A comparative study of two retaining enzymes of *Trichoderma reesei*: transglycosylation of oligosaccharides catalysed by the cellobiohydrolase I, Cel7A, and the β-mannanase, Man5A

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Abstract HPLC, MALDI-TOF MS and NMR spectroscopy were used to investigate the hydrolysis of cello- and manno-oligosaccharides by Cel7A and Man5A from *Trichoderma reesei*. The experimental progress curves were analysed by fitting the numerically integrated kinetic equations, which provided cleavage patterns for oligosaccharides. This data evaluation procedure accounts for product inhibition and avoids the initial slope approximation. In addition, a transglycosylation step had to be included in the model to reproduce the experimental progress curves. For the hydrolysis of manno-oligosaccharides, Man_{4-6}, by Man5A no mannose was detected at the beginning of the reaction showing that only the internal linkages are hydrolysed. For cellotriose and cellotetraose hydrolysis by Cel7A, the main product is cellobiose and glucose is released from the non-reducing end of the substrate. Intermediary products longer than the substrates were detected by MALDI-TOF MS when oligosaccharides (Glc_{4-6} or Man_{4-6}) were hydrolysed by either Cel7A or Man5A. Interestingly, two distinct transglycosylation pathways could be observed. Cel7A produced intermediates that are one unit longer than the substrate, whereas Man5A produced intermediates that are two units longer than the substrate.

Key words: Cellulase; Mannanase; Oligosaccharide; Transglycosylation; *Trichoderma reesei*

1. Introduction

Cellulose and hemicelluloses are the major structural components of plant cell walls. Cellulose is an inert and insoluble polysaccharide composed of long, linear chains of β-1,4-linked glucose units whereas hemicelluloses form a heterogeneous family of both linear and branched polysaccharides. Xylans and mannans are the main hemicelluloses in plants containing a backbone of β-1,4-linked xylose and mannose units, respectively, and having different side groups depending on the origin. In nature a set of cellulolytic and hemicellulolytic enzymes, i.e. cellulases and hemicellulases, each with a slightly different role, are needed in the total hydrolysis of these natural substrates [1,2]. The mechanism of enzymatic degradation of cellulose and hemicelluloses has attracted considerable interest in recent years because of its evident ecological and industrial importance. A fundamental understanding of the mode of action of enzymes involved is required in order to optimise their role in commercial applications.

The filamentous fungus *Trichoderma reesei*, with one of the best characterised enzyme systems, produces a wide variety of different cellulolytic and hemicellulolytic activities, e.g. two cellobiohydrolases (Cel), five endoglucanases (Cel), two major xylanases (Xyn) and a β-mannanase (Man) [3–7]. Most *Trichoderma* cellulases and some hemicellulases are two-domain proteins composed of a catalytic domain connected via a linker peptide to a small cellulose-binding domain (CBD) [8]. The fungal CBDs share high sequence identity whereas the catalytic domains usually belong to different sequence families having different folds. Despite different folds there is limited variation in the active site topologies of all glycosyl hydrolases [9]. Pocket-like active sites are encountered in true exoenzymes, such as β-glucosidases which cleave monosaccharide units from chain ends. Endoenzymes have active sites situated in clefts on enzyme surfaces allowing the cleavage to happen in the middle of the chain. Endoglucanases, xylanases and β-mannanases belong to this type of enzymes. Cellobiohydrolases belong to the third class of enzymes which have a basically similar active site as homologous endoglucanases but the loop structures covering the active site are longer and thus the active site is situated in a closed tunnel rather than in an open cleft. It is common in all three classes of enzymes that the active site contains several binding subsites for the substrate. The numbering of the subsites is indicated in such a way that the point of cleavage is located between the subsites −1 and +1, with increasing numbering (+1, +2, ..., +*n*) towards the reducing end and decreasing (−1, −2, ..., −*n*) towards the non-reducing end of the carbohydrate chain.

