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Investigation of endoglucanase selectivity on carboxymethyl cellulose by mass spectrometric techniques

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Abstract The benefits of applying cellulose selective enzymes as analytical tools for chemical structure characterization of cellulose derivatives have been frequently addressed over the years. In a recent study the high selectivity of cellulase Cel45A from *Trichoderma reesei* (Tr Cel45A) was utilized for relating the chemical structure to the flow properties of carboxymethyl cellulose (CMC). However, in order to take full advantage of the enzymatic hydrolysis the enzyme selectivity on the cellulose substrate must be further investigated. Therefore, the selectivity of Tr Cel45A on CMC was studied by chemical sample preparation of the enzyme products followed by mass spectrometric chemical structure characterization. The results strongly suggest that, in accordance with recent studies, also this highly selective endoglucanase is able to catalyze hydrolysis of glucosidic bonds adjacent to mono-substituted anhydroglucose units (AGUs). Furthermore, the results also indicate that substituents on the nearby AGUs will affect the hydrolysis.

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Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOFMS) is a valuable tool for chemical structure elucidation of biopolymers. MALDI–TOFMS has been used for chemical structure characterization of partially hydrolyzed methyl cellulose (Momcilovic et al. 2003b), hydroxypropyl cellulose (HPC) (Richardson et al. 2003) and ethylhydroxyethyl cellulose (Andersson et al. 2004). Recently, MS and tandem MS methods for characterization of cellulose ethers were evaluated (Mischnick et al. 2005). The study showed that electrospray ionization mass spectrometry (ESI-MS) and MALDI–TOFMS, are powerful techniques for investigation of the substituent distribution in the intact polymer. In addition, even more detailed chemical structure information can be obtained by tandem mass spectrometry (MS/MS) (Tuting et al. 2004b; Adden and Mischnick 2005; Adden et al. 2006).

In CMC, variations in the chemical structure greatly affect the product properties (e.g. rheological properties), hence making it more or less suitable in a specific application. These properties

are known to be affected by, not only the molar mass and molar mass distribution, but also the degree of substitution (DS) and the substituent distribution in the polymer. The determination of the substituent distribution in CMC is still not a straightforward task.

One attractive approach for characterization of the substituent distribution in CMC is to use cellulose selective enzymes, e.g. endoglucanases, for selective hydrolysis of the glucosidic bonds in the substrate. Due to that the carboxymethyl groups constitute obstacles (e.g. sterical or electrostatic) or hinder the formation of hydrogen bonds between the enzyme and the substrate, the hydrolysis will be restricted to regions on the molecules containing no or few substituents. The chemical structure and concentration of the enzyme products will thus depend on the selectivity of the enzyme and on the CMC-substrate structure (e.g. DS and substituent distribution). There are several publications available where enzymatic degradation in combination with different analytical techniques has been applied for the characterization of the substituent distribution in CMC. Gelman combined enzymatic degradation with viscosity measurements and reducing sugar determination (Gelman 1982) while more recent studies included chromatographic techniques (e.g. size-exclusion chromatography, anion-exchange chromatography and nuclear magnetic resonance spectroscopy of the enzymatic hydrolysates) (Horner et al. 1999; Saake et al. 2000).

We have recently shown that by enzymatic hydrolysis with Tr Cel45A, it is possible to differentiate the substitution pattern in two CMCs displaying large variations in the product properties (Enebro et al. 2007).

In order to take full advantage of the selective hydrolysis, more information on the endoglucanase sensitivity for substituents on the AGUs involved with the active site is required (Fitzpatrick et al. 2006).

It has been suggested that endoglucanases require at least two non-substituted AGUs in order to hydrolyze the CMC molecules (Reese 1957; Wirick 1968; Eriksson and Hollmark 1969). However, in a recent study Horner et al. (1999) reported that an endoglucanase from *Humicola insolens* was able to hydrolyze the glucosidic bond adjacent to a substituted AGU if the substituent was located at the *O*-6 position.

