reesei* Cel7B hydrolysate.

The observed pattern of non-reducing and reducing end pyranosyl units in the *Tr* Cel5A hydrolysate shows lower specificity of the enzyme in comparison to mannanase and *Tr* Cel7B. As the used *Tr* Cel5A preparate is obtained from a culture filtrate of a wild type *Trichoderma reesei* and not from a Man^{-} strain, it needs to be noted that the produced linear mannooligosaccharides are different from that of purified mannanase. Mannanase released mannose (7.7% of substrate) and mannobiose (16%) as main linear manno-oligosaccharides whereas *Tr* Cel5A released only little mannose (1.5%) and the main products were mannobiose (5.2%), mannotriose (8.3%) and mannotetraose (3.8%), which suggests that the observed man-man hydrolysis is truly carried out by *Tr* Cel5A rather than mannanase impurity in the enzyme preparate.

3. Discussion

The hydrolysis efficiency of the three enzymes was evaluated based on the analysis of released reducing sugars during a 48-h hydrolysis. All the three studied *Trichoderma reesei* enzymes hydrolysed konjac mannan efficiently, reducing sugars over 50% of the substrate (Figure 1). The most efficient enzyme in hydrolysis of konjac mannan was, in terms of both hydrolysis reaction velocity and overall hydrolysis yield, was mannanase. Based on the shape of the hydrolysis curve, the hydrolysis by EGI and mannanase had reached maximal level after 48 h. However, the hydrolysis by EGII was still slowly progressing after 48 h and further hydrolysis of the detected oligosaccharides might thus occur.

Although *Trichoderma reesei* mannanase was very efficient in the hydrolysis of konjac mannan, it also was selective at its -1 subsite as almost all the detected oligosaccharides had a non-reducing end mannose unit. Similar degradation pattern of konjac mannan has previously been observed by mannanases from *Aspergillus niger*[9,14] and *Streptomyces* sp..[9] Hydrolysis of galactoglucomannan present in pine kraft pulp by *T. reesei* mannanase revealed that it is able to hydrolyse β-D-1,4 –pyranosyl linkage between two mannose units and between mannose and glucose but not between glucose and mannose leading to the formation of e.g. GM disaccharides.[15] The observed cleavage pattern by *T. reesei* mannanase belonging to GH family 5 is in accordance with the conclusions made on the substrate specificity of GH family 5 and GH family 26 mannanases.[18] Based on site-directed mutagenesis of different mannanases it has been concluded that the GH 5 mannanases can accommodate glucose in their -2 and +1 subsites but not in -1 subsite of the active site.[18] This property leads to the formation of oligosaccharides with either glucose or mannose in non-reducing end and exclusively mannose in reducing end as hydrolysis products.

Endoglucanases in general are able to hydrolyze the linkage β-D-1,4 –linkage between two glucose units and additionally all endoglucanases from *T.reesei* are known to be able to cleave the β-D-1,4 –linkage between a glucopyranosyl and mannopyranosyl unit in glucomannan.[4] *T. reesei* Cel7B is known to hydrolyse xylan, but not homopolymeric mannans.[19] Based on the work by Vlasenko et al. 2010,[6] this hydrolysis pattern is typical for GH family 7 endoglucanases as none of the seven studied GH family 7 endoglucanases hydrolyse linear mannan or galactomannan, although some of them are relatively efficient in their activity towards xylans. Unexpectedly some linear manno-oligocaccharides were present in the *T. reesei* Cel7B hydrolysate. The M_n molecular weight of the utilised konjac mannan substrate is relatively low, 13 000 g mol^{-1} (~ DP 70),[20] and the linear manno-oligosaccharides may have been released from konjac mannan chains possessing mannose in the reducing end. The observed hydrolysis product pattern of *T. reesei* Cel7B resembles that obtained previously with *Trichoderma viride* EG IV (GH family 7),[17] i.e. release of glucose and oligosaccharides with reducing end glucose and non-reducing end mannose, even though linear manno-oligosaccharides were not observed in *T. viride* EG IV hydrolysate.

Similar hydrolysis products to *T.reesei* Cel5A were observed in the konjac mannan hydrolysate of *Penicillium funiculosum* endoglucanase.[9] Identification of dimeric and trimeric oligosaccharides from konjac mannan hydrolysed by *Penicillium funiculosum* β-endoglucanase revealed that MG, MM, MMG and MMM were the dominant di- and trisaccharides present in the hydrolysate. Glucose and mannose are present in the reducing end of the oligosaccharides in 1:1 ratio and the non-reducing end ratio presents the Man:Glc ratio of the original konjac mannan substrate. *Trichoderma reesei* Cel5A belongs to GH family 5 and as described by Vlasenko et al. 20106, several GH family 5 endoglucanases are able to hydrolyse mannans. In addition, unlike other *T. reesei* endoglucanases, Cel5A (Cel 5a) (previously known as EGIII) has been found to possess mannanase activity.[21] The determined *Km* values of the enzyme were similar towards both Locust bean gum galactomannan and carboxymethylcellulose, but the *kcat* was tenfold higher to carboxymethylcellulose than galactomannan.

