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Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*

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Abstract

Trichoderma reesei produces five known endoglucanases. The most studied are Cel7B (EG I) and Cel5A (EG II) which are the most abundant of the endoglucanases. We have performed a characterisation of the enzymatic properties of the less well-studied endoglucanases Cel12A (EG III), Cel45A (EG V) and the catalytic core of Cel45A. For comparison, Cel5A and Cel7B were included in the study. Adsorption studies on microcrystalline cellulose (Avicel) and phosphoric acid swollen cellulose (PASC) showed that Cel5A, Cel7B, Cel45A and Cel45Acore adsorbed to these substrates. In contrast, Cel12A adsorbed weakly to both Avicel and PASC. The products formed on Avicel, PASC and carboxymethylcellulose (CMC) were analysed. Cel7B produced glucose and cellobiose from all substrates. Cel5A and Cel12A also produced cellotriose, in addition to glucose and cellobiose, on the substrates. Cel45A showed a clearly different product pattern by having cellotetraose as the main product, with practically no glucose and cellobiose formation. The kinetic constants were determined on cellotriose, cellotetraose and cellopentaose for the enzymes. Cel12A did not hydrolyse cellotriose. The k_{Cat} values for Cel12A on cellotetraose and cellopentaose were significantly lower compared with Cel5A and Cel7B. Cel7B was the only endoglucanase which rapidly hydrolysed cellotriose. Cel45Acore did not show activity on any of the three studied cello-oligosaccharides. The four endoglucanases' capacity to hydrolyse β-glucan and glucomannan were studied. Cel12A hydrolysed β-glucan and glucomannan slightly less compared with Cel5A and Cel7B. Cel45A was able to hydrolyse glucomannan significantly more compared with β-glucan. The capability of Cel45A to hydrolyse glucomannan was higher than that observed for Cel12A, Cel5A and Cel7B. The results indicate that Cel45A is a glucomannanase rather than a strict endoglucanase. © 2002 Published by Elsevier Science B.V.

Keywords: Endoglucanase; *Trichoderma reesei*; Enzymatic hydrolysis; Cellulases

1. Introduction

Cellulose is one of the most abundant biopolymers on earth. It has been estimated that 7.2×10^{11} tonnes of cellulose is stored in plants and

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that the annual production of cellulose is 4×10^{10} tonnes (Coughlan, 1985). Cellulose degradation is thus a very important process. One putative industrial use is to convert cellulose to glucose and then ferment the glucose to ethanol (Duff and Murray, 1996). The ethanol can then be utilised as fuel for vehicles as a substitute for fossil fuels.

Enzymes which hydrolyse cellulose are produced by several bacteria and fungi (Béguin and Aubert, 1994). One of the most studied fungal cellulase systems is produced by *Trichoderma reesei*. The fungus produces a complete set of cellulases which can hydrolyse cellulose to soluble sugars. The cellulases are repressed when glucose is present and expressed when the organism starves or when certain inducers are present (Ilmén, 1997). *T. reesei* expresses at least two cellobiohydrolases, Cel6A (CBH II) (Teeri et al., 1987) and Cel7A (CBH I) (Shoemaker et al., 1983a; Teeri et al., 1983), (EC 3.2.1.91) and five endoglucanases, Cel5A (EG II) (Saloheimo et al., 1988), Cel7B (EG I) (Penttilä et al., 1986), Cel12A (EG III) (Ward et al., 1993; Okada et al., 1998), Cel45A (EG V) (Saloheimo et al., 1994) and Cel61A (EG IV) (Saloheimo et al., 1997) (EC 3.2.1.4). Here, we have adopted the nomenclature for classification of the catalytic domains into families based on sequences (Henrissat et al., 1998). All *T. reesei* cellulases, except Cel12A, are modular enzymes and consist of a cellulose binding module (CBM) and a catalytic core domain. Cel12A consists only of a catalytic core. The 3D-structures for the catalytic core domain of Cel6A (Rouvinen et al., 1990), Cel7A (Divne et al., 1994), Cel7B (Kleywegt et al., 1997) and the cellulose binding domain of Cel7A (Kraulis et al., 1989) and Cel7B (Mattinen et al., 1998) are solved. Recently, the 3D structure of Cel12A was determined (Sandgren et al., 2001). For the cellobiohydrolases Cel6A and Cel7A, the active site is located in a tunnel whereas the endoglucanases, Cel7B and Cel12A, has the active site in an open cleft.

The aim of this study was to perform a comparative characterisation of the enzymatic properties of the less well studied endoglucanases Cel12A and Cel45A. The catalytic core domain of Cel45A was included to compare with Cel12A, the endoglucanase of *T. reesei* which does not have a CBM. The two more well studied endoglucanases Cel5A and Cel7B were included for comparison. Both Cel12A and Cel45A have a lower molecular mass compared with the other cellulases of *T. reesei*, 25 and 23 kDa, respectively. A wide range of substrates for the enzymes have been studied, including microcrystalline and amorphous cellulose, soluble carbohydrate polymers and cello-oligosaccharides. Only a few studies have been published on the low molecular mass endoglucanases Cel12A (Håkansson et al., 1978; Ülker and Sprey, 1990; Hayn et al., 1993; Okada et al., 1998, 2000) and Cel45A (Saloheimo et al., 1994). In these studies, both Cel12A and Cel45A showed activity against carboxymethylcellulose (CMC) and filter paper but not against Avicel. A number of studies have been performed on the enzymatic properties of the more well known Cel5A (Shoemaker and Brown, 1978; Henrissat et al., 1985; van Tilbeurgh and Claeyssens, 1985) and Cel7B (Håkansson et al., 1979; Shoemaker et al., 1983b; Bhikhabhai et al., 1984; Henrissat et al., 1985). However, there is a lack of systematic studies of the product formation during hydrolysis of microcrystalline, amorphous and soluble cellulose substrates. One important aspect of our study has been to investigate if there are any distinct differences between the enzymes which could explain why *T. reesei* produces several endoglucanases. There is currently ongoing work on structure determination of Cel45A (Jerry Ståhlberg, personal communication). In this context it is vital to have information about the enzymatic properties of the enzymes. Furthermore, a more thorough understanding of the activity of these endoglucanases is important for the applications of the enzymes, e.g. in constructing a more efficient cellulase mixture for total hydrolysis of cellulose.

2. Materials and methods

2.1. Enzymes

Cel12A (EG III) from *T. reesei* was purified by chromatographic methods from the culture filtrate produced and processed as described in Pere et al.

