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Enzyme-aided characterisation of carboxymethylcellulose

S. Horner[a], J. Puls[a,*], B. Saake[a], E.-A. Klohr[b], H. Thielking[b]

[a]*Federal Research Centre of Forestry and Forest Products, Institute of Wood Chemistry, D-21031 Hamburg, Germany*
[b]*Wolff Walsrode AG, D-29655 Walsrode, Germany*

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Abstract

Two conventionally prepared carboxymethylcelluloses (CMCs) with differing degrees of substitution (DS: 0.6 and 1.2) were fragmented by endoglucanase treatment until no further degradation was possible. This treatment brought about a decisive improvement in the water solubility of the polymers. The degraded samples were separated into 18 fractions by size exclusion chromatography (SEC). The quality of the preparative SEC was monitored by analytical SEC equipped with a multiple detector system. After acidic hydrolysis of each individual fraction, the carboxymethyl derivatives of the anhydroglucose units were determined by anion exchange chromatography and pulsed amperometric detection. The accessibility of endoglucanase action was clearly a function of the degree of substitution (DS). As the DS increased the efficiency of the enzyme became more limited. It could also be demonstrated that the polysaccharide chain of both CMC samples included some regions that were highly substituted and some other regions of low substitution. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Carboxymethylcellulose; Enzymes; Size exclusion chromatography

1. Introduction

Carboxymethycellulose (CMC) is technically the most important cellulose ether; it is widely used in detergents, oil exploration, and in the food, paper and textile industries. The most informative factor in characterising CMC is the degree of substitution (DS). Additional valuable information on the polysaccharide can be obtained from a knowledge of the distribution of the substituents within the anhydroglucose units and of their distribution along the cellulose chain. Until now it has only been possible to give reliable statements on the amount of the individual eight building blocks in CMC. Our knowledge on the evenness of distribution of the substituents along the polysaccharide chain is still incomplete, although information on this subject would be of major importance for the determination of structure–property relationships.

The classical methods for the determination of substituents within the anhydroglucose units are methylation analysis (Lindberg, Lindquist, & Stenberg, 1987) and ^{13}C NMR-spectroscopy (Nehls, Wagenknecht, Philipp, & Stscherbina, 1994). A simpler method, which has recently been introduced (Heinze, Erler, Nehls, & Klemm, 1994), consists of an acid hydrolysis step, followed by cation exchange chromatography to separate the glucose and mono-substituted, di-substituted and tri-substituted anhydroglucose units. In the meantime a direct HPLC-method for the separation of all possible carboxymethylated glucose units has become available (Kragten, Kamerling, & Vliegenthart, 1992). Instead of total hydrolysis, Arisz, Kauw, and Boon (1995) partially hydrolysed methylcellulose (MC) to obtain a mixture of monosaccharides, disaccharides and trisaccharides. The substituent distribution of the trimers was determined by FAB/MS. The results suggest that there are regions of high and low substitution within the MC samples investigated. According to our knowledge similar investigations have not yet been carried out for CMCs.

Based on our earlier work (Saake, Horner, & Puls, 1998), our method includes enzymatic fragmentation by endoglucanases instead of partial acid hydrolysis. Enzymatic methods have the advantage that they specifically cleave the polysaccharide within those regions that are either not substituted or only partially substituted. Moreover, this method allows for the option of subsequent treatment with exoglucanases to obtain more information on regions with a lower DS or to prepare highly substituted cellulose fragments. These fragments can then be analysed by means of the existing classical methods.

* Corresponding author. Bundesforschungsanstalt fur Forst- und Holzwirtschaft Institut fur Holzchemie und chemische Technologie des Holzes, 21027 Hamburg, Germany. Tel.: + 49-040-73962-0; fax: + 49-040-73962-296.