4. Conclusions

In this paper we present a detailed analysis of glucomannooligosaccharides obtained by hydrolysis of konjac glucomannan with two *T. reesei* endoglucanases (*Tr* Cel5A and *Tr* Cel7B, formerly known as EGII and EGI, respectively) and one mannanase (GH family 5). By enzymatic hydrolysis of konjac mannan DP2-6 oligosaccharides with varying sequence were produced, isolated and identified by the combination of NMR and secondary enzyme hydrolysis. The analysis of the hydrolysis products provides knowledge on the substrate specificity of the hydrolytic enzymes of one of the most studied plant biomass degrading fungi. Based on our results *Tr* Cel5A hydrolyses the cleavage of all the glycosidic bonds present in glucomannan main chain, whereas *Tr* Cel7B and *T.reesei* mannanase have higher substrate specificity, especially in their -1 subsite of the active site. One of the challenges in studying enzymatic hydrolysis of mannans with varying mannose:glucose:galactose ratio is the lack of commercial standard samples for analysis of the hydrolysis products. The combination of specific enzymatic hydrolysis and fractionation with size exclusion gives the possibility to produce oligosaccharides with defined structure to be used as analytical standards, for determination of bioactivity of oligosaccharides with defined structure or as substrates for defining specificity of novel carbohydrate hydrolyzing enzymes.

5. Experimental

5.1 Materials

Enzyme treatments were carried out with mannanase, endoglucanase I (*Tr* Cel7a, GH family 7) and endoglucanase II (*Tr* Cel5A, GH family 5) purified from *Trichoderma reesei* culture

filtrate as described by Pere *et al.* (1995)[22] and Rättö *et al.* (1993).[23] Mannanase and endoglucanase activities were determined as described by Pere *et al.* (1995)[22] and the activities were expressed as katals. One nanokatal (nkat) of enzyme catalyses the release of 1 nmol of reducing sugars from the substrate polymer (locust bean gum for mannanase and HEC for endoglucanase) in one second. Protein content of the preparates was determined with BIORAD protein assay. For the secondary hydrolysis of the oligosaccharides commercial β-mannosidase from *Cellulomonas fimi* (Megazyme, Ireland) was used. Commercial enzyme product Novozyme 188 was used as a source of *Aspergillus niger* β-glucosidase.

Low viscosity native konjac glucomannan (Megazyme, Ireland) was used as substrate in the enzyme hydrolysis. The carbohydrate composition of the substrate was determined after acid hydrolysis with 4% H_2SO_4 at 120°C for 1 h by HPAEC (Dionex ICS-3000) with pulse amperometric detection using CarboPac PA1 column. The determined Man:Glc ratio of the substrate was 1.64:1 and it contained traces of galactose (0.9% of dw) and arabinose (0.1% of dry weight). The used substrate has been characterized in detail by Chua *et al.* (2012)[20] and based on their analysis the M_w of the substrate is 5.5 x 10^4g/mol and the degree of acetylation 1.8%.

5.2 Enzymatic hydrolysis of konjac mannan

Enzyme hydrolysis was performed in 1% substrate concentration in 50-mM ammonium acetate buffer (pH 5), 45°C for 48 hours with mixing. Enzyme dosage was 2 mg of protein/g of substrate. After hydrolysis the solutions were boiled for 10 min to inactivate the enzyme. In order to isolate formed oligosaccharides the hydrolysis was performed to 4 g batches of konjac mannan.

The enzyme hydrolysis was followed by analysing the increase of reducing sugars.The concentration of reducing sugars was determined on a 96-well plate using a colorimetric DNS (dinitrosalicylic acid) method described by Bernfeld (1955).[24] The colored nitro ammonium compound resulting from reaction of DNS with reducing sugars was quantified by reading the absorbance at 540 nm using a Multiscan MS spectrophotometer. Distilled water was used as a blank and 0.1-0.5 mg/ml *D-(+)*-glucose was used as standard.

5.3 Fractionation of konjac mannan hydrolysates by size exclusion chromatography

The hydrolysate was fractionated by size exclusion using preparative BioGel P2 (BioRad) column as described by Tenkanen *et al.* (1997).[15] Freeze-dried hydrolysates were dissolved into distillated water for fractionation by gel filtration. Samples ca. 5 g were diluted using 50 ml of water to gain ca. 10% (w/v) of end concentration. Whole 50 ml of diluted sample was injected into preparative column of BioGel P2. P2 was eluted with water using 10 ml/min flow, totally 200 fractions were collected using collection time of 3 min for each fraction. The formed fractions were analysed by HPAEC-PAD and samples containing the target oligosaccharides

were combined. The oligosaccharides in the combined samples were characterized using acid hydrolysis and HPAEC-PAD (carbohydrate composition), HPLC-RI (to determine the DP of purified oligosaccharides), enzymatic hydrolysis (determination of non-reducing end carbohydrate) and NMR (to verify structure and purity of purified compounds).