(1995). The strain used for enzyme production (kindly provided by Roal Oy, Finland) was genetically modified as described by Suominen et al. (1993) so that the genes coding for Cel5A (EG II) and Cel6A (CBH II) were deleted. Purity of the enzyme preparations was monitored by gel electrophoresis techniques using 12.5% polyacrylamide gels (Amersham Biosciences, Uppsala, Sweden) for sodiumdodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and IEF 3–9 gels (Amersham Biosciences) for isoelectric focusing in PhastSystem (Amersham Biosciences) according to manufacturer's instructions. Protein amounts during the chromatographic purification were estimated on the basis of absorbance at 280 nm. Protein concentration of the raw enzyme concentrates and purified enzyme preparations were assayed by the method of Lowry et al. (1951).

The concentrated culture filtrate (227 g protein) was treated with bentonite as described by Zurbriggen et al. (1990) to adsorb impurities and to remove the precipitates formed in the concentration. The clarified sample was equilibrated with 7 mM sodium phosphate buffer, pH 7.2 by gel filtration (Sephadex G-25 coarse, Amersham Biosciences) and applied to an anion exchange column of DEAE Sepharose FF (Amersham Biosciences), equilibrated with the same buffer. Elution was performed first with the starting buffer to remove unadsorbed proteins and thereafter 12.5 mM sodium acetate, pH 7.2 with increasing concentration of sodium chloride. A pool containing Cel12A (5.26 l, 10.9 g protein) was collected from the fractions that were neither adsorbed nor retarded in the column by the equilibration buffer.

The pool containing Cel12A was applied to a column of Phenyl Sepharose FF (Amersham Biosciences) equilibrated with 20 mM sodium phosphate at pH 6.0 containing 0.35 mol l^{-1} $(NH_4)_2SO_4$, after it had been adjusted to the corresponding pH and conductivity by ammonium sulphate. The unadsorbed material was eluted by equilibrating buffer and the other adsorbed proteins by 20 mM sodium phosphate and water. Cel12A was tightly adsorbed to the column and was eluted by 5 M urea, after which the column was washed by 6 M urea. The collected pool (4.4 l, ca. 0.7 g protein) was equilibrated to the conductivity and pH of 12 mM sodium acetate, pH 4.8 and applied to a cation exchange column (CM Sepharose FF, Amersham Biosciences), equilibrated with the same buffer. The bound proteins were eluted with a combined pH and ionic strength gradient from 12 mM sodium acetate, pH 4.8–25 mM sodium acetate, pH 5.4, and after that by 1 M NaCl. Fractions containing Cel12A, which eluted at the end of the gradient were combined (ca. 0.34 g protein in 0.57 l) for further purification.

Final purification was achieved by gel filtration. A part of the collected pool from the cation exchange chromatography was concentrated by ultrafiltration (Amicon, PM 10 membranes) to 12 ml (ca. 0.21 g protein) and applied to a column of Sephacryl S-100 HR (5.0×88 cm, Amersham Biosciences) pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 100 mM NaCl. The elution was carried out with the same buffer. The fractions containing Cel12A were combined in two pools and concentrated by ultrafiltration (Amicon, PM 10 membranes) to final volumes 103 ml (pool I) and 20 ml (pool II) with 180 g (I) and 27 mg (II) of protein. The purified Cel12A showed a single band in SDS-PAGE and IEF, corresponding to the reported molecular mass and isoelectric point of the protein (Okada et al., 2000).

Cel45A (EG V) was purified from the culture filtrate of a *T. reesei* strain lacking the genes expressing the two major endoglucanases Cel7B (EGI) and Cel5A (EG II). The strain was genetically modified as described by Suominen et al. (1993). The culture filtrate was pH adjusted to pH 6.0 and applied to a column with DEAE Sepharose FF. The protein was eluted with a NaCl gradient from 0 to 0.2 M. The fractions containing Cel45A were pooled and the buffer was adjusted to 0.02 M sodium acetate (pH 5.0) and 0.5 M $(NH_4)_2SO_4$. The pooled fractions were applied to a column with Phenyl Sepharose FF. For elution a gradient of decreasing concentration of sodium acetate and $(NH_4)_2SO_4$ was used where Cel45A was eluted with 1 mM sodium acetate. The pooled fractions with Cel45A were pH adjusted to pH 3.0 with 5 mM acetic acid/sodium acetate and applied to a SP Sepharose FF column. Cel45A was eluted with a NaCl gradient from 0 to 0.5 M. The final step in the purification was gel filtration per-

formed with a Sephacryl S100 HR column equilibrated with 0.05 M sodium acetate and 0.15 M sodium chloride at pH 5.0. The purified Cel45A showed a single band in SDS-PAGE after silver staining.

Cel45Acore was purified from the culture filtrate of a *T. reesei* strain constructed for expression of the protein in glucose-containing medium, as described by Nakari-Setälä and Penttilä (1995). In glucose medium all other cellulases are repressed (Ilmén, 1997). The purification of Cel45Acore followed a similar procedure as for the purification of the full length Cel45A. The full length Cel45A and the core protein Cel45Acore were eluted at different salt concentrations in the chromatography step with Phenyl Sepharose FF column. The final gel filtration step was not performed; the Cel45Acore preparation was pure after the cation exchange step on SP Sepharose FF column at pH 3.0 (5 mM acetic acid/sodium acetate) and elution with NaCl gradient from 0 to 0.5 M. The purity of the Cel45Acore preparation was verified with SDS-PAGE where the preparation showed a single band after silver staining.

Cel5A (EG II) was purified according to Rahkamo et al. (1996). The purity of the Cel5A preparation was verified with SDS-PAGE after silver staining. Cel7B (EG I) was purified from a culture filtrate of *T. reesei* QM9414. The culture filtrate was buffer exchanged to 20 mM ammonium acetate pH 7.0 in a Sephadex G25 column (Amersham Biosciences). The purification was performed in two anion-exchange steps. In the first step a Source Q column (Amersham Biosciences) was used with a linear gradient of ammonium acetate, pH 7.0, from 20 mM to 1 M. The second step was performed as above except that the pH was decreased to 6.5. The purification was performed at room temperature using a FPLC system (Amersham Biosciences). The purity of the Cel7B preparation was verified with SDS-PAGE after silver staining.

2.2. Substrates and chemicals

Avicel and glucose were obtained from Sigma (St. Louis, MI, USA). Phosphoric acid swollen cellulose (PASC) was prepared from Avicel according to Wood (1988). CMC was from ICN (Costa Mesa, CA, USA). Barley β-glucan and konjac glucomannan were from Megazyme (Bray, Ireland). Konjac glucomannan composition is approximately 60% mannan and 40% glucose and the polymer is partially acetylated which makes it water soluble. Cellobiose, cellotriose, cellotetraose and cellopentaose were from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was from ICN (Costa Mesa, CA, USA). Other chemicals were of analytical grade.