E-mail address: jpuls@aixh0501.holz.uni-hamburg.de (J. Puls)

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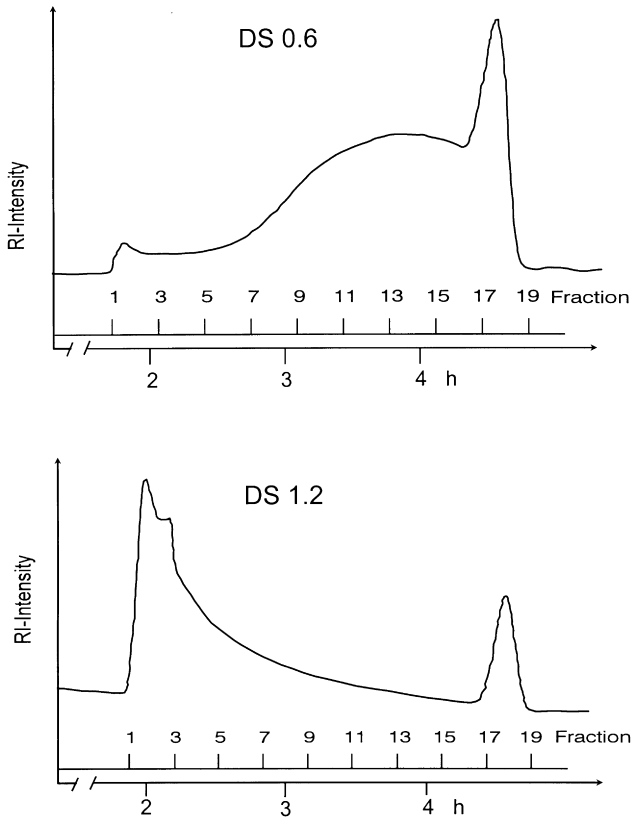

Fig. 1. Preparative size exclusion separation of endoglucanase fragmented CMCs.

2. Materials and methods

2.1. Carboxymethylcelluloses

The CMC preparations investigated were laboratory samples from Wolff-Walsrode AG (Walsrode, Germany). The CMCs were prepared by a heterogeneous reaction of alkaline cellulose with chloroacetic acid in 2-propanol. The DS was determined by titration.

2.2. Enzyme-aided fragmentation

The endoglucanase (EG) from *Humicola insolens* was a research preparation from Novo-Nordisk (Bagsværd, Denmark). The CMCs were incubated in 0.2% solutions with 10 000 nkat of EG per mg of sample for 92 h at 45°C. Enzyme activity was destroyed by boiling. The enzyme was cooled and then removed by centrifugation.

2.3. Analytical size exclusion chromatography

Hundred microlitres of 0.2% CMC samples were injected into thermostatically controlled (40°C) TSK-SEC columns (Toso Haas) coupled in line (TSK G5000PW_{xl}, G4000PW_{xl}, G3000PW_{xl}, each 300 × 7.8 mm^2 and a G2500PW_{xl} guard column, 40 × 6 mm^2). The eluent was 0.1 M $NaNO_3$ in water (0.4 ml/min). The multi-detection system consisted of a refractive index detector (Shodex RI-71, Showa-Denko), a two-angle light scattering detector (Precision Detector PD 2000), and a viscosity detector (Viscotek H502). The molar mass of the starting materials was determined by light scattering. This detector was also used for monitoring the presence of aggregates in samples fragmented by endoglucanase, whereas molar masses of fragmented materials were determined with the viscosity detector by applying the universal calibration curve based on dextran standards (Fluka). The software WINGPC 4.0 from Polymer Standard Service (PSS) was used for data capture and evaluation of the results. Low-molar-mass fragmentation products of CMC with indistinct viscosity signals were characterised by the molar masses of dextran equivalents from a conventional calibration curve. Fragmented samples were distinguished by extremely broad dispersities. In order to give adequate consideration to the low-molar-mass portions in the evaluation of these samples, the light scattering and viscosity signals were extrapolated by applying compensation curves in the low-molar-mass regions.

2.4. Preparative size exclusion chromatography

Five millilitres of the EG-fragmented CMCs were injected into an size-exclusion chromatography (SEC) column (HiLoad 26/60 Superdex 75, Parmacia Biotech). The mobile phase was 0.1 M ammonium acetate (1.1 ml/min). A refractive index detector was used (ERC-7511, Erma). The original sample was separated into 17–18 fractions.