All above-mentioned cellulases and hemicellulases hydrolyse β-1,4-glycosidic linkages within the main chain of the substrate either by inversion or by retention of the anomeric configuration. The inverting enzymes utilise a single-displacement mechanism where an attacking nucleophilic water molecule inverts the configuration at the anomeric centre during hydrolysis. The retaining enzymes instead utilise a double-displacement mechanism, containing a covalent glycosyl-enzyme intermediate (at subsite −1) where the nucleophile in the second step can be either a water molecule or another carbohydrate molecule [10,11]. The latter reaction is known as transglycosylation and it has been reported for many retaining

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Abbreviations: HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; NMR, nuclear magnetic resonance; Cel, endoglucanase/cellobiohydrolase/cellulase; Man, β-mannanase; Xyn, xylanase; Glc_1–Glc_6, glucose–cellohexaose; Man_1–Man_6, mannose–mannohexaose

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glycosyl hydrolases [12–16]. Thus these enzymes possess a transfer activity in addition to the hydrolytic activity and this behaviour can be very useful in the production of different carbohydrates which are difficult to synthesise by conventional methods.

In the present work the hydrolysis of cello-oligosaccharides (Glc_{3-6}) catalysed by *T. reesei* cellobiohydrolase Cel7A and the hydrolysis of manno-oligosaccharides (Man_{3-6}) catalysed by *T. reesei* β-mannanase Man5A has been studied by means of HPLC, proton NMR spectroscopy and MALDI-TOF MS in order to determine the degradation pattern and transglycosylation products of the hydrolysis of pure soluble oligosaccharides.

2. Materials and methods

2.1. Enzymes and substrates

β-Mannanase was produced in a *T. reesei* strain lacking the *cel5A* and *cel7A* genes. The concentrated culture filtrate kindly provided by Primalco Oy (2.7 l, containing 0.28 kat mannanase activity) was clarified and some of the impurities removed by bentonite treatment at pH 4.0 [17]. The buffer exchange was carried out by Sephadex G-25 c and the enzyme preparation was then applied to an DEAE Sepharose FF column (Pharmacia, total volume 6 l and bed height 12 cm) equilibrated with 0.008 M sodium phosphate, pH 7.2. The column was eluted with equilibration buffer with stepwise additions of NaCl up to 1 M concentration to remove the adsorbed material. The mannanase activity-containing fractions which eluted by equilibrating buffer were pooled (20.2 l) and 5 l of the pool was subjected to further purification by hydrophobic interaction chromatography. Phenyl-Sepharose FF (Pharmacia, total volume 5 l and bed height 50 cm) was equilibrated with 0.020 M sodium phosphate, pH 6.0, containing 0.4 M $(NH_4)_2SO_4$. The sample was applied to the column and first eluted with 0.020 M sodium phosphate buffer, pH 6.0, containing 0.1 M $(NH_4)_2SO_4$ (7.3 l), then with 0.015 M sodium phosphate buffer, pH 6.0, and a decreasing gradient (total volume 8 l) from 15 mM to 0.2 mM sodium phosphate at pH 6.0. The elution was continued by 0.2 mM phosphate (11.2 l), and after that the remaining adsorbed material was eluted from the column by distilled water and 6 M urea. The pool containing intact mannanase (catalytic domain+CBD) protein (10.86 l) was eluted during the end of the gradient and by 0.2 mM phosphate buffer. The third purification step was CM Sepharose FF (Pharmacia, total volume 2 l and bed height 20 cm) equilibrated with 0.010 M sodium acetate, pH 4.0. The sample (pH and conductivity adjusted) was applied to the column and eluted with an increasing gradient from 0.010 M acetate at pH 4.0 to 0.02 M sodium acetate at pH 5.4. The elution was continued by 0.02 M sodium acetate, pH 5.4 (11.2 l). The remaining adsorbed material was eluted from the column by 0.02 M sodium acetate, pH 5.4 containing 1 M NaCl. The fractions containing the two major isoenzymes of Man5A (with p*I* 4.8 and p*I* 5.4) were eluted at the end of the gradient and by 0.02 M acetate, pH 5.4. The collected pool contained totally 0.0224 kat mannanase activity and 13.1 g protein. The purity and the isoforms present in the fractions were analysed by isoelectric focusing in gel (Pharmacia PhastSystem, IEF 3–9, according to the manufacturer's instructions). The fractions containing the pure isoform with p*I* 5.4 were pooled and the pool was used in this study. During the purification, protein was assayed by the method of Lowry et al. [18] using bovine serum albumin (Sigma) as the standard and mannanase activity according to Ståhlbrand et al. [5].