2.3. Effect of pH on enzymatic activity

The pH effect on enzymatic activity for the enzymes was determined on β-glucan, 5 g l^{-1}, at 40 °C. The substrate was hydrolysed for 30 min and the production of reducing ends was analysed with the DNS method using glucose as standard (Bailey and Nevalainen, 1981). BSA was present at 0.1 g l^{-1} to stabilise the enzymes. The buffers used were, pH 3, Na-citrate; pH 4, Na-acetate; pH 5, Na-acetate; pH 6, MES; pH 7, MOPS; pH 8, Tris; pH 9, Glycine; pH 10, Glycine. The pH was set with either NaOH or HCl.

2.4. Temperature effect on enzymatic activity

The effect of temperature on endoglucanase activity was determined on β-glucan, 5 g l^{-1}, in 50 mM Na-acetate at pH 5.0. The substrate was hydrolysed for 30 min and the production of reducing ends was determined with the DNS method. BSA was present at 0.1 g l^{-1} to stabilise the enzymes.

2.5. Adsorption

The adsorption to Avicel (10 g l^{-1}) and PASC (5 g l^{-1}) was studied with enzyme concentrations of 0.5 μM endoglucanase in 50 mM Na-acetate at pH 5.0. BSA was included at 1 g l^{-1} to stabilise the enzymes and to reduce unspecific adsorption. The adsorption experiments were performed at 4 °C. The adsorption time was between 3 min and 12 h. The substrate with adsorbed enzyme was filtered off using a 0.22 μm filter. The enzyme

activity was analysed in the filtrate using CMC for Cel5A and Cel7B, and β-glucan for Cel12, Cel45 and Cel45Acore. The activity was determined using the DNS assay.

2.6. Product formation

The formation of products after hydrolysis on different polymeric substrates was investigated after a 1 h hydrolysis, at 40 °C. Enzyme at 0.25 μM was incubated with the substrate, 10 g l^{-1} Avicel, 5 g l^{-1} PASC and 5 g l^{-1} CMC, in 50 mM Na-acetate at pH 5.0. BSA was included at 0.1 g l^{-1} in all experiments to stabilise the enzymes. The Avicel and PASC hydrolysis were terminated by filtering off the unhydrolysed substrate with a 0.22 μm filter. The CMC hydrolysis was not terminated but product formation was determined directly after 1 h. All experiments were analysed for production of soluble sugars immediately, within 1 min. The soluble sugars (glucose to cellopentaose) were analysed on a Dionex HPLC system with an anion exchange Carbopac PA100 column and pulsed amperometric detection (Dionex, Sunnyvale, CA, USA). The oligosaccharides were eluted isocratically with 50 mM NaOH and with a simultaneous linear gradient of Na-acetate from 50 to 300 mM. The detector was set with the following pulse potentials and durations: $E_1 = 0.05$ V, 200 ms (sampling); $E_2 = 0.75$ V, 200 ms (cleaning); $E_3 = -0.15$ V, 400 ms (reduction), and a response time of 1 s. Glucose, cellobiose, cellotriose, cellotetraose and cellopentaose were used as standards.

2.7. Kinetic studies of oligosaccharide hydrolysis

The kinetic constants were determined for hydrolysis of cellotriose, cellotetraose and cellopentaose. The enzyme and substrate concentrations used are listed in Table 1. The substrate was incubated with the enzyme for a specific time at 40 °C in 50 mM Na-acetate at pH 5.0, and 0.1 g l^{-1} BSA was present. The hydrolysis was terminated by adding NaOH to a final concentration of 0.4 M. The hydrolysate was analysed immediately on a HPLC system as described above. The kinetic constants were calculated with a Lineweaver–Burke plot.

2.8. Hydrolysis of β-glucan and glucomannan

The hydrolytic activity on β-glucan and konjac glucomannan for Cel12A, Cel45Acore, Cel5A and Cel7B was studied. The enzyme concentrations were 0.5 μM for Cel12A and Cel45Acore and 0.25 μM for Cel5A and Cel7B. The substrate (5 g l^{-1}) was dissolved in 50 mM Na-acetate at pH 5.0, BSA (0.1 g l^{-1}) was present to stabilise the enzymes. The hydrolysis was performed at room temperature for 9 h. The hydrolysate was analysed on a size exclusion chromatography (SEC) system with 200 mM ammonium-acetate, pH 5.0, as elution buffer at 0.5 ml min^{-1}. Two TSK G3000 SW columns (Tosohaas, Stuttgart, Germany) were connected in series and the eluate was analysed with a refractive index detector ERC-7515A (ERMA Inc., Tokyo, Japan). Dextran standards with molecular mass of 5.2, 25, 50 and 80 kDa were used to calibrate the system.

3. Results

The enzymatic properties were studied for the low molecular mass endoglucanases Cel12A and Cel45A of *T. reesei*. For Cel45A, the studies included both the full length enzyme and the catalytic core domain (Cel45Acore) produced after deletion of the CBM by genetic engineering. The more well-studied endoglucanases Cel5A and Cel7B were included for comparison.

3.1. Effect of pH on enzymatic activity

The effect of pH on hydrolysis was studied for Cel12A, Cel45Acore and Cel5A with the soluble polymeric substrate β-glucan during a 30 min hydrolysis, Fig. 1. At acidic pH values, Cel12A showed a distinctly different behaviour compared with the other enzymes. Cel12A had maximum activity at pH 5. The activity decreased more rapidly when decreasing the pH than when the pH was increased; more than 50% of the maximal activity was retained at pH 7 compared with only

Table 1
The enzyme and substrate concentrations used for determination of the kinetic constants

Enzyme	Enzyme concentration (μM)	Substrate	Substrate concentration (μM)
Cel12A	0.5	Cellotriose	500
Cel12A	0.5	Cellotetraose	30–1000
Cel12A	0.01	Cellopentaose	30–1000
Cel45Acore	0.5	Cellotriose	500
Cel45Acore	0.5	Cellotetraose	500
Cel45Acore	0.5	Cellopentaose	500
Cel5A	0.5	Cellotriose	500
Cel5A	0.02	Cellotetraose	50–500
Cel5A	0.001	Cellopentaose	10–150
Cel7B	0.05	Cellotriose	50–5000
Cel7B	0.001	Cellotetraose	30–250
Cel7B	0.001	Cellopentaose	10–200

25% at pH 4. Also Cel45Acore showed maximal activity at pH 5, however, Cel45Acore retained more than 75% of its maximal activity between pH 3 and 6. Above pH 6 the activity dropped rapidly and no activity could be detected at pH 8 and higher. For Cel5A the highest activity was obtained at pH 4. The maximum activity range was broad, with 85% of maximum at pH 3 and 5. The activity decreased to 50% at pH 7, and reached 0 at pH 10.