2.5. Acid hydrolysis

0.02 ml 70% $HClO_4$ was added to 1 mg of each individual fraction from the preparative SEC. The pre-hydrolysis step was conducted at room temperature for 10 min. The hydrolysate was later diluted with 0.2 ml water and post-hydrolysed at 120°C for 60 min. After it had been cooled, the sample was neutralised with 2 M KOH. The precipitated $KClO_4$ was separated by centrifugation. The supernatants were transferred into 1 ml volumetric flasks.

2.5.1. HPLC of monosaccharides:

The eight building blocks of CMC were analysed by HPLC (Dionex Corp.) using a CarboPak™ PA 1 column (9 × 250 mm). In order to achieve adequate separation, a linear gradient was applied, which changed from 95%: 5% A (0.1 M NaOH)–B (1 M NaOAc in 0.1 M NaOH) to 100% B within 15 min. After completion of this profile, 100% B was pumped for 2 min, before the column was re-equilibrated to the starting buffer. The flow rate was 3 ml/min. A gold electrode was used in the pulsed amperometric detector, and the following potential differences were applied: $E1$ 0.05 V and 300 ms; $E2$ 0.64 V and 120 ms, $E3$ to − 0.78 V and 120 ms.

Table 1
Molecular weight (M_w) and intrinsic viscosity [η] of CMC before and after incubation with purified endoglucanase

CMC	Starting material			Endoglucanase treated material		
	LS-M_w (g/mol)	[η] (ml/g)	M_w/M_n	Viscotek M_w (g/mol)	[η] (ml/g)	M_w/M_n
DS 0.6	249 000	750	1.4	10 700	8.4	1.5
DS 1.2	248 000	690	2.2	72 000	108	6.1

3. Results and discussion

3.1. Preparative size exclusion chromatography

Two CMC samples of DS 0.6 and 1.2 underwent intensive fragmentation by the action of endoglucanase. Neither extending the incubation period nor adding fresh enzyme led to any further degradation of the polysaccharides. The degraded samples were then separated into 18 fractions by preparative SEC (Fig. 1). A comparison of both elution curves suggest that the accessibility for endoglucanase attack is a function of the DS. As DS increases, the accessibility is reduced. The low-DS sample (DS 0.6) was fragmented so intensively that only a small polymeric portion was left (fractions 1–3). Short fragments, which also included monosaccharides, predominated (fractions 15–18). In contrast to the sample with DS = 0.6, the polymeric portion of the more highly substituted CMC sample (DS 1.2) prevailed. A comparative view of the elution curves confirmed the reduced efficiency of the enzyme towards the DS 1.2 CMC preparation, although low-molar-mass degradation products could be detected. This finding is a clear indication that regions with either no substitution or a lesser degree may exist within the polymer chain. The individual fractions from the preparative HPLC were used for further chemical and physical characterisations.

Table 2
Molecular weight (M_w) and intrinsic viscosity [η] of the individual fractions of CMC DS 1.2 after EG fragmentation and preparative SEC

Fraction	CMC DS 1.2		
	Viscotek M_w (g/mol)	[η] (ml/g)	M_w/M_n
1	122 600	248	1.93
2	58 200	110	1.72
3	45 900	52	1.26
4	32 800	24.6	1.52
5/6	29 800[a]	17.2	1.3
7/8/9	19 610[a]	11.8	1.2
10/11/12	10 500[a]	6.3	1.2
13/14/15	2600[a]	3.6	1.1
16	720[a]	3.9	1.2
17/18/19	690[a]	3.7	1.2

[a] Calculated with dextran calibration.

3.2. Analytical size exclusion chromatography

3.2.1. Starting materials

The CMC samples (DS 0.6 and 1.2) had a relatively narrow molar mass distribution without any low-molar-mass portions or impurities. There was only a small difference in the elution profiles (figure not shown here). The DS 0.6 sample was distinguished by a small shoulder in the low-molar mass flank, whereas a certain degree of tailing could be observed in the low molar mass flange of the DS 1.2 CMC. The average molar mass of both samples was about 250 000 g/mol. This value corresponds to a degree of polymerisation (DP) of 1180 for the DS 0.6 CMC and 980 for the DS 1.2 CMC. The intrinsic viscosities of the DS 0.6 and 1.2 samples amounted to 750 and 690 ml/g, respectively (see Table 1).