Cel7A was purified either from *T. reesei* Rut C-30 strain or from a *T. reesei* strain lacking both major endoglucanase genes (*cel5A* and *cel7B*). The purification was performed basically as described earlier using affinity chromatography as the last purification step [19]. As a third production host, a *T. reesei* strain lacking genes for *cel5A* and *cel6A* was used. The purification of Cel7A was then performed according to the method described by Rahkamo et al. [20]. Additional steps of anion exchange chromatography and gel filtration were performed in order to remove the remaining minor impurities in the preparation. The sample was applied to the column (98 ml) of DEAE Sepharose FF equilibrated by 0.025 M sodium acetate, pH 5.0. The column was eluted by a gradient from equilibration buffer to 0.025 M sodium acetate, pH 4.0, containing 0.25 M NaCl. The elution was completed by the equilibration buffer containing 1 M NaCl. The fractions containing the highest protein concentration were pooled and part of the pool (10 ml) was applied to a Sephacryl S-100 HR column (total volume 1670 ml, bed height 85 cm), equilibrated with 0.050 M sodium acetate, pH 5.0, containing 0.1 M NaCl. The elution was carried out with the same buffer at a flow rate of 1.5 ml/min. The fractions containing the highest protein concentration were pooled and used as the Cel7A preparation in this study. In all cases the purified protein was run on SDS-PAGE and the contaminating activities on small chromophoric substrates and on hydroxyethylcellulose (HEC) were also determined as described earlier [21]. Protein concentration for each Cel7A preparation was measured by ultraviolet absorption at 280 nm using epsilon value $\varepsilon^{280} = 83\,000\ M^{-1}\ cm^{-1}$ measured with total amino acid analysis (A. Koivula, unpublished results).

Glucose (Glc_1) and cello-oligosaccharides (Glc_{2-6}) were purchased from Merck (Darmstadt, Germany) and Seikagaku (Japan); mannose (Man_1) and manno-oligosaccharides (Man_2-Man_6) from Megazyme (Australia); deuterium oxide (D_2O, 99.8% isotopic purity), deuterated acetic acid (CD_3CO_2D, 99.8% isotopic purity) and 2,5-dihydroxybenzoic acid (DHB) from Aldrich, and Dextran 1000 from Fluka AG (Switzerland).

2.2. HPLC experiments

The hydrolysis experiments on cello-oligosaccharides were carried out in a 10 mM sodium acetate buffer at pH 5.0 and 27°C and on manno-oligosaccharides in a 10 mM sodium acetate buffer at pH 4.5 and 50°C. The substrate and enzyme concentrations varied between 3×10^{-5} and 1×10^{-3}, and 3.5×10^{-8} and 1.6×10^{-6} M, respectively. In all experiments samples were taken at different time points, the enzymatic reaction was stopped and the oligosaccharide composition analysed by HPLC (Waters, USA) as described earlier [22].

2.3. NMR experiments

For the proton NMR experiments, the buffer exchange from H_2O to D_2O for Cel7A and Man5A was performed by repeated concentration by ultrafiltration (Ultrafree-MC, cut-off 10 kDa, Millipore) and dilution in 10 mM acetate buffer of pH 5.0 and 4.5 in D_2O, respectively. The substrates were then dissolved in the same buffers and the enzymatic reactions were carried out directly in the NMR tube at 27°C for Cel7A and at 50°C for Man5A. The substrate and enzyme concentrations used varied between 1×10^{-4} and 3×10^{-3}, and 1.5×10^{-8} and 8.5×10^{-6} M, respectively. Proton NMR spectra were recorded at 599.86 MHz using a Varian UNITY 600 MHz spectrometer. Spectral acquisition and NMR resonance assignments were performed as described earlier by Harjunpää et al. [16,22].

2.4. MS experiments

The hydrolysis experiments on cello-oligosaccharides were carried out in 10 mM sodium acetate buffer at pH 5.0 and 27°C and on manno-oligosaccharides in 10 mM sodium acetate buffer at pH 4.5 and 50°C. The substrate and enzyme concentrations used varied between 1×10^{-4} and 1×10^{-3}, and 1×10^{-7} and 1.6×10^{-6} M, respectively. In all experiments samples were taken at different time points, the enzyme reaction was stopped and the oligosaccharide composition was analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS).