3.2. Temperature effect on enzymatic hydrolysis

The effect of temperature on hydrolysis of β-glucan at pH 5 was determined for Cel12A, Cel45Acore and Cel5A, Fig. 2. The incubation time for each enzyme was 30 min. For Cel12A maximal activity was shown at 50 °C. At 60 °C the enzyme retained almost 90% of maximal activity but less than 10% of maximal activity was obtained at 70 °C. In contrast, Cel45Acore

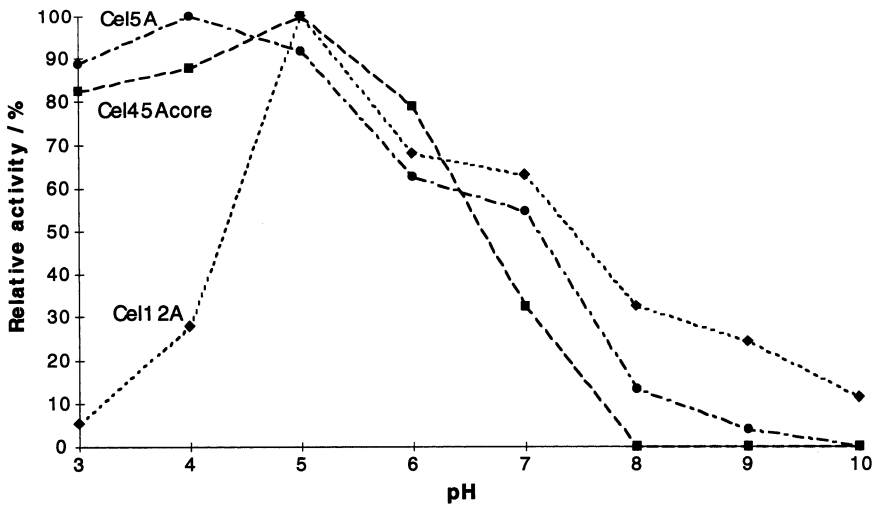

Fig. 1. The effect of pH on hydrolysis of β-glucan by the endoglucanases. The activity was determined by incubating the endoglucanases (0.5 μM) for 30 min with β-glucan, 5 g l^{-1}, at 40 °C at different pH values. The experiments were performed in triplicates with a S.D. of below 10%. The hydrolysis of the substrate was analysed with the DNS assay. ◆, Cel12A; ■, Cel45Acore; ●, Cel5A.

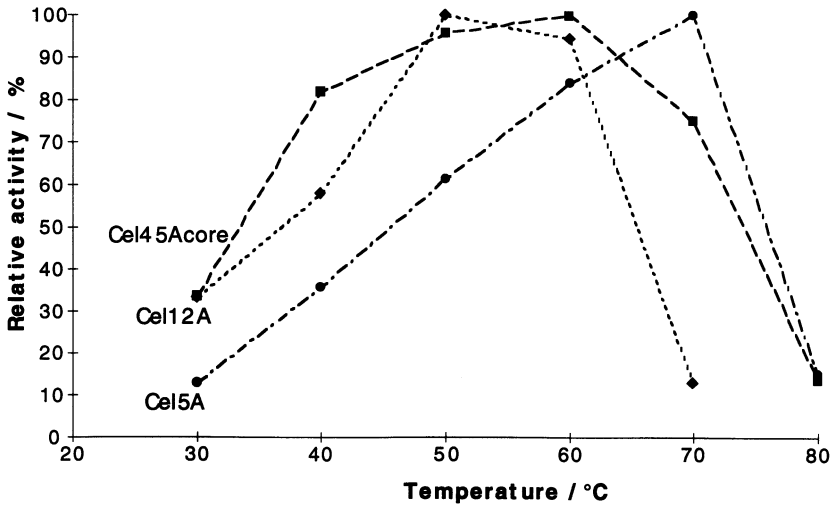

Fig. 2. The effect of temperature on hydrolysis of β-glucan by the endoglucanases. The activity was determined by incubating 0.5 μM of the endoglucanases for 30 min in 50 mM Na-acetate, pH 5.0, at different temperatures with β-glucan, 5 g l^{-1}. The experiments were performed in triplicates with a S.D. of below 10%. The hydrolysis of the substrate was analysed with the DNS assay. ◆, Cel12A; ■, Cel45Acore; ●, Cel5A.

retained activity over a wide temperature range, clearly wider than for the other endoglucanases. Maximal activity was observed at 60 °C but more than 70% of maximal activity was retained between 40 and 70 °C. For Cel5A the activity at 70 °C was higher than for the other endoglucanases. The activity increased up to 70 °C where maximal activity was retained during the 30 min incubation. At 80 °C only 10% of the activity was retained.

3.3. Adsorption to cellulose substrates

The adsorption of Cel12A, Cel45A, Cel45Acore, Cel5A and Cel7B to Avicel and PASC was studied, Fig. 3A and B. The adsorption was studied at 4 °C to minimise substrate change due to enzymatic activity. Furthermore, BSA (1 g l^{-1}) was included to reduce unspecific adsorption of the enzymes. The substrate with bound enzyme was filtered off and the filtrate was analysed for enzyme activity.

The weakest adsorption to Avicel was shown by Cel12A (Fig. 3A). About 20% of Cel12A adsorbed to Avicel within 3 min, and no major changes in adsorption were detected over time. Cel45A adsorbed strongly to Avicel, with 90% adsorbed after 60 min. Cel45A continued to adsorb to Avicel at longer adsorption times with 100% of the enzyme adsorbed at 12 h. The same enzyme lacking CBM, Cel45Acore, adsorbed as Cel45A up to 30 min where more than 85% was adsorbed. Cel45Acore desorbed from the substrate at longer incubation times, but after 12 h 75% of the enzyme was still adsorbed to Avicel. Both Cel5A and Cel7B showed strong adsorption on Avicel, with 85% adsorbed after 30 min, and the adsorption remained constant during the 12 h.

The majority of the studied endoglucanases, Cel5A, Cel7B, Cel45A and Cel45Acore, showed stronger adsorption to PASC compared with Avicel (Fig. 3B). No desorption of these enzymes could be detected up to 12 h. In contrast, Cel12A adsorbed to 60% after 3 min and a desorption was observed up to 12 h when 30% of Cel12A was still adsorbed to the substrate.