There have been a few studies on the selectivity of Tr Cel45A endoglucanase on cellulose derivatives in recent years. Schagerlof et al. (2006a, b) suggested, from studies on HPC and hydroxypropyl methyl cellulose (HPMC), that the endoglucanase cannot tolerate hydroxypropyl groups in the active site and that it, in addition, is severely hindered by methyl groups. Furthermore, Cohen et al. (2004) presented a study where CMC was hydrolyzed using six different endoglucanases, including Tr Cel45A, and the oligomeric products analyzed by liquid chromatography (LC)-MS. It was concluded that all six endoglucanases are able to hydrolyze the glucosidic bond adjacent to a substituted AGU. This conclusion was based on the detection of substituted monomers in the hydrolysates. However, according to their results no substituted monomers were detected for Tr Cel45A. Furthermore, it was found that fully substituted cellotriose was a product from Tr Cel45A hydrolysis. This implies that the active site of Tr Cel45A is insensitive to carboxymethyl substituents, which should lead to substantial hydrolysis of the CMC. However, according to their results from size-exclusion chromatography and reducing sugar analysis it was found that the CMC was not extensively hydrolyzed by Tr Cel45A, which has also been shown in another study (Karlsson et al. 2002a).

To gain further knowledge on the enzyme selectivity the substituent locations on the oligomeric enzyme products must be analyzed. Therefore, the objective of this study was to investigate the selectivity of Tr Cel45A on CMC by detailed chemical structure characterization. This could be achieved by chemical sample preparation of the enzyme products followed by MALDI–TOFMS and ESI-MS/MS. From our findings we are able to present more detailed information regarding the selectivity of Tr Cel45A on CMC.

Materials and methods

Chemicals

A high viscosity grade CMC (DS 0.61, CEKOL® 50,000, Lot no. Z0607) was provided by CP Kelco (Skoghall, Sweden). The dimethylsulfoxide (DMSO, anhydrous), NaOH (pellets), $NaBH_4$ and all solvents, analytical grades, were from Sigma-Aldrich

(Stockholm, Sweden). The strong ion exchange resin Dowex® 50 W × 8 (H^+-form, 50–100 mesh), iodomethane (MeI) and the MALDI matrices 2,5-dihydroxybenzoic acid (DHB) and 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP) were purchased from Fluka (Buchs, Switzerland). LiCl, NaCl and $(NH_4)_2SO_4$ were from Merck (Darmstadt, Germany). Deionized water was used in the chemical sample preparations while MilliQ grade water from a Synergy 185 UV Ultrapure Water System (18.2 MΩcm, Millipore AB, Solna, Sweden) was used in the MALDI preparations.

Enzymatic hydrolysis of CMC

The CMC was dialyzed using a dialysis tubing with a molecular weight cut-off (MWCO) of ≈12,000 (globular proteins) (Sigma-Aldrich) and deionized water in order to remove any low mass molecules in the sample. The endoglucanase Cel45A from *Trichoderma reesei*, purified according to Karlsson et al. (2002b), was used for hydrolysis of the CMC according to Enebro et al. (2007). The endoglucanase showed no side activities.

Chemical sample preparation

A small volume of the enzymatic hydrolysate (containing approximately 5 mg of CMC) was filtrated through a micro centrifuge filter (MWCO 10,000) (Pall Norden AB, Lund, Sweden) in order to remove any remaining high mass products. When most of the solution had passed through the filter, additional water was added (three times) in order to recover as high fraction as possible of the low-mass analytes. The solution was freeze-dried before subjected to the chemical derivatization.

The hydrolysate was first reduced in a 3 mL micro-reaction vessel (Supelco, Sigma-Aldrich) by addition of 1 mL of 2 g L^{-1} $NaBH_4$ in aqueous ammonia (2 mol L^{-1}) for 5 h under stirring. The solution was evaporated followed by repeated coevaporation (five times) with 15% methanolic acetic acid under a stream of nitrogen in order to remove borate as its methyl ester (Adden et al. 2006).

The enzymatic hydrolysates were permethylated according to Ciucanu and Kerek (1984) by using alkali powder in DMSO and MeI as reagents. The reduced sample was dissolved in 1 mL of DMSO before addition of 100 mg of powdered NaOH. After 30 min, 20 μL of MeI was added and the solution was stirred at room temperature (RT) over night. In order to maximize the methylation yield of the hydroxyl groups, 50 mg of powdered NaOH and, after 30 min, 10 μL of MeI was added three times in 24 h intervals.