5.4 Liquid chromatographic analysis

Oligosaccharides present in the hydrolysate were analysed with HPAEC (Dionex ICS-3000) with pulse amperometric and MSQ detection using CarboPAC 200 column with a configuration described by Bruggink et al. (2005).[25] The system was equilibrated with 3 mM NaOH. After injection of a 25µl sample 3 mM NaOH was run through the column for 43 minutes. Two linear gradients first from 3 mM to 100 mM NaOH during 37 minutes and then from 100 mM NaOH to 100 mM NaOH + 150 mM Na-acetate during 30 minutes were then created. Finally the column was washed with 100 mM NaOH + 300 mM Na-acetate and then with 300 mM NaOH. The flow rate was 0.3 ml min^{-1}. The formed oligosaccharides were identified based on elution of commercial standards (linear manno-oligosaccharides from MegaZyme, linear cello-oligosaccarides from Serva, Seikagu, and Merck) and elution of in-house standards (GM, GGM, GMM) described in Tenkanen et al. (1997).[15] Yield of each oligosaccharide was calculated as percentage from the substrate after anhydrocorrection with coefficient calculated from the equation: $m_{\text{hydrolysis product}} \div (m_{\text{hydrolysis product}} + m_{H2O})$. HPAEC-MS was used to verify and to identify unidentified compound as Li –adducts $[M + Li]^+$ at $[M + 7]^+$ in the positive mode.

HPLC-RI was also utilized to identify the purity of purified compound and to determine the DP of those. Used equipment was HPLC-RI (PerkinElmer, Flexar) system equipped with Phenomenex RSO-oligosaccharide 4 % Ag+, 200x10 mm -column. Running conditions were isocratic 0.3 ml/min of milliQ -water. The DP range and elution order was calibrated by using linear manno- and cellooligosaccharide standard mixtures of DP 1-6.

5.5 NMR Spectroscopy

For NMR experiments the oligosaccharide samples were dissolved in 600 µl of D_2O (99.9% D, Aldrich) containing 0.05 wt. % 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid as an internal standard. All NMR experiments were carried out at 8ºC on a 600 MHz Bruker Avance III NMR spectrometer equipped with a Q-CPN cryoprobe. The residual water signal was suppressed by presaturation using the noesy1d pulse sequence. DQFCOSY, TOCSY and ^{13}C multiplicity edited HSQC 2D spectra were recorded with standard pulse sequnces. In TOCSY the mixing time was 80 ms and in HSQC the $^1J_{HC}$ coupling constant was estimated to 150 Hz. The chemical shifts were referenced to internal TSP.

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References

1. Moreira, L.R.S.; Filho, E.X.F. *Appl. Microbiol. Biotechnol.*, **2008**, 79, 165-178.
2. Katsuraya, K.; Okuyama, K.; Hatanaka, K.; Oshima, R.; Sato, T.; Matsuzaki, K.; *Carbohydr. Polym.*, **2003**, 53, 183-189.
3. Willför, S.; Sjöholm, R.; Laine, C.; Roslund, M.; Hemming, J.; Holmbom, B. *Carbohydr. Polym.*, **2003**, 52, 175–187.
4. Biely, P.; Tenkanen, M. Enzymology of hemicellulose degradation. In *Trichoderma and Gliogladium*. Harman, G.E., Kubicek, C.P., Eds.; Taylor & Francis Ltd, London & Bristol, **1998**; Vol. 2, pp 25-47.
5. van Zyl, W.; Rose, S.H.; Trollope, K.; Görgens, J.F. *Process Biochem.*, **2010**, 45, 1203-1213.
6. Vlasenko, E.; Schülein, M.; Cherry, J.; Xu, F. *Biores. Technol.*, **2010**, 101, 2405-2411.
7. Al-Ghazzewi, F.H.; Khanna, S.; Tester, R.F.; Piggott, J. *J. Sci. Food Agric.*, **2007**, 87, 1758-1766.
8. Akipnar, O.; Erdogan, K.; Bakir, U.; Yilmaz L. *LWT – Food Sci Technol.*, **2010**, 43, 119-125.
9. Cescutti, P.; Campa, C.; Delben, F.; Rizzo, R. *Carbohydr. Res.*, **2002**, 337, 2505-2511.
10. McCleary, B.V.; Charnock, S.J.; Rossiter, P.C.; O´Shea, M.F.; Power, A.M., Lloyd, R.M. *J. sci. Food Agric.*, **2006**, 86, 1648-1661.
11. Martinez, D.; Berka, R.M.;Henrissat, B.;Saloheimo, M.; Arvas, M.; Baker, S.E.; Chapman,J.; Kubicek, C.P.; Han, C.S.; Ho, I.; Larrondo, L.F.; de Leon, A.L.; Magnuson, J.K.; Merino, S.; Misra, M.; Nelson, B.; Putnam, N.; Robbertse, R.; Salamov, A.A.; Schmoll, M.; Terry, A.; Thayer, N.; Westerholm-Parvinen, A.; Schoch, C.L.; Yao, J.; Barabote, R.; Nelson, M.A.; Detter, C.; Bruce, B.; Kuske, C.R.; Xie, G.; Richardson, P.; Rokhsar, D.S.;Lucas, S.M.; Rubin, E.M.; Dunn-Coleman, N.; Ward, M.; Brettin, T.S. *Nature Biotech.*, **2008**, 26, 553–560.
12. Saloheimo, M., Nakari-Setälä, T., Tenkanen, M. and Penttilä, M. Eur. J. Biochem. 1997, **249**, 584–591.
13. Stålbrand, H.;Saloheimo, A.;Vehmaanperä, J.; Henrissat, B.; Penttilä,. M. *Appl. Environ. Microbiol.*, **1995**, 1090-1097.