3.4. Product formation after hydrolysis of polymeric substrates

The products formed after hydrolysis of Avicel, PASC and CMC were analysed with HPLC (Fig. 4). Samples were analysed after 1 h hydrolysis at 40 °C. The degree of conversion for each sub-

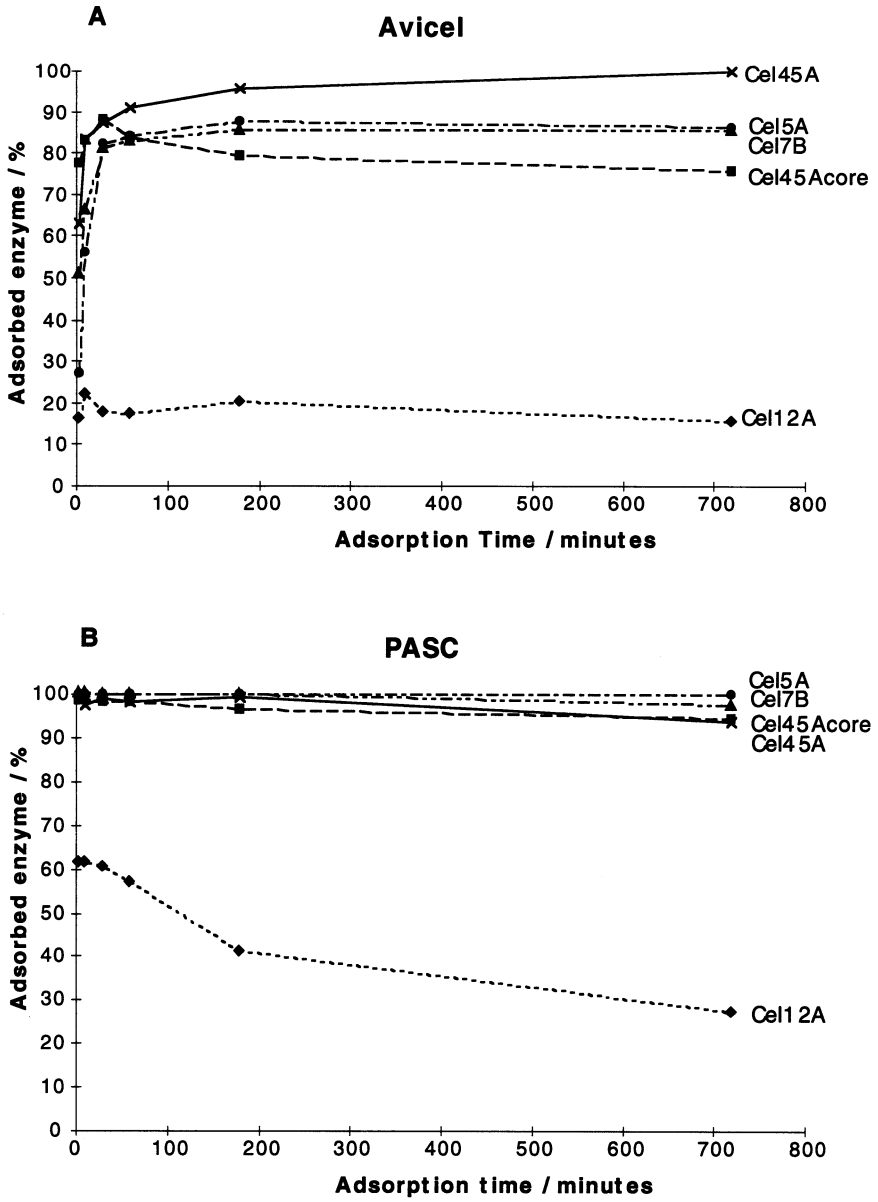

Fig. 3. Adsorption of the endoglucanases to Avicel and PASC. The endoglucanase (0.5 μM) was incubated with the substrate at 4 °C in 50 mM Na-acetate, pH 5.0. The adsorption was terminated by filtering of the substrate with adsorbed endoglucanase with a 0.2 μm filter. The filtrate was then analysed for specific endoglucanase activity and the amount of adsorbed enzyme was subsequently calculated. The substrate concentration was 10 g l^{-1} Avicel, and 5 g l^{-1} for PASC. **A**, adsorption to Avicel; **B**, adsorption to PASC; ◆, Cel12A; **X**, Cel45A; ■, Cel45Acore; ●, Cel5A; ▲, Cel7B.

strate and enzyme is shown in Table 2. After 24 h hydrolysis (data not shown) the product pattern was similar to what was observed after 1 h hydrolysis for all the enzymes studied.

For Cel12A, the products formed on all substrates were glucose, cellobiose, cellotriose and cellotetraose (Fig. 4A). Trace amounts of cellopentaose were also detected on PASC. The main

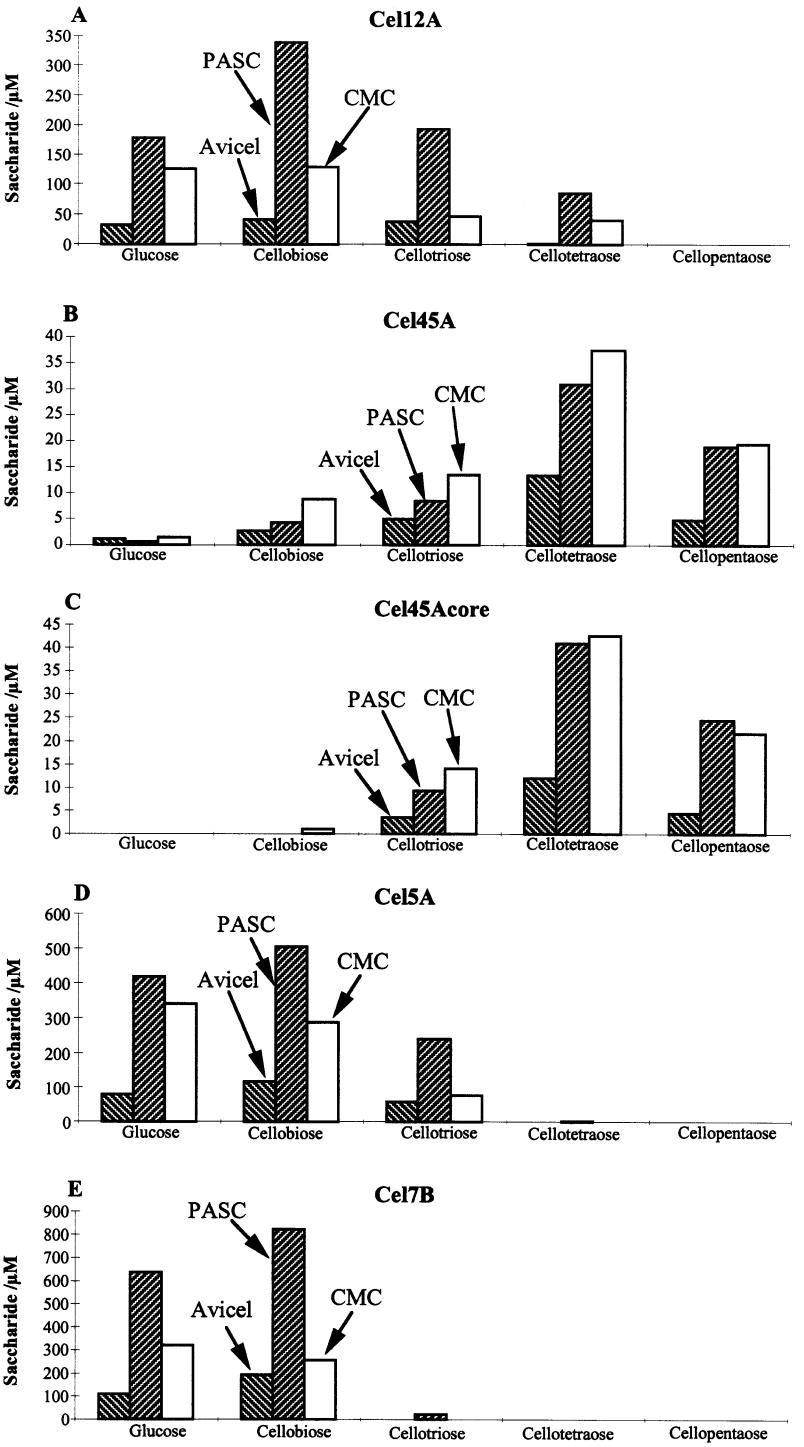
A
Cel12A
Saccharide /µM
PASC
CMC
Avicel
Glucose
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
B
Cel45A
C
Cel45Acore
D
Cel5A
E
Cel7B