Twenty-four hours after the last addition of reagents, the excess of MeI was evaporated under a stream of nitrogen at RT for 30 min. The reaction mixture was then transferred to a separation funnel containing water and the solution was made acidic (pH 1–1.5) by addition of a few drops of HCl. This was done in order to retain the CM-oligomers in their acidic form. The permethylated CM-oligomers were extracted in dichloromethane three times followed by washing of the organic phase with slightly acidic water three times. The dichloromethane was then evaporated under nitrogen and the residue was dissolved in 1 mL of MeOH. A small aliquot of the solution was diluted ten times before addition of a few strong ion exchange beads to the solution and stirring the solution for a few days. The solution was then used without any further purification. Three separate reactions were performed.

Acid hydrolysis

A small volume of the reduced and permethylated, but not Dowex-treated, sample was subjected to acid hydrolysis. The methanol was evaporated under a stream of nitrogen before addition of aqueous trifluoroacetic acid (TFA, 2 mol L^{-1}), giving a sample concentration of approximately 2.5 g L^{-1}. The hydrolysis was performed by heating the solution to 90 °C for 6 h with continuous stirring. Thereafter the acid was evaporated under a stream of nitrogen and to the residue toluene was added for co-evaporation with the TFA. This procedure was repeated five times. The hydrolysate was then dissolved in water to a concentration of 1 g L^{-1} and used without further purification.

MALDI-TOFMS

MALDI-TOFMS was conducted on a Bruker UltraFlex MALDI-TOF mass spectrometer with a SCOUT-MTP Ion Source (Bruker Daltonics, Bremen, Germany) equipped with a N_2-laser (337 nm), a

gridless ion source and a reflector. Two spectrum acquisition methods were used, both in the reflector-positive mode. Spectra in the mass-to-charge ratio (m/z) range of 500–2,000 were acquired with an acceleration voltage of 25 kV and a reflector voltage of 26.3 kV, while spectra from m/z range of 220–400 were acquired with an acceleration voltage of 10 kV and a reflector voltage of 11.3 kV. The laser intensity was set to the lowest value possible to acquire high resolution spectra.

The MALDI target spot preparation for the permethylated oligomers was performed by mixing equal volumes of analyte (0.5 g L^{-1} in MeOH) with the THAP-matrix (10 g L^{-1} in MeOH). Additional sodium was added to the mixture by addition of a minute volume of NaCl (1.5 g L^{-1} in MeOH) in order to ensure ionization by sodium.

The target spot preparation of the unmodified enzyme products and the monomers from acid hydrolysis was performed by mixing the analyte with aqueous DHB (10 g L^{-1}) in a mass ratio of 1:10 (analyte:matrix). A minute fraction of aqueous $(NH_4)_2SO_4$ was added in order to reduce the high intensity peaks from matrix clusters and analyte molecules with sodium exchanged carboxylate groups (Enebro and Karlsson 2006). Matrix reference samples, using the same preparation but without sample, were also prepared in order to avoid misinterpretation of the mass spectra due to matrix clusters.

The analyte/matrix/salt mixtures were vortexed using a Vortex Genie (Scientific Industries, Bohemia, NY, USA). Approximately 0.5 μL of the mixture was added on the target plate and was allowed to dry in ambient temperature before insertion into the instrument.

Spectra were acquired by irradiating 10–20 positions on the sample spot with a total of 500–2,500 laser shots. The spectra were processed using FlexAnalysis software (Bruker Daltonics).

ESI-MS/MS

ESI-MS and MS/MS was performed on a Finnigan LCQ (Thermo Fisher Scientific, Waltham, NA, USA). The analyte solution (0.25 g L^{-1} in MeOH containing 2 mmol L^{-1} of LiCl) was infused into the ESI source via a syringe at a flow of 5 μL min^{-1}. The spray voltage was approximately 5 kV and the sheat gas was set to 10 (arb. units). The ion-transfer capillary was set to 175 °C and mass spectra were recorded in the positive ion mode. For collision induced dissociation (CID) helium was used as collision gas. Mass spectra were recorded and processed using Xcalibur 2.0 SR2 software (Thermo Electron Corp., San Jose, CA, USA).

Results and discussion

The main objective of this study was to investigate the selectivity of Tr Cel45A on CMC by mass spectrometric structure analysis of the enzyme products. Initially, MS/MS was performed on CM-oligomers not subjected to chemical sample preparation. Interpretation of the spectra was very difficult due to that several different fragment types were obtained and due to low sensitivity. In order to overcome these limitations, we decided to perform chemical sample preparation of the CM-oligomers by reduction and permethylation. Derivatization of carbohydrates prior to MS/MS increases the sensitivity due to that more volatile organic solvents can be used and facilitates tandem mass spectrometry interpretations (Ikonomou et al. 1991; Reinhold et al. 1995; Kang et al. 2008). In addition, transformation of the charged CM-oligomers to neutral species should significantly enhance the analysis in the positive ion mode (Kang et al. 2008).