14. Takahashi, R.; Kusakabe, I.; Kusama, S.; Sakurai, Y.; Murakami, K.; Maekawa, A.; Suzuki, T. *Agric. Biol. Chem.*, **1984**, 48, 2943-2950.

15. Tenkanen, M.; Makkonen, M.; Perttula, M.; Viikari, L.; Teleman, A. *J. Biotechnol.*, **1997**, 57, 191-204.

16. Bock, K.; Pedersen C.; Pedersen, H. *Adv. Carboh. Chem. Biochem.*, **1984,** 42, 93-225

17. Albrecht, S.; van Muiswinkel, G.C.J.; Xu, J.; Schols, H.A.; Voragen, A.G.J.; Gruppen, H. *J. Agric. Food Chem.*, **2011**, 59, 12658-12666.

18. Tailford, L., Ducros, V. M.-A.; Flint, J.E.; Roberts, S.M.; Morland, C.; Zechel, D.L.; Smith, N.; Bjornvad, M.E.; Borchert, T.V.; Wilson, K.S.; Davies, G.J.; Gilbert, H.J. *Biochemistry*, **2009**, 48, 7009-7018.

19. Bailey, M.; Siika-aho, M.; Valkeajärvi, A.; Penttilä, M. *Biotechnol. Appl. Biochem.* **1993**, 17, 65-76.

20. Chua, M.; Chan, K.; Hocking, T.J.; Williams, P.A.; Perry, C.J.; Baldwin, T.C. *Carbohydr. Polym.*, **2012**, 87, 2202-2210.

21. Macarrón, R.; Acebal, C.; Castillón, M.P.; Clyessens, M. *Biotechnol. Lett.*, **1996**, 18, 599-602.

22. Pere, J.; Siika-aho, M.; Buchert, J.; Viikari, L. *Tappi J.*, **1995**, 78, 71-78.

23. Rättö, M.; Poutanen, K. *Biotechnol. Lett.*, **1988**, 10, 661-664.

24. Bernfeld, P. Amylases, α and β. In: Colowick, S. P. and Kaplan, N. O. (eds.). Methods in enzymology, vol 1. New York, NJ, USA: Academic Press. **1955.** pp. 149-158.

25. Bruggink, C.; Maurer, R.; Herrmann, H.; Cavalli, S.; Hoefler, F. *J. Chromatogr. A*, **2005**, 1085, 104 -109.

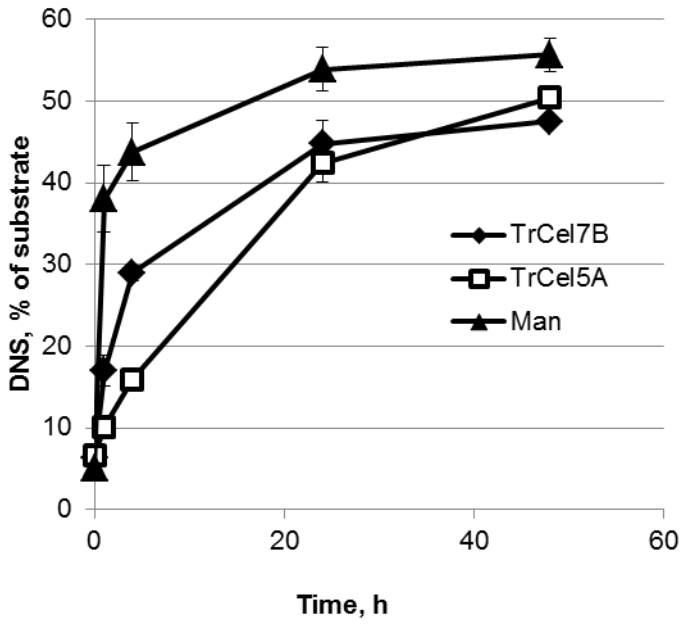

Figure 1. Enzymatic hydrolysis of konjak mannan by *Trichoderma reesei* endoglucanases (Tr Cel7B and TrCel5A) and mannanase (man) was followed by release of reducing sugars using dinitrosalicylic acid method (DNS). The data represents average and standard deviation of three parallel reducing sugar analyses.