Fig. 4

Table 2
The conversion, in percent, of Avicel, PASC and CMC with the different enzymes

Substrate	Cel5A	Cel7B	Cel12A	Cel45A	Cel45Acore
Avicel	0.8	0.8	0.4	0.1	0.1
PASC	7.0	7.1	5.6	0.8	0.9
CMC	3.7	2.7	2.2	0.9	0.9

The substrate was incubated with the enzyme for 1 h and the produced oligosaccharides were analysed with HPLC. The experiments were performed in triplicates with a S.D. of below 10%. The experimental conditions were the same as in Fig. 4.

product was cellobiose from all substrates. The cellotetraose concentration was significant for all substrates, except for Avicel. The conversion of PASC to soluble sugars by Cel12A was 5.6%. The corresponding figures for Avicel and CMC were 0.4 and 2.2%, respectively.

A notable difference in product formation pattern relative to the other endoglucanases was observed for the Cel45A enzymes. The main product was cellotetraose for both Cel45A and Cel45Acore for all substrates after 1 h hydrolysis (Fig. 4B–C). Both enzymes produced significant amounts of cellopentaose and cellotriose. Cel45A also produced low amounts of glucose and cellobiose, whereas Cel45Acore only produced trace amounts of cellobiose from CMC. The conversion of the substrates was low for both Cel45A and Cel45Acore, and there was no significant difference between the core and the full length enzyme on any substrate (Table 2).

The products formed by Cel5A were glucose, cellobiose and cellotriose on all substrates, and trace amounts of cellotetraose were also detected from PASC (Fig. 4D). The main product was cellobiose on Avicel and PASC, and glucose from CMC. The cellotriose concentration was significant for all substrates. The product formation pattern for Cel7B showed mainly glucose and cellobiose from all substrates (Fig. 4E). The main product was cellobiose from Avicel and PASC, and glucose from CMC. Cel7B produced trace amounts of cellotriose from PASC. The conversion of the substrates was similar for Cel5A and Cel7B on Avicel and PASC where 0.8 and about 7.0% were converted, respectively. A difference in conversion was observed for CMC: Cel5A converted 3.7% whereas Cel7B converted only 2.7%.

3.5. Kinetic studies for oligosaccharide hydrolysis

The steady state kinetic constants for Cel12A, Cel45core, Cel5A and Cel7B were determined for hydrolysis of cellotriose, cellotetraose and cellopentaose, Table 3. The kinetic constants were determined at 40 °C. The substrate concentrations used (Table 1) were both higher and lower than the calculated k_M value.

For cellopentaose, Cel12A, Cel5A and Cel7B hydrolysed this substrate efficiently, whereas no hydrolysis was observed for Cel45Acore. Cel12A had a four times higher k_M (230 μM) and about ten times lower k_{Cat} (14 s^{-1}) compared with Cel7B. The k_M values for Cel5A and Cel7B were very similar (≈ 50 μM), but the k_{Cat} for Cel7B was a factor of two higher (118 s^{-1}).

Cellotetraose was a substrate for Cel5A, Cel7B and Cel12A but not for Cel45Acore. The k_M for Cel12A and Cel5A was in the same range (≈ 400 μM). Cel12A had a k_{Cat} more than ten times lower (0.8 s^{-1}) than Cel5A. Cel7B had a lower k_M and a higher k_{Cat} compared with Cel5A.

Neither Cel12A nor Cel45Acore showed any detectable hydrolysis on cellotriose. No hydrolysis was observed with 0.5 μM enzyme and 0.5 mM

Fig. 4. Production of oligosaccharides on different polymeric cellulose substrates. The endoglucanase (0.25 μM) was incubated with the substrate, and the hydrolysate was analysed by HPLC for the formation of oligosaccharides. The experiments were performed in triplicates with a S.D. of below 10% for each oligosaccharide. The substrate concentration was 10 g l^{-1} Avicel, 5 g l^{-1} PASC and CMC. The hydrolysis was performed at 40 °C for 1 h in 50 mM Na-acetate pH 5.0. **A**, Cel12A; **B**, Cel45A; **C**, Cel45Acore; **D**, Cel5A; **E**, Cel7B.

Table 3
Kinetic constants for Cel12A, Cel45Acore, Cel5A and Cel7B on cellopentaose, cellotetraose and cellotriose

Enzyme	Substrate	k_M (μM)	k_{Cat} (s)	k_{Cat} (k_M)
Cel12A	Cellopentaose	230	14	0.03
Cel12A	Cellotetraose	430	0.8	0.002
Cel12A	Cellotriose	No hydrolysis detected		
Cel45Acore	Cellopentaose	No hydrolysis detected		
Cel45Acore	Cellotetraose	No hydrolysis detected		
Cel45Acore	Cellotriose	No hydrolysis detected		
Cel5A	Cellopentaose	53	65	1
Cel5A	Cellotetraose	356	22	0.08
Cel5A	Cellotriose	Very slow hydrolysis		
Cel7B	Cellopentaose	55	118	2
Cel7B	Cellotetraose	113	120	1
Cel7B	Cellotriose	Kinetic constants could not be determined		

An appropriate amount of enzyme was incubated with the oligosaccharide for a specific time at 40 °C (see Table 1). The hydrolysis was terminated with NaOH and immediately analysed with HPLC.

substrate incubated for 120 min. Only Cel7B hydrolysed cellotriose at a significant rate. The problem with Cel7B was that the kinetic constants could not be determined due to analytical problems when the substrate concentration was increased. However, the k_M seemed to be in the mM range. Cel5A hydrolysed cellotriose very slowly, only 10% of the substrate was hydrolysed in 120 min (0.5 μM enzyme and 0.5 mM cellotriose). Due to the low hydrolysis rate, the kinetic constants were not determined for Cel5A hydrolysis of cellotriose.