MALDI-TOF MS was performed with a BIFLEX mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany), using a 337 nm nitrogen laser. 0.5–1 μl of sample (5–20 pmol) and 2 μl of 2,5-dihydroxybenzoic acid matrix (DHB, 10 mg/ml in water) were mixed on the target plate and dried with a gentle stream of air. Dextran 1000 was used as an external calibrant.

2.5. Data evaluation

The full kinetic equations were solved by direct forward integration with a time step of 0.1–0.5 s to produce complete progress curves [22]. Since the binding equilibria are not necessarily fast compared to the hydrolysis rates, the binding step had also to be included in the reaction model. The calculated progress curves were fitted to the experimental data by varying the involved kinetic and equilibrium constants. The goodness of fit was judged by the error square sum as described earlier [16,22].

3. Results and discussion

3.1. Cleavage pattern of cello- and manno-oligosaccharides

A series of hydrolysis experiments were carried out using either cello-oligosaccharides (Glc_{3-6}) or manno-oligosaccharides (Man_{4-6}) to determine the cleavage patterns. From the NMR data of cellotriose hydrolysis by Cel7A (see Fig. 1), it can be deduced that the cleavage takes place almost exclusively (>90%) at the first glycosidic linkage. This cleavage produces cellobiose from the reducing end in an anomeric equilibrium and β-glucose from the non-reducing end. For cellotetraose the major cleavage (75%) takes place at the second glycosidic linkage producing two molecules of cellobiose. In addition, a minor cleavage (25%) at the first glycosidic linkage results in a cellotriose from the reducing end, in an anomeric equilibrium, and β-glucose (data not shown). Cleavage patterns of cello-oligosaccharide hydrolysis by Cel7A are shown in Fig. 2.

For the longer cello-oligosaccharides, i.e. cellopentaose and cellohexaose, the main hydrolysis products were Glc_1, Glc_2 and Glc_3 and only trace amounts of Glc_4 and Glc_5 (<1%) could be detected. We have previously shown for *T. reesei* cellobiohydrolase Cel6A, using NMR, that the degradation pattern of cellopentaose can be obtained from the progress curve of the initially formed cellotriose [22]. When we repeated this experiment with *T. reesei* Cel7A, we found that at the high enzyme concentrations compulsory for this experiment, some of the proton resonances of cellotriose were selectively broadened, making the resolution of the internal anomeric proton resonances from α- and β-cellotrioses impossible. Thus we were unable to determine whether the second or third glycosidic linkage in cellopentaose is the preferred one for hydrolysis. However, the observed broadening, some 3 Hz, shows that the off-rate of Glc_3 from Cel7A is in the order of 10^3 s^{-1}. Whether this is the real off-rate from the active site is currently not clear. It may as well be the exchange from an external site or from the entrance of the active site tunnel.

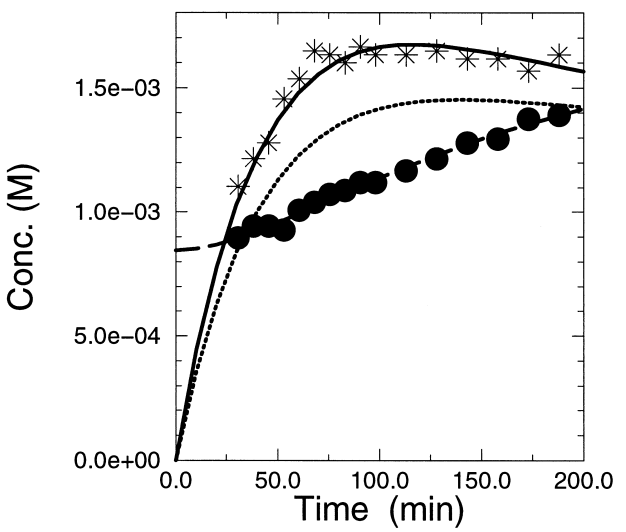

Fig. 1. Cellotriose (2 mM) hydrolysis by Cel7A (36 μM). The changes in the concentrations of α-anomeric (●) and β-anomeric (+) protons of glucose are shown as determined by ^1H NMR spectroscopy. The solid and dotted lines show the calculated concentrations of β-glucose assuming (—) cleavage exclusively at the non-reducing end or (···) random cleavage. The dashed line shows the calculated concentrations of α-anomeric protons.