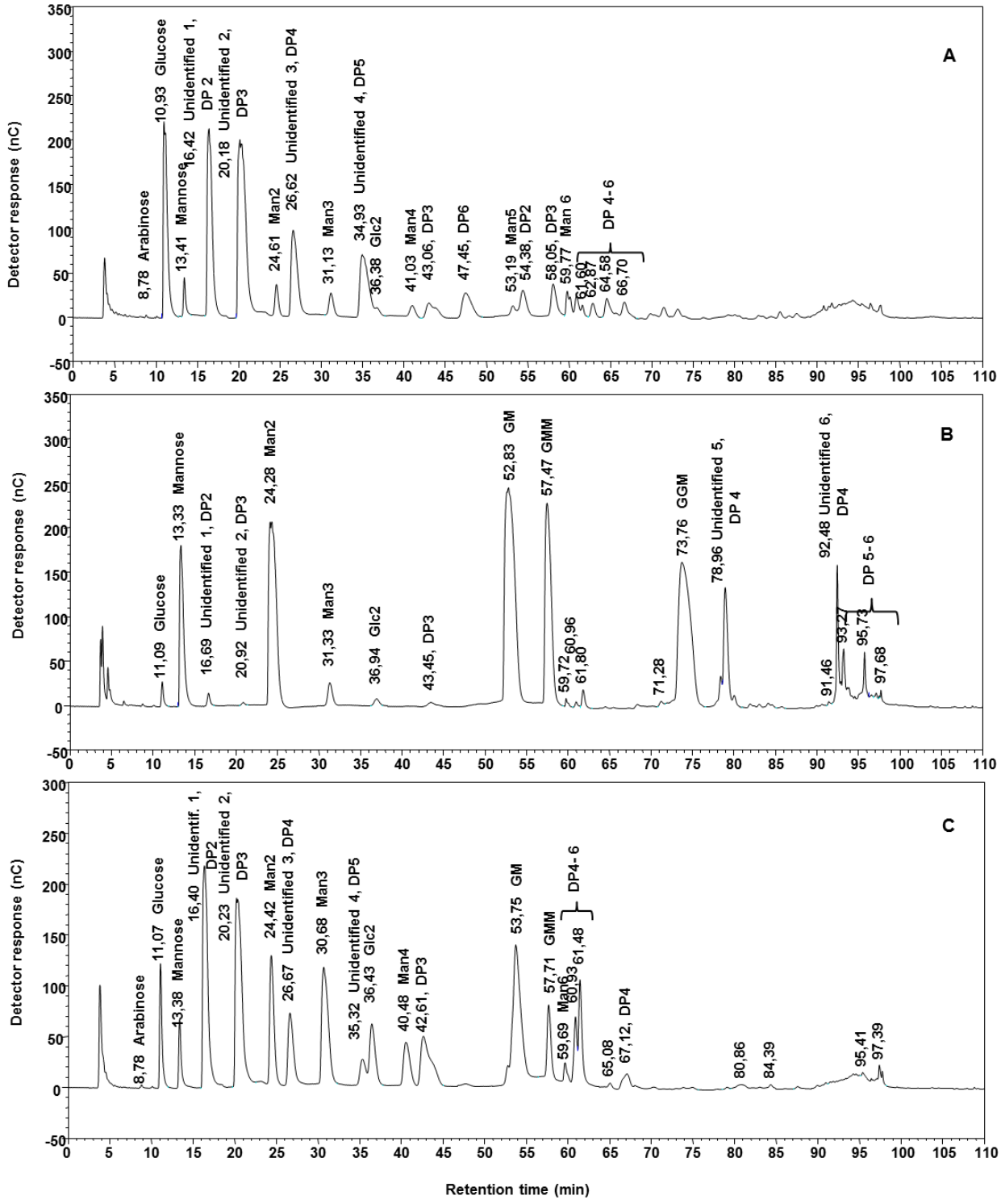

Figure 2. HPAEC –PAD chromatograms of konjac mannan hydrolysed by *Trichoderma reesei* endoglucanase Cel7B (A), mannanase (B) and endoglucanase Cel5A (C). The degree of polymerization (DP) of the unidentified peaks were determined using MSQ –detection.