3.6. Hydrolysis of β-glucan and glucomannan

The hydrolytic capacity of Cel12A, Cel45Acore, Cel5A and Cel7B were determined on the soluble polymers β-glucan and konjac glucomannan, Fig. 5. The polymers were analysed after 9 h hydrolysis using a SEC system. The hydrolysate was not analysed with the HPLC system due to the lack of standards.

β-Glucan was hydrolysed to a significant extent with Cel12A, Cel5A and Cel7B as can be seen in the increase in elution volume compared with the non-hydrolysed substrate, Fig. 5A. The mean molecular mass of the formed products was in the range of 5 kDa when compared with the dextran standards. The opposite was observed for Cel45Acore; there was only a limited increase in elution volume compared with the non-hydrolysed substrate. However, Cel45Acore was able to hydrolyse glucomannan to a high extent, Fig. 5B. The products formed exhibit a peak in the chromatogram from below 5 up to 50 kDa compared with the dextran standards. The other three endoglucanases were also able to hydrolyse glucomannan, but to a lesser extent than Cel45Acore. Cel12A was less efficient than Cel5A and Cel7B on both substrates.

4. Discussion

4.1. Adsorption to cellulose substrates

Cel12A adsorbed to both Avicel and PASC (Fig. 3). However, the affinity was lower than for the other cellulases which can be explained by the lack of CBM in Cel12A. Most surprising was that both the full length Cel45A and Cel45Acore adsorbed significantly to Avicel and PASC. A difference between the core and the full length Cel45A was observed on Avicel where the core desorbed with time; about 75% of the core was adsorbed after 12 h compared with 100% for the full length enzyme. However, the adsorption on PASC was very similar for Cel45A and Cel45Acore. Thus, a significant difference in adsorption was observed between Cel12A and Cel45Acore,

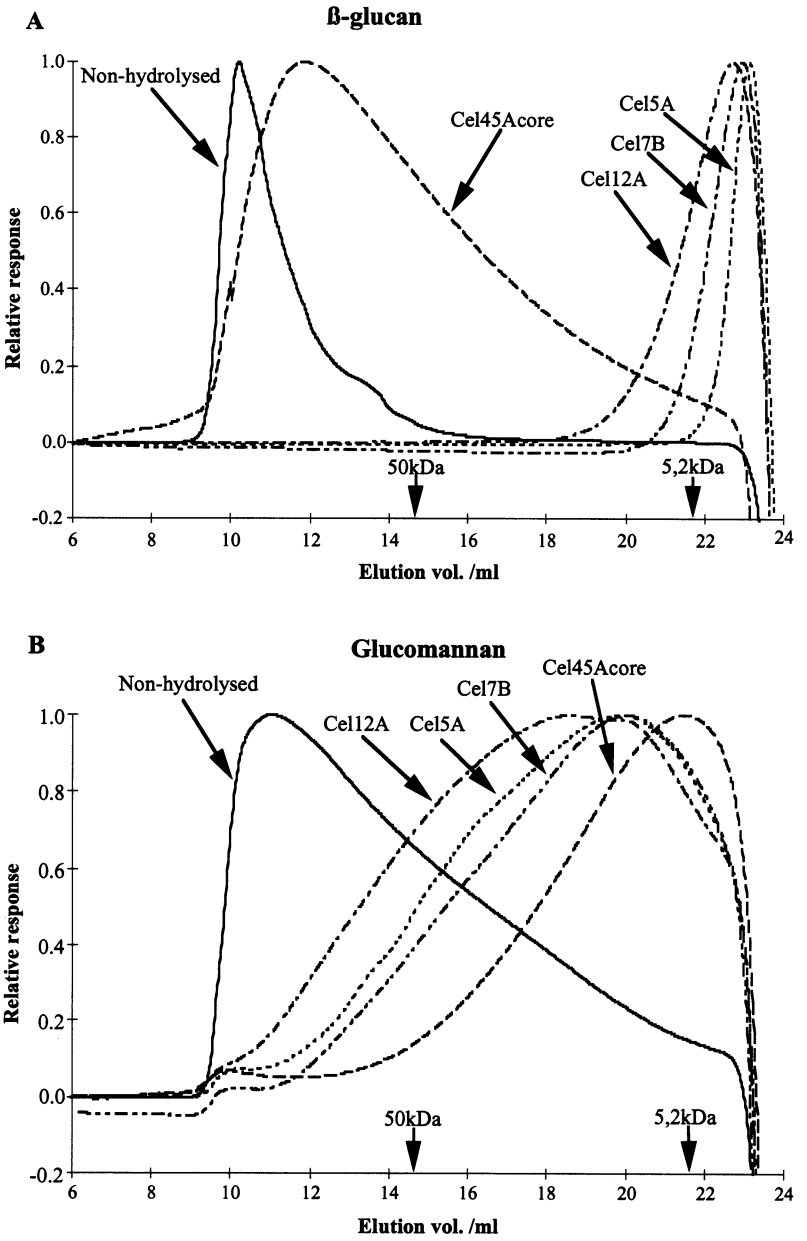

Fig. 5. Hydrolysis of barley β-glucan (**A**) and soluble konjac glucomannan (**B**) by Cel5A, Cel7B, Cel12A and Cel45Acore. The substrate (5 g l^{-1} in 50 mM Na-acetate, pH 5.0) was incubated with the enzyme (0.5 μM Cel12A and Cel45Acore; 0.25 μM Cel5A and Cel7B) at room temperature for 9 h. The hydrolysates were analysed using SEC. Dextran standards are marked for a mean molecular mass comparison.

which both lack the CBM. However, it has been shown that adsorption to steam pretreated spruce by the full length and core domain of Cel5A was similar, whereas the same experiment for Cel7A showed a decrease in adsorption for the core domain (Kotiranta et al., 1999). The core domains of Cel6A and Cel7A have been shown to adsorb to Avicel and bacterial microcrystalline cellulose

(BMCC) but with lower affinity than the full length enzyme (Tomme et al., 1988; Ståhlberg et al., 1991; Palonen et al., 1999). Similar results were obtained on BMCC and cotton for Cel7B core domain and full length enzyme (Srisodsuk, 1994). However, Tomme et al. (1988) showed that Cel7A core domain adsorbed almost as much as the full length enzyme on PASC, whereas Cel6A core domain showed significantly decreased adsorption compared with the full length enzyme on the same substrate. Hence, the core domains of the cellulases are able to adsorb to cellulose, but often with lower affinity than the full length enzyme. Furthermore, the core domains for the different cellulases adsorb with different affinity to cellulose substrates.