Chemical sample preparation

The chemical sample preparation was performed in three subsequent reactions. Initially, the anomeric carbon atom in the CM-oligomers was reduced. This was done in order to allow for differentiation between the C/Y and B/Z fragments that can be obtained in MS/MS (Fig. 1). The reduction should also prevent degradation that otherwise may occur under the alkaline conditions of the permethylation (Richtzenhain et al. 1954). The second step involved permethylation of the hydroxyl groups on the oligomers. In MALDI-TOF mass spectra of the permethylated reaction products it was seen that the most intense peaks corresponded to undermethylated oligomers. Furthermore, the number of undermethylations correlated with the number of CM-groups, which was an indication that the carboxylic acids had not been transformed to methyl esters. Therefore, a third step was introduced where the carboxylic acids were methyl esterified in acidic

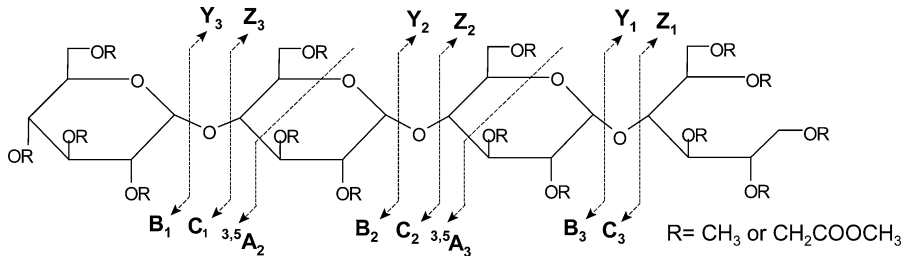

Fig. 1 Fragment nomenclature according to Domon and Costello (1988) when applied to cellotetraose oligomers from chemical sample preparation

MeOH, which was prepared by addition of strong cation exchange beads to MeOH.

MALDI-TOFMS

In Fig. 2 MALDI mass spectra of the enzyme products before, Fig. 2a, and after chemical sample preparation, Fig. 2b, are shown. The relative peak intensities within each DP are similar in both spectra. This indicates that no DS-selective loss of analyte components occurred during the chemical sample preparation.

Furthermore, it can be seen in Fig. 2b that the chemical sample preparation procedure was successful as all major peaks in the mass spectrum correspond to fully permethylated oligomers. The identical oligomers, with respect to DP and number of substituents, were detected in both mass spectra.