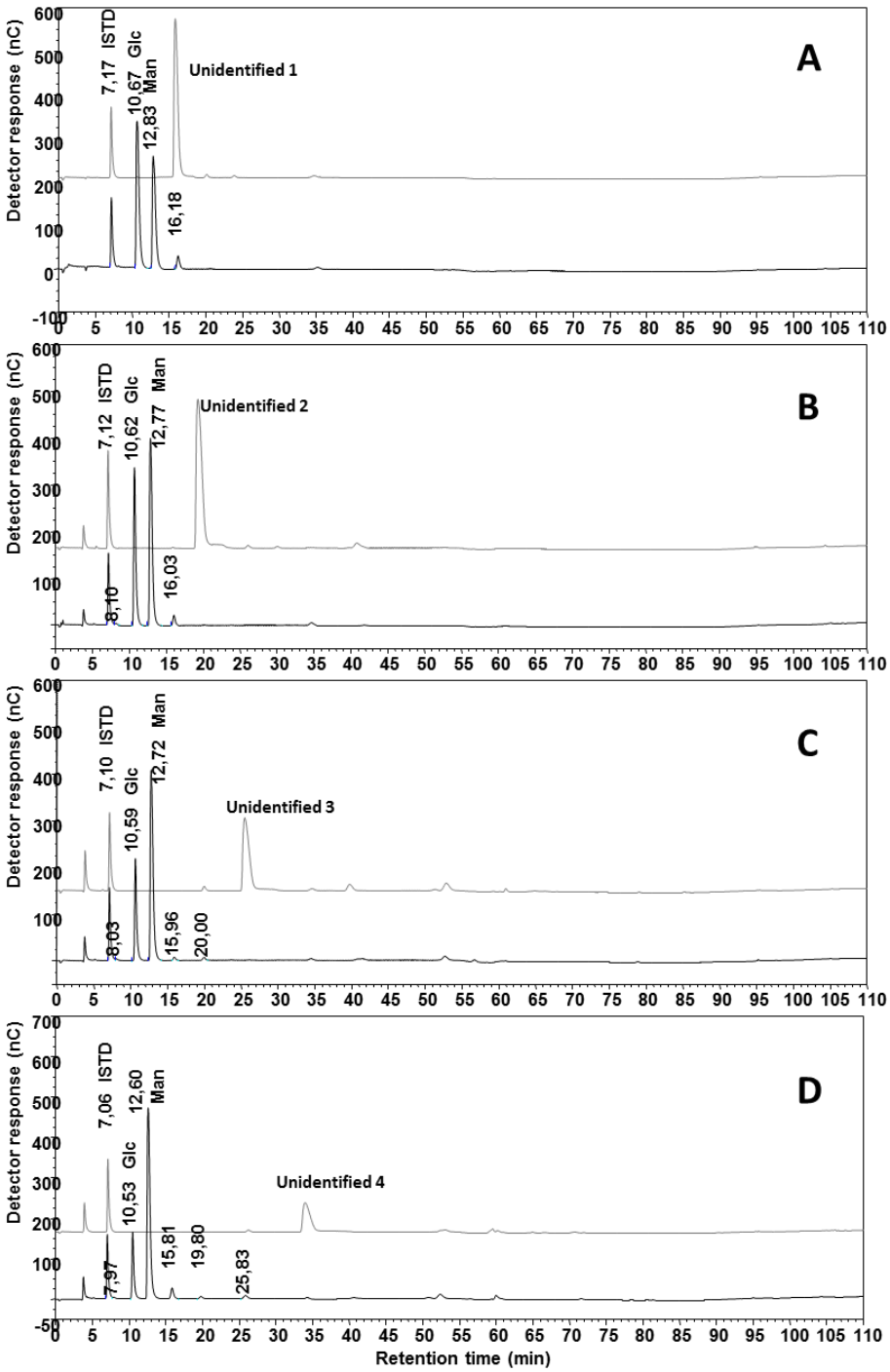

Figure 3. HPAEC-PAD chromatogram of fractions isolated by gel filtration from *Trichoderma reesei* Cel7B hydrolysate of Konjac mannan A) Unidentified oligosaccharide 1(MG), B) Unidentified oligosaccharide 2 (MMG), C) Unidentified oligosaccharide 3 (MMMG), D) Unidentified oligosaccharide 4 (MMMMG). The chromatograms on top in each panel are the fractions as such, the chromatogram below are the fractions after secondary hydrolysis with β-mannosidase from *Cellulomonas fimi*.

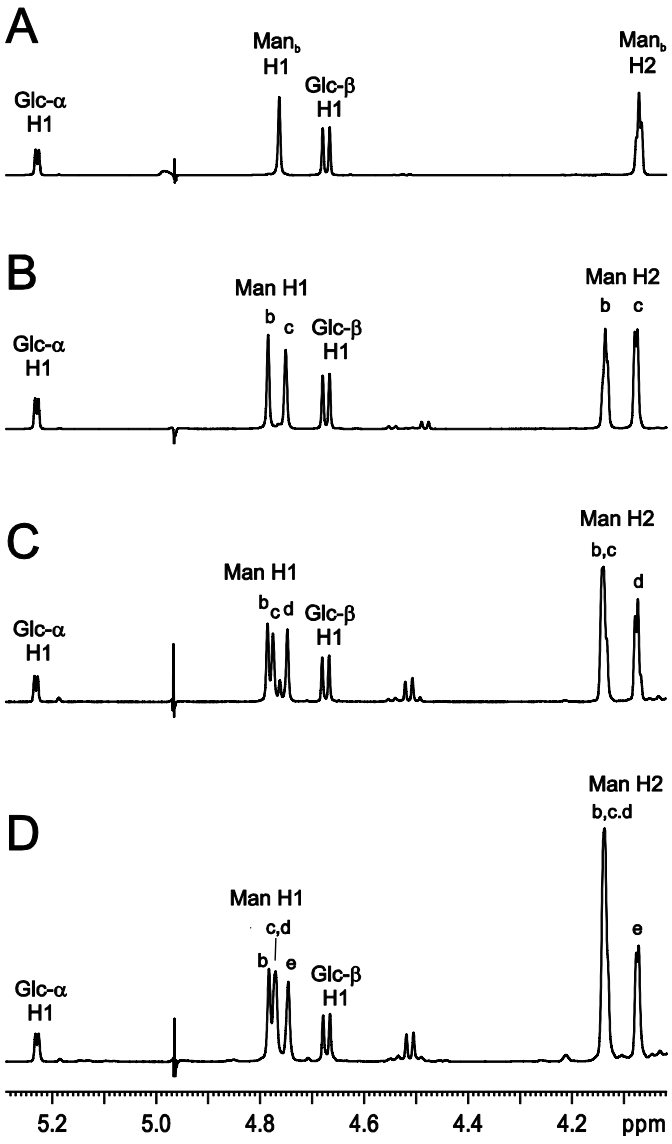

Figure 4. Structural reporter group regions of the 600 MHz ^{1}H NMR spectra of A) MG (unidentified 1), B) MMG (unidentified 2), C) MMMG (unidentified 3), D) MMMMG (unidentified 4). The most important structural reporter groups observed in these spectra are H1 of the reducing end α and β glucose (doublets at 5.23 and 4.67 ppm, respectively), H1 of the mannoses (between 4.7 and 4.8 ppm) and H2 of the mannoses (around 4.1 ppm). The letters b-e refer to the different mannose units of the saccharides, starting from the reducing end. The spectra were recorded at 8ºC in D_2O and the chemical shifts were referenced to internal TSP (0 ppm).