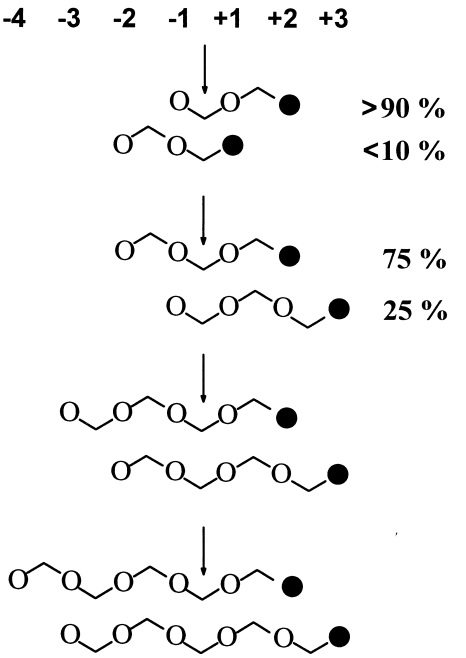

Fig. 2. Cel7A-catalysed cleavage pattern of cello-oligosaccharides (Glc_{3-6}) showing the possible orientations of different oligosaccharide in the active site tunnel (only subsites −4↔+3 are shown). Arrows indicate the point of cleavage between subsites −1 and +1. The up and down orientations of the glycosidic linkage are denoted by ∧ and ∨, respectively; ○, glucose unit; ●, reducing end.

The facts that in the hydrolysis of cellohexaose by Cel7A, essentially no Glc_4 is produced and that the formed glucose is almost exclusively in its β-anomeric form, strongly indicate that the reducing end of the substrate enters to the active site tunnel from the −7 site end, as suggested from the crystal structure by Divne et al. [23]. Assuming that the substrate enters the active site from the + end makes it difficult to explain why there is no formation of cellotetraose since Glc_4 should be formed as a primary product of the enzymatic reaction and it has to leave the active site tunnel before the reaction can proceed any further. The lack of Glc_4 also shows that after the release from the covalent enzyme intermediate, Glc_4-E, it is quickly hydrolysed to two molecules of cellobiose before leaving the active site. This implies that the hydrolysis of the formed Glc_4 is faster than the off-rate of this substrate and it is more favourable for this substrate to move forward from subsites −4↔−1 to −2↔+2 to be cleaved a second time than to back out from the tunnel. This behaviour is often referred to as processivity and our results are consistent with results of Nidetsky et al. [24] showing that only Glc_1, Glc_2, and Glc_3 are released during the hydrolysis of longer cello-oligosaccharides Glc_{4-8} by Cel7A.

The cleavage patterns reported here agree with others in the literature obtained using tritium-labelled, 4-methylumbelliferyl-substituted or free cello-oligosaccharides [13,25] in the sense that the central glycosidic linkages are preferred over the terminal ones. On the other hand, the present results regarding the preference for the non-reducing end glycosidic linkage, as shown in Fig. 2, do not agree with previous reports. We have no explanation for this discrepancy unless the previously used