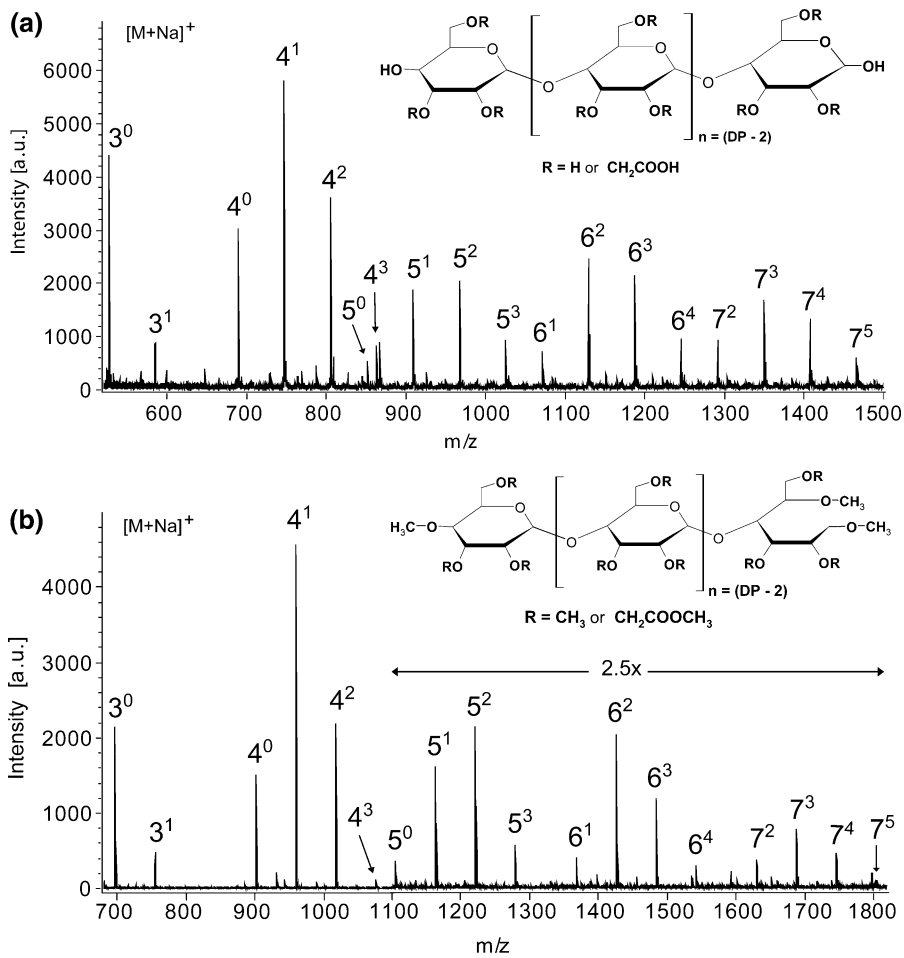

Fig. 2 MALDI-TOF mass spectra of CM-oligomers from enzymatically hydrolyzed CMC (**a**) before the chemical sample preparation and (**b**) after chemical sample preparation. All peaks were identified as sodium ion adducts $[M + Na]^+$. Peaks corresponding to oligomers with DP 3-7 were identified and labeled. The labeling 4^1 corresponds to the sodium ion adduct with DP4 (large number) containing one carboxymethyl group (number in superscript). The incorporated figures display the chemical structures of the CM-oligomers in each spectrum

In Fig. 2 it can be seen that cellotriose (3^0), cellotetraose (4^0) and cellopentaose (5^0) are enzyme products but not cellohexaose (6^0). This observation is in accordance with previous studies where CMC has been hydrolyzed by Tr Cel45A and indicates that the endoglucanase requires a substrate longer than five AGUs in order catalyze a cleavage (Karlsson et al. 2002a). It is also seen that the main products are oligomers with few substituents, which is an indication on a high endoglucanase selectivity on the CMC.

ESI-MS/MS

In order to obtain information on the substituent positions in the CM-oligomers, ESI-MS/MS was performed. In MS/MS ions of a specific m/z (i.e. DP and number of substituents) are selected, fragmented, e.g. by collision induced dissociation (CID) and subsequently the charged fragments are analyzed with respect to their m/z. The measured m/z and the fragmentation scheme given in Fig. 1 were used for the identification of the fragments.

Figure 3 displays the CID-spectrum of cellotetraose (4^0), from chemical sample preparation, where the fragments have been identified and labeled according to Fig. 1.

It can be seen that the most intense peaks in the CID-spectrum correspond to Y- and B fragments. In addition there are also peaks corresponding to, what we believe are A- and C- fragments. Obviously C-fragments are formed in CID which shows that a reduction of the oligomers is required in order to differentiate between C- and Y-fragments. If the reduction was not performed, it would not be possible to determine whether these fragments contain the non-reducing or reducing end of the original ion. It has been suggested that cross-ring fragments, e.g. ^{3,5}A-fragments, are formed via B- or C-fragments that contain a reducing end (Spengler et al. 1990; Tuting et al. 2004a).

Detectable signal in MS/MS was only obtained for oligomers with m/z < 1,500. From the MS/MS data on the substituted oligomers, it was possible to deduce information regarding the substituent positions. Due to that all B- or Y-fragments still contain either the non-reducing or reducing end of the intact CM-oligomer it is possible to obtain substituent information on the terminal AGUs.

Figure 4 displays a CID-spectrum of di-substituted cellotetraose (4^2) where peaks corresponding to fragments of varying DP and number of substituents have been labeled.