4.2. Hydrolytic properties of Cel12A

Cel12A hydrolysed Avicel, PASC and CMC, Fig. 4A. The conversion was highest on PASC and lowest on Avicel, Table 2. However, the conversion is lower than that observed for Cel5A and Cel7B, but significantly higher than for Cel45A and Cel45Acore. The main product of Cel12A hydrolysis of the substrates was cellobiose. After 24 h the cellotetraose, which was observed after 1 h of hydrolysis, had been hydrolysed but cellotriose was still a significant product (data not shown). This was confirmed in the kinetic studies, Table 3, where cellotriose was not a substrate for Cel12A but the longer oligosaccharides were. Contrary to earlier reports (Ülker and Sprey, 1990) stating that Cel12A does not hydrolyse Avicel, our results show that Cel12A adsorbs to Avicel, and that Avicel is hydrolysed by Cel12A. The low activity of Cel12A on Avicel, compared with Cel5A and Cel7B, can partly be explained by the low adsorption. Cel12A was highly active on β-glucan and it was also able to hydrolyse glucomannan, Fig. 5, but Cel12A was less active than Cel5A and Cel7B on both substrates. Thus, the hydrolytic properties of Cel12A is similar to Cel5A on all substrates tested. However, Cel5A is an endoglucanase with higher activity compared with Cel12A.

The activity of Cel12A on cellotetraose and cellopentaose was significantly lower than that of Cel5A and Cel7B, as shown by the k_{Cat} values in Table 3. The kinetic constants for Cel5A and Cel7B were similar. The turnover numbers on the chromophoric substrates 2-chloro-4-nitrophenyl-β-D-cellobioside ($CNPG_2$) and 2-chloro-4-nitrophenyl-β-D-cellotrioside ($CNPG_3$) have been determined for Cel12A to be 0.11 and 1.1 s^{-1}, respectively (Hayn et al., 1993). Furthermore, the turnover number for Cel7B on $CNPG_2$ has been reported to be 44 s^{-1} and for Cel5A on $CNPG_3$ to be 4.5 s^{-1} (Nidetzky and Claeyssens, 1994). It was surprising that Cel12A was able to hydrolyse $CNPG_2$ since this substrate is supposed to mimic cellotriose, a substrate for which we did not observe any Cel12A activity. However, the turnover number for Cel12A was significantly lower than for Cel7B on $CNPG_2$. The turnover number for Cel5A on $CNPG_3$ was four times higher than that determined for Cel12A. On cellotetraose we observed an approximately 20 times higher turnover number for Cel5A compared with Cel12A (Table 3). Thus, the kinetic constants on chromophoric substrates showed similar differences between the endoglucanases as the kinetic studies reported here, i.e. Cel5A and Cel7B had higher turnover number than Cel12A. The kinetic constants have also been determined for *Humicola insolens* endoglucanases (Schou et al., 1993). Hi Cel12A hydrolysed cellotetraose and longer oligosaccharides. The turnover number on cellopentaose was 0.0016 s^{-1} for Hi Cel12A and 0.029 and 40 s^{-1} for Hi Cel5A and Hi Cel7B, respectively. Hence, the Cel12A endoglucanase is the least efficient endoglucanase in both *T. reesei* and *H. insolens* when compared with Cel5A and Cel7B.

The kinetic constants (k_{Cat}/k_M) obtained here for Cel7B on cellotetraose (1 s^{-1} μM^{-1}) and cellopentaose (2 s^{-1} μM^{-1}) were significantly higher than the k_{Cat}/k_M values determined at 25 °C reported by Biely et al. (1991) (0.04 and 0.02 s^{-1} μM^{-1}, respectively). The difference in temperature can partly explain the discrepancy, as we determined the kinetic constants at 40 °C. Furthermore, we used substrate concentrations between 30 and 250 μM for cellotetraose and 10–200 μM for cellopentaose whereas Biely et al. determined the constant at 25 μM. The transglycosylation activity increases with an increase in substrate concentration. Determining the kinetic

constants using steady-state kinetics might not be optimal for a retaining enzyme, i.e. an enzyme with transglycosylation activity.

4.3. Hydrolytic properties of Cel45A

Cel45A and Cel45Acore behaved very similarly in their hydrolysis of all substrates studied. The main product from Avicel, PASC and CMC was cellotetraose with significant amounts of cellotriose and cellopentaose, Fig. 4B and C. The product formation pattern after 1 h hydrolysis was similar to what was observed after 24 h hydrolysis. The product formation pattern for Cel45A was thus distinctly different from the other *T. reesei* endoglucanases. The conversion was low on all substrates, between 0.1 and 0.9% for Avicel and CMC, respectively (Table 2). Interestingly, the conversion was higher on CMC compared with PASC, where the opposite was observed for the other endoglucanases. However, after 24 h hydrolysis both the full length and the core domain produced more soluble sugars from PASC than from CMC, 5.0 and 2.1%, respectively. Apparently, CMC has some sites which can rapidly be hydrolysed by Cel45A, which then become depleted. On the other hand PASC has more hydrolysable sites than CMC; however, the hydrolysis is slower at these sites. Cel45Acore readily hydrolysed soluble konjac glucomannan and it was the most efficient endoglucanase on this substrate, Fig. 5. All *T. reesei* endoglucanases have been reported to be able to hydrolyse glucomannan (Biely and Tenkanen, 1998), which is in agreement with our results. We have observed that Cel45Acore does not hydrolyse mannan or galactomannan and ^{1}H-NMR studies on konjac glucomannan hydrolysed by Cel45Acore showed only glucose reducing end protons (unpublished results). Thus, Cel45Acore was able to hydrolyse glucomannan between two glucose units or possibly between a glucose and a mannose unit where the glucose was in the -1 subsite in the active site of the enzyme. Moreover, these NMR studies also clearly showed that Cel45A is not a mannanase since mannose reducing end protons were not observed in the glucomannan hydrolysate. For Cel45Acore we obtained higher hydrolysis of glucomannan compared with β-1,4-glucan substrates and no activity was found on other mannans. Thus, these results taken together indicate that Cel45A is a glucomannanase rather than a strict endoglucanase.

No activity was observed for Cel45Acore on cellotriose, cellotetraose and cellopentaose, Table 3, which is in agreement with the production pattern on Avicel, PASC and CMC where these oligosaccharide products were not further degraded. However, Cel45A of *H. insolens* was able to hydrolyse cellotetraose and longer oligosaccharides (Schou et al., 1993). Furthermore, Hi Cel45A showed a higher turnover number than both Hi Cel5A and Hi Cel12A but lower than Hi Cel7B. Apparently, the Cel45A endoglucanases of *T. reesei* and *H. insolens* are significantly different even though they both belong to the same glycoside hydrolase family.

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