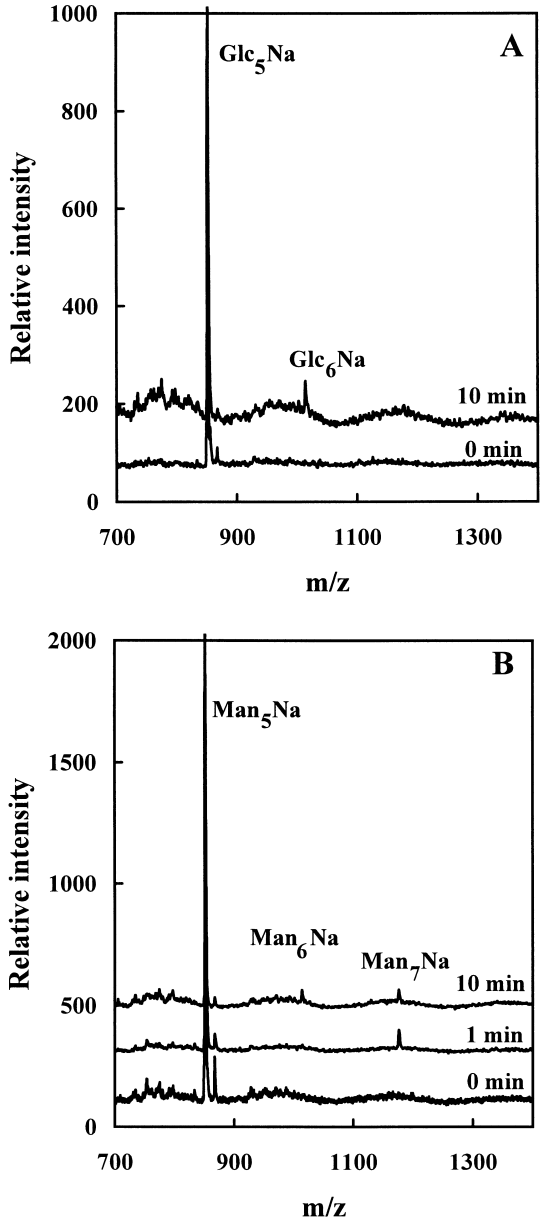

Fig. 3. A: Mass spectra for cellopentaose (1 mM) hydrolysis catalysed by Cel7A (5 μM). The lower spectrum represents the mannopentaose solution before enzyme addition (0 min) and the upper one 10 min after the addition. B: Mass spectra for mannopentaose-catalysed (1 mM) hydrolysis by Man5A (0.035 μM) before enzyme addition (bottom), 1 (middle) and 10 (top) min after the addition. Cello- and manno-oligosaccharides are detected as corresponding sodium adducts (M+23).

enzymes were contaminated with minor amounts of an efficient endoglucanase in a similar manner as has been demonstrated for *T. reesei* Cel6A [26].

We have reported earlier for *T. reesei* Man5A that there is no strong preference for either of the two mannosidic linkages in the hydrolysis of mannotriose [16]. These results were obtained using a simple model for the hydrolysis of mannotriose. Here, during the hydrolysis of mannotetraose, mannopentaose and mannohexaose by Man5A no mannose was detected in the initial phase of the hydrolysis and thus no terminal linkages are cleaved. Thus, mannotetraose is cleaved in the middle and mannopentaose at the second (30%) and third (70%) mannosidic linkages as reported earlier by Harjunpää et al. [16]. Even if no mannose is formed initially in the hydrolysis of mannotetraose, an appreciable amount of mannotriose is formed as a result of transglycosylation. Contrary to the hydrolysis of Glc_6 by Cel7A where essentially no Glc_4 could be detected, for the hydrolysis of Man_6 by Man5A an appreciable amount of Man_4 was detected. This also indicates that Man5A is a non-processive endoenzyme. The cleavage patterns of Man5A agree well with the results reported for the β-mannanases isolated from *Pseudomonas fluorescens* ssp. *cellulosa*, *Aspergillus niger* and *Streptomyces* [27–29]

3.2. Transglycosylation of cello- and manno-oligosaccharides

A number of cello-oligosaccharide and manno-oligosaccharide hydrolysis experiments were performed and analysed by MALDI-TOF MS to determine the possible transglycosylation product(s). For cellotriose hydrolysis catalysed by *T. reesei* Cel7A no longer cello-oligosaccharides than the substrate itself were detected, whereas for cellotetraose, cellopentaose, and cellohexaose a (n+1) cello-oligosaccharide was detected (see Fig. 3A), where n denotes the number of glucose units in the original substrate. For mannotriose hydrolysis catalysed by *T. reesei* Man5A no longer manno-oligosaccharides were detected. For mannotetraose, mannopentaose and mannohexaose, we initially detected (n+2) manno-oligosaccharides and somewhat later also (n+1) manno-oligosaccharides as shown in Fig. 3B. This kind of transglycosylation product composition has earlier been reported in mannopentaose and mannopentaitol hydrolyses catalysed by β-mannanase from *A. niger* [12].