In the CID-spectrum of 4^2 (Fig. 4) peaks corresponding to $Y_1{}^1$ and $B_3{}^1$ were detected. Evidently, mono-substituted AGUs can be located at the reducing end of the oligomers. Furthermore, a peak corresponding to $Y_3{}^1$ was also detected. Thus, mono-substituted AGUs can also be found at the non-reducing end. These observations were recurrent when performing MS/MS on all substituted oligomers, with the exception for mono-substituted cellohexaose (6^1) where no fragments corresponding to substituents at either of the end groups were detected. This may be due to that such oligomers contain a sequence of five non-substituted AGUs, and therefore may be further degraded by the enzyme. Although there was evidence on mono-substituted AGUs at the end groups, there were no indications on

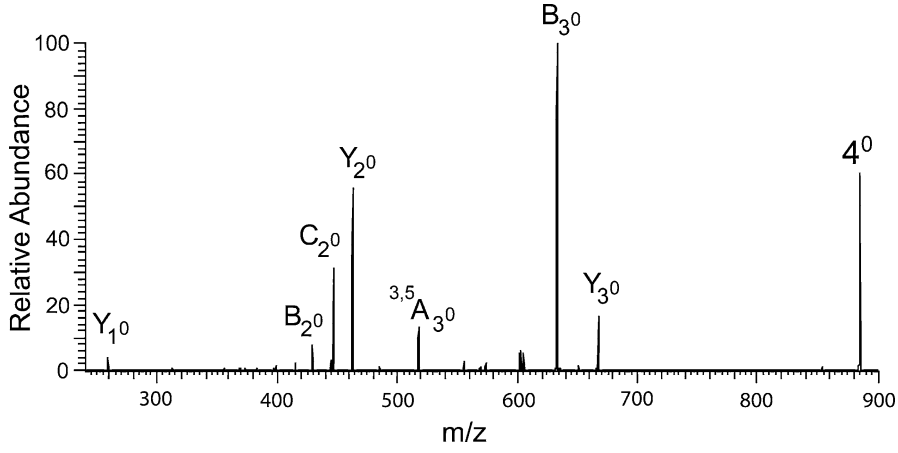

Fig. 3 CID-spectrum of reduced and permethylated cellotetraose as recorded by ESI-MS/MS. The fragments have been labeled according to Fig. 1 while the number in superscript denotes the number of substituents located at the fragment

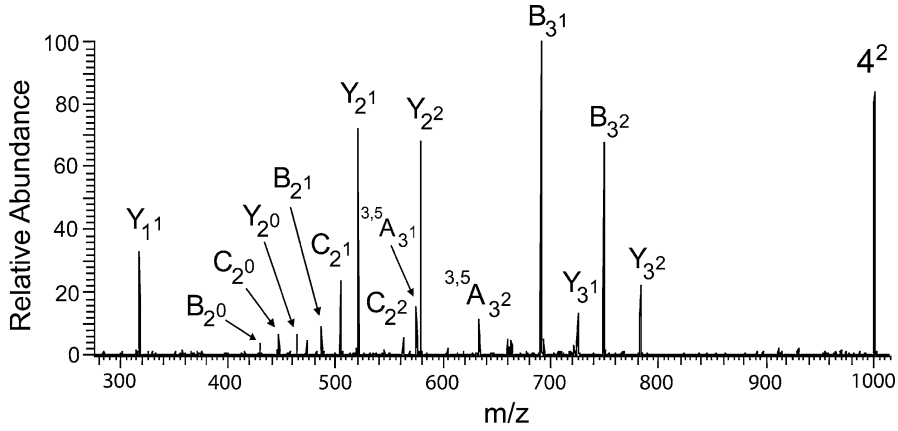

Fig. 4 CID-spectrum of reduced and permethylated cellotetraose carrying two substituents (4^2) recorded by ESI-MS/MS. The fragments have been labeled according to Fig. 3

di- or tri substituted AGUs at either of the end groups. In addition, in MS/MS of oligomers with three substituents no B_2^3 or Y_2^3 fragments, or peaks corresponding to neutral loss of tri-substituted dimers, were detected. This indicates that no more than two substituents were present on the two AGUs at either end of the oligomers. In order to further investigate the chemical structure of the reducing and non-reducing ends, acid hydrolysis of the oligomers was performed followed by MS of the monomers thus obtained.