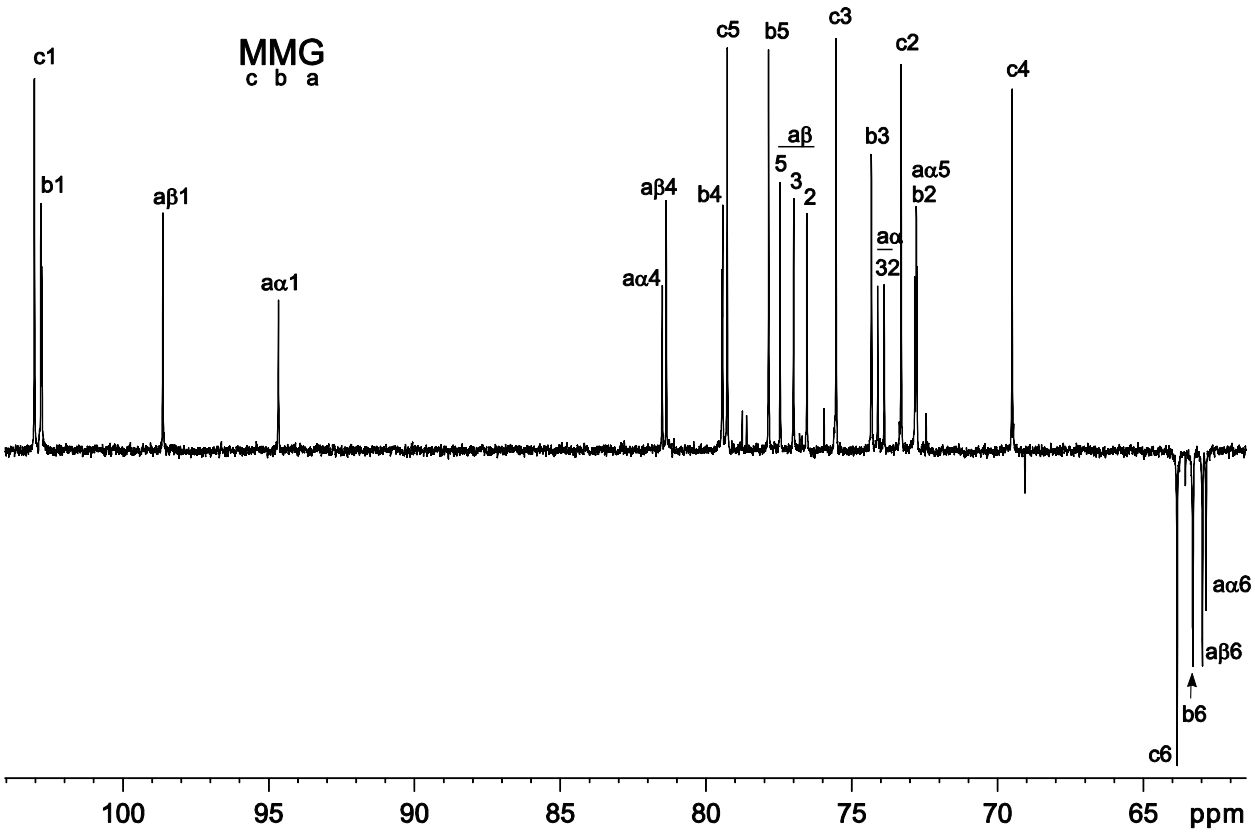

Figure 5. 150 MHz ^{13}C NMR DEPT(135) spectrum of the trisaccharide MMG at 8ºC. The clear glycosylation shifts of aα4, aβ4 and b4 ^{13}C signals compared to the non-glycosylated c4 confirm the sites of the glycosidic bonds. As illustrated, the residues are named by letters a-c, starting from the reducing end glucose. The assignments are based on 2D COSY, TOCSY and HSQC spectra and the exact chemical shifts are given in table 4 with reference to internal TSP, 0 ppm..

Table 1 Identification of oligosaccharides isolated by size exclusion chromatography from *Trichoderma reesei* Cel7B konjac mannan hydrolysate

	Proposed sequence	DP[a,b]	MSQ detection (SIM ions m/z)[b]	Man:Glc ratio (acid hydrol.)	Man:Glc ratio (enzyme hydrol.)	Yield (mg)	Yield (% of subst.)	Estimated purity (%)
Unidentified 1	MG	2	349	0.9:1	0.9:1	180	4.5	96
Unidentified 2	MMG	3	511	1.6:1	1.7:1	174	4.4	95
Unidentified 3	MMMG	4	673	2.2:1	3.1:1	238	6.0	86
Unidentified 4	MMMMG	5	421[c], 836	2.5:1	5.1:1	323	8.1	81

[a]HPLC-RI: DP was measured with Phenomenex RSO-oligosaccharide column which DP range was calibrated using linear manno- and cellooligosaccharides having degree of polymerization of 1-6.

[b]MSQ detection: SIM (single ion monitoring) mas per charge values for sugars can be detected as Li-adducts $[M + Li]^+$ at $[M + 7]^+$ in the positive mode. For oligohexoses m/z values were checked using linear manno- and cello-oligosaccharides (DP 1-6)

[c]The adduct of saccharide and two lithium ions has two overall charges, therefore the total mass has to be divided by a factor 2 in order to obtain the mass per charge ratio (m/z).