Even though no transglycosylation products could be detected for mannotriose by MALDI-TOF MS analysis, NMR data show that the transglycosylation must be involved. The increase in the intensity of the terminal anomeric proton resonance (having contributions from both Man_2 and Man_3) cannot be explained without transglycosylation because a simple degradation of mannotriose into Man_2 and Man_1 would result in a constant sum of Man_2 and Man_3. The hydrolysis of the longer manno-oligosaccharides, Man_{4-6}, is, however, much faster (>100-fold) than the hydrolysis of Man_3 and therefore these transglycosylation products are never accumulated in measurable amounts.

The NMR progress curves obtained from the hydrolysis of mannotriose by Man5A can be accurately fitted in two different ways. First, the primary hydrolysis of mannotriose produces Man_2 and Man_1-E and the covalent mannosyl-enzyme intermediate is transglycosylated into Man_4 which is then rapidly hydrolysed to two molecules of mannobiose. Second, the primary hydrolysis of mannotriose produces Man_1 and Man_2-E and the covalent mannobiosyl-enzyme intermediate is instead transglycosylated into Man_5. The mannopentaose formed can be hydrolysed in two different ways, either into Man_3 and Man_2-E that is the non-detectable reverse reaction of transglycosylation, or into Man_2 and Man_3-E and the mannotriosyl-enzyme intermediate can be transglycosylated into Man_6. Mannohexaose can be hydrolysed in three different ways producing Man_2+Man_4, Man_3+Man_3, and Man_4+Man_2. The Man_3+Man_3 reaction is just the reverse reaction of transglycosylation and therefore cannot be detected whereas the two other reactions will ultimately result in the formation of three molecules of mannobiose. We have previously

shown that both mannosidic linkages in mannotriose are cleaved almost equally fast by Man5A showing only a slight preference for the second mannosidic linkage [16]. This indicates that the full degradation mechanism of mannotriose by Man5A has to include both of the above discussed transglycosylation pathways. The relative weight of these two pathways strongly depends on the hydrolysis rates of Man_1-E and Man_2-E as well as on the two initial transglycosylation rates. We may argue that the fact that for longer substrates only the (n+2) transglycosylation product is observed initially would indicate that also for mannotriose this should be the major product. Therefore we have to consider that only the central glycosidic linkages are hydrolysed to a detectable degree in the longer substrates and no (n+1) products can be formed and both transglycosylation pathways remain for the mannotriose case.

It is interesting to compare the transglycosylation of the two enzymes of *T. reesei* when using substrates longer than trisaccharide. For Man5A only (n+2) products are observed initially because no Man_1-E intermediate is formed. For Cel7A on the other hand only (n+1) products are observed even though e.g. in cellotetraose hydrolysis, the formation of Glc_2-E is faster than for Glc_1-E. One plausible explanation to this could be that the occupation of binding site -2 may cause a minor conformational change along the tunnel making the entrance of a transglycosylating substrate unlikely. Such an allosteric effect would be advantageous since an efficient transglycosylation would of course slow down the cellulose degradation.

4. Conclusions

Cel7A and Man5A of *T. reesei* are retaining enzymes and thus able to exhibit transglycosylation. For Man5A an efficient transglycosylation was seen in the hydrolysis of mannotriose even though no longer manno-oligosaccharides could be detected. On the other hand, for cellotriose hydrolysis by Cel7A the transglycosylation was too slow to be detected, if it existed at all. For the longer substrates, Glc_{4-6}, and Man_{4-6}, both enzymes exhibit transglycosylation and longer intermediates could be detected. However, there is an interesting difference between the two enzymes, namely Cel7A produces an intermediate that is one unit longer than the original substrate, whereas the initially formed intermediate by Man5A is two units longer than the original substrate. This is in part a reflection of the different degradation patterns of these two enzymes since in the case of Man5A a Man_1-E intermediate is never formed with substrates longer than trisaccharide. The lack of (n+2) products for Cel7A may be a trait developed to retain an efficient cellulose degradation. On the other hand, the very efficient transglycosylation of Man5A makes this enzyme an interesting candidate for synthesis of longer oligosaccharides using solvents with lower water activities.

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