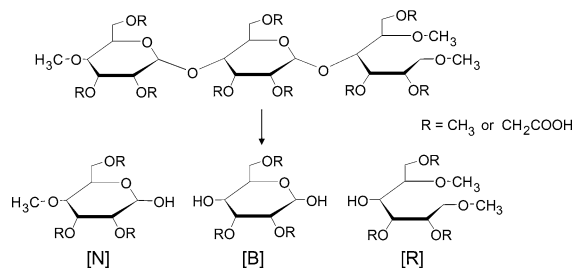

Fig. 5 The monomers formed during acidic hydrolysis of the reduced and permethylated oligomers. The labels indicate the original position of the monomer in the intact oligomers (non-reducing end [N]; between the chain ends [B]; reducing end [R])

Monomer analysis

A small fraction of the reduced and permethylated sample (not Dowex-treated) was subjected to extensive acid hydrolysis in order to obtain monomers. Due to that the monomer residues in the oligomers differ in chemical structure after the chemical sample preparation, the monomers obtained from hydrolysis will have different masses depending on their original position in the intact oligomers. Figure 5 displays the chemical structure of the monomers initially located at the non-reducing end (labeled [N]), the reducing end ([R]) and from between the two chain-ends ([B]). The mass of monomers denoted [R] is 16 Da higher than those denoted [N], which in turn is 14 Da higher than [B]. Due to these mass differences the three monomer types can be separated and identified in MS.

Figure 6 displays the MALDI mass spectrum where the peaks have been labeled according to Figs. 2 and 5. Due to that CM-oligomers with non-methyl esterified carboxyl groups were hydrolyzed, additional peaks originating from H/Na exchanged carboxyl groups were detected. These peaks (labeled + Na) were found at $\Delta m/z + n \cdot 22$ ($n = 1, 2, \ldots$) relative to the main series. However, the additional peaks did not interfere with the identification of the analytes.

In Fig. 6, intense peaks corresponding to non-substituted (1^0) and mono-substituted (1^1) monomers from all three monomer types were detected. In addition, intense peaks corresponding to di-substituted [B]-monomers were seen. However, no peaks corresponding to di-substituted [N]- or [R]-monomers were detected. The high intensity of the peaks corresponding to di-substituted monomers (1^2[B], 1^2[B] + Na and 1^2[B] + 2Na) compared to the non- and mono-substituted peaks is probably partly due to a high relative abundance and partly due to that the ionization yield increases with the number of substituents (Momcilovic et al. 2003a). Therefore, no conclusions on the quantities of the different monomers can be drawn from the mass spectrum. Nevertheless, the results from the monomer analysis

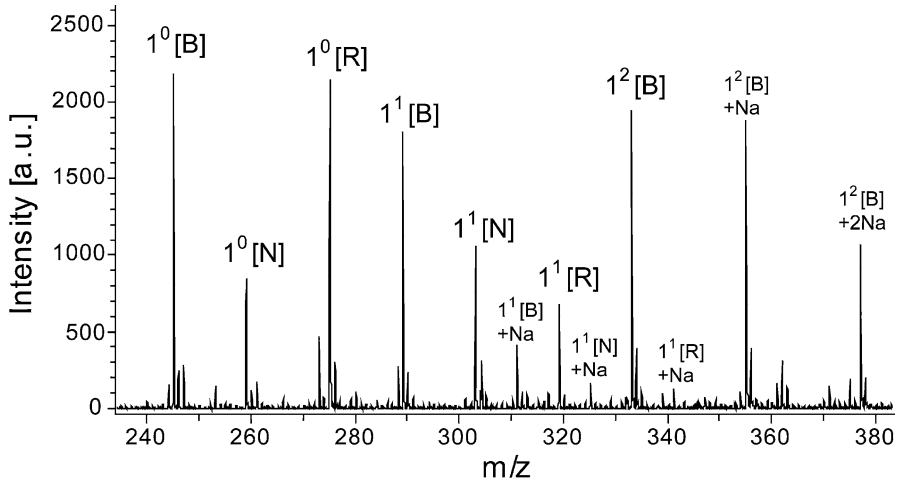

Fig. 6 MALDI mass spectrum of the monomers obtained from extensive acid hydrolysis (TFA, 2 mol L^{-1}, 90 °C, 6 h) of the reduced and permethylated CM-oligomers. The peaks have been labeled according to Figs. 2 and 5

of acidically hydrolyzed CM-oligomers supported the findings from MS/MS, i.e., that the reducing and non-reducing ends were at most mono-substituted.

Endoglucanase selectivity

A high viscosity grade (high molar mass) CMC was chosen for this study in order to make it possible to neglect the effect of the original end groups in the polymer (i.e., those present prior to the enzymatic hydrolysis). Therefore, it can be assumed that all end groups in the sample have been formed during the enzymatic hydrolysis and have, therefore, been in contact with the active site of the endoglucanase. Figure 7 illustrates the active site of an endoglucanase and the nomenclature of the sugar-binding subsites according to Davies et al. (1997).