3.3. Endoglucanase fragmentation

The DS 0.6 CMC was degraded much more thoroughly than the DS 1.2 sample due to the better accessibility of endoglucanase owing to the reduced number of substituents (Saake et al., 1998). There was a strong shift to the low-molar-mass region for the fragmented DS 0.6 sample, so that strong signals of oligomeric degradation products could be visualised. The average molar mass of the sample was reduced to 10 700 g/mol by the endoglucanase treatment. The molar mass of the DS 1.2 sample was also reduced. The corresponding value was 72 000 g/mol (see Table 1).

3.4. SEC analysis of the individual fractions

The quality of the preparative SEC was checked by carrying out analytical SEC on each individual fraction. It was indeed found that the polymeric fractions of the fragmented DS 1.2 CMC were separated according to SEC principles. On-line viscosimetry was able to demonstrate that these values were halved from one fraction to the next (Table 2). The individual fractions of this CMC had rather narrow elution curves; the RI signals are shown in Fig. 2. The amount of CMC material collected in the medium fractionation range of the preparative column was too low for an additional check using analytical SEC. Consequently, beyond fraction 5 up to three fractions had to be combined. Up to fraction 4 it was possible to base evaluation of the analysis on the viscosity signal: the CMC material of

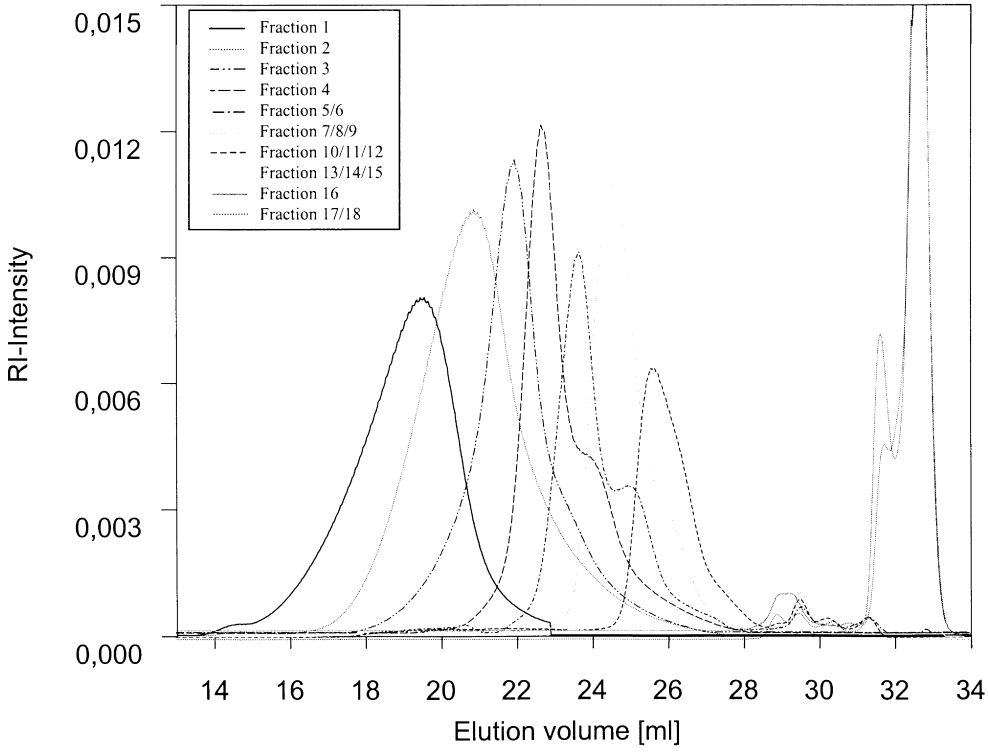

Fig. 2. Analytical SEC analysis of individual fractions of CMC (DS 1.2) after EG fragmentation and preparative SEC.