Table 2 Secondary hydrolysis products obtained from unidentified oligosaccharides 5 and 6 enriched from *T. reesei* mannanase konjac mannan hydrolysate by size exclusion chromatography into fractions 120 – 128.

	Unident. 5 (%)	Unident. 6 (%)	GMM (%)	GGM (%)	Man2 (%)	Man (%)	Glc (%)
Fraction 120-128	42	29	28	-	-	-	-
β-mannos.[a]	-	31	30	35	-	5	-
β-glucos.[b]	43	-	23	-	3	2	19

Secondary hydrolysis performed by [a]*Cellulomonas fimi* β-mannosidase and [b]*Aspergillus niger* β-glucosidase

Table 3 ^{1}H chemical shifts of the glucomannooligosaccharides produced by enzymatic hydrolysis of konjac glucomannan recorded at 600 MHz.

Compound	Residue[a]	Chemical shift[b]						
		H1	H2	H3	H4	H5	H6	H6'
MG	α-Glc_a	5.228	3.588	3.861	3.680	3.931	3.822	3.767
	β-Glc_a	4.673	3.291	3.679	3.692	3.586	3.878	3.738
	β-Man_b	4.763	4.071	3.664	3.585	3.433	3.950	3.735
MMG	α-Glc_a	5.229	3.588	3.864	3.684	3.935	3.828	3.773
	β-Glc_a	4.673	3.291	3.683	3.70	3.590	3.882	3.740
	β-Man_b	4.785	4.136	3.818	3.843	3.553	3.926	3.769
	β-Man_c	4.751	4.077	3.665	3.561	3.452	3.962	3.730
MMMG	α-Glc_a	5.229	3.585	3.866	3.684	3.934	3.828	3.770
	β-Glc_a	4.672	3.298	3.684	3.70	3.590	3.880	3.739
	β-Man_b	4.784	4.138	3.827	3.841	3.564	3.928	3.768
	β-Man_c	4.774						
	β-Man	4.746	4.074	3.664	3.566	3.446	3.960	3.731
MMMMG	α-Glc_a	5.229	3.587	3.865	3.684	3.936	3.828	3.771
	β-Glc_a	4.672	3.291	3.684	3.70	3.591	3.882	3.740
	β-Man_b	4.783	4.138	3.826	3.843	3.565	3.930	3.765
	β-Man_{c-d}	4.770						
	β-Man_e	4.757	4.074	3.666	3.564	3.451	3.963	3.731

[a] The residues are named by letters a-e, starting from the reducing end glucose.
[b]In ppm relative to internal TSP at 0 ppm (D_2O, 8ºC)

Table 4. ^{13}C chemical shifts of the glucomannooligosaccharides produced by enzymatic hydrolysis of konjac glucomannan recorded at 600 MHz.

Compound	Residue[a]	Chemical shift[b]					
		C1	C2	C3	C4	C5	C6
Man-Glc	α-Glc_a	94.64	73.90	74.14	81.51	72.77	62.90
	β-Glc_a	98.58	76.52	77.06	81.45	77.46	63.02
	β-Man_b	102.85	73.40	75.61	69.45	79.29	63.75
Man_2-Glc	α-Glc_a	94.65	73.88	74.10	81.47	72.77	62.85
	β-Glc_a	98.63	76.56	77.00	81.36	77.53	63.00
	β-Man_b	102.80	72.78	74.34	79.45	77.88	63.32
	β-Man_c	103.05	73.31	75.58	69.49	79.28	63.85
Man_3-Glc	α-Glc_a	94.64	73.92	74.09	81.48	72.75	62.85
	β-Glc_a	98.62	76.58	77.00	81.35	77.57	62.95
	β-$Man_{b,c}$	102.87	72.76	74.31	79.40	77.87	63.27
	β-Man_d	103.00	73.30	75.57	69.47	79.28	63.82
Man_4-Glc	α-Glc_a	94.65	73.89	74.09	81.43	72.77	62.85
	β-Glc_a	98.61	76.54	77.00	81.32	77.5	62.94
	β-Man_{b-d}	102.84	72.78	74.32	79.35	77.85	63.26
	β-Man_e	103.00	73.30	75.57	69.47	79.28	63.83

[a] The residues are named by letters a-e, starting from the reducing end glucose.
[b] In ppm relative to internal TSP at 0 ppm (D_2O, 8ºC)

Table 5 Share of reducing and non-reducing end gluco- and mannopyranosyl units in the konjac mannan hydrolysates of *T.reesei* Cel7B, Cel5A and mannanase

	Tr Cel7B hydrolysate (%)[a]	*Tr* Cel5A hydrolysate (%)[a]	*Tr* mannanase hydrolysate (%)[a]
Reducing end Glc	83.3	49.8	1.0
Reducing end Man	16.7	50.2	99.0
Non-reducing end Glc	12.5	29.1	71.2
Non-reducing end Man	87.5	70.9	28.8
Sum of identified hydrolysis product[b] (%)	67.4	75.9	100.9

[a]Percentage of identified hydrolysis products

[b]Sum of identified hydrolysis products was calculated using anhydrocorrection