The endoglucanase used in the present study (Tr Cel45A) has been suggested to be highly selective towards CMC (Karlsson et al. 2002a). In addition, the endoglucanase must bind to a cellulose segment longer than five AGUs in order to hydrolyze the substrate (Karlsson et al. 2002b). This latter suggestion was also supported by our findings, as peaks corresponding to cellotriose, cellotetraose and cellopentaose were detected in the MALDI mass spectrum (Fig. 2), but no peak corresponding to cellohexaose.

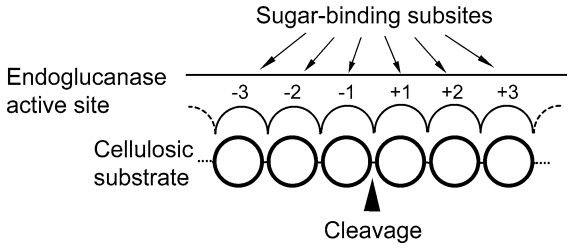

Fig. 7 Schematic illustration of the endoglucanase active site and nomenclature of the sugar-binding subsites involved with the enzyme–substrate interaction

Due to that the substrate–enzyme interaction can be hindered by the substituents, the endoglucanase hydrolysis of the glucosidic bonds in the CMC-substrate will be restricted to locations where there are no substituents, or where the substituents do not interfere with the enzyme–substrate interactions. Therefore, from chemical structure analysis of the enzyme product end groups we can obtain information on to what extent, the endoglucanase allows for carboxymethyl substituents at AGUs involved with the sugar-binding subsites.

MS/MS as well as acid hydrolysis followed by monomer analysis of the CM-oligomers strongly indicate that the terminal AGUs of the oligomers may contain up to one substituent. This suggests that the endoglucanase used in our study is able to hydrolyze the glucosidic bond adjacent to at least one mono-substituted AGU. However, we cannot determine from these results whether the endoglucanase is able to hydrolyze the glucosidic bond between two mono-substituted AGUs. Furthermore, there seems to be restrictions in the total number of substituents on the two terminal AGUs at either side of the cleavage point.

From the results we can conclude that Tr Cel45A does not require continuous non-substituted AGUs in order to hydrolyze the CMC-substrate, but rather has restrictions regarding the number of substituents

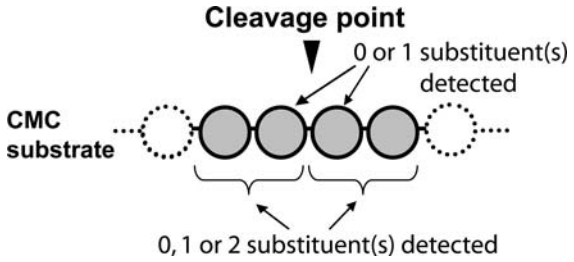

Fig. 8 Schematic illustration of the restriction in number of substituents at the AGUs closest to the glucosidic bond in order for the Tr Cel45A to hydrolyze the CMC-substrate, according to our results

allowed at (at least) the four AGUs closest to the glucosidic bond. The indicated restrictions regarding the number of substituents at these AGUs are illustrated in Fig. 8.

Conclusions

In this study we present a chemical sample preparation method for facilitated MS/MS of CM-oligomers from endoglucanase hydrolysis. We show that reduction of the anomeric carbon atom at the reducing end of the oligomers is required in order to differentiate between fragments containing the non-reducing and reducing ends of the intact oligomers. Reduction and permethylation followed by extensive acidic hydrolysis also allows for detailed monomer analysis where monomers located at different positions in the intact oligomers can be differentiated by mass spectrometry.

Tr Cel45A is able to hydrolyze glucosidic bonds adjacent to a mono-substituted AGU. This is in agreement with a recent study, where it was shown that some endoglucanases are able to hydrolyze glucosidic bonds adjacent to a mono-substituted AGU, if the substituent is located at the *O*-6 position (Horner et al. 1999). Furthermore, by taking into account the low number of substituents detected on the oligomers by both MALDI–TOFMS and ESI-MS/MS, it is conceivable to assume that several of the Tr Cel45A subsites are sensitive to substituted AGUs. This is a possible explanation for the apparent high selectivity of Tr Cel45A on CMC compared to other endoglucanases (Karlsson et al. 2002a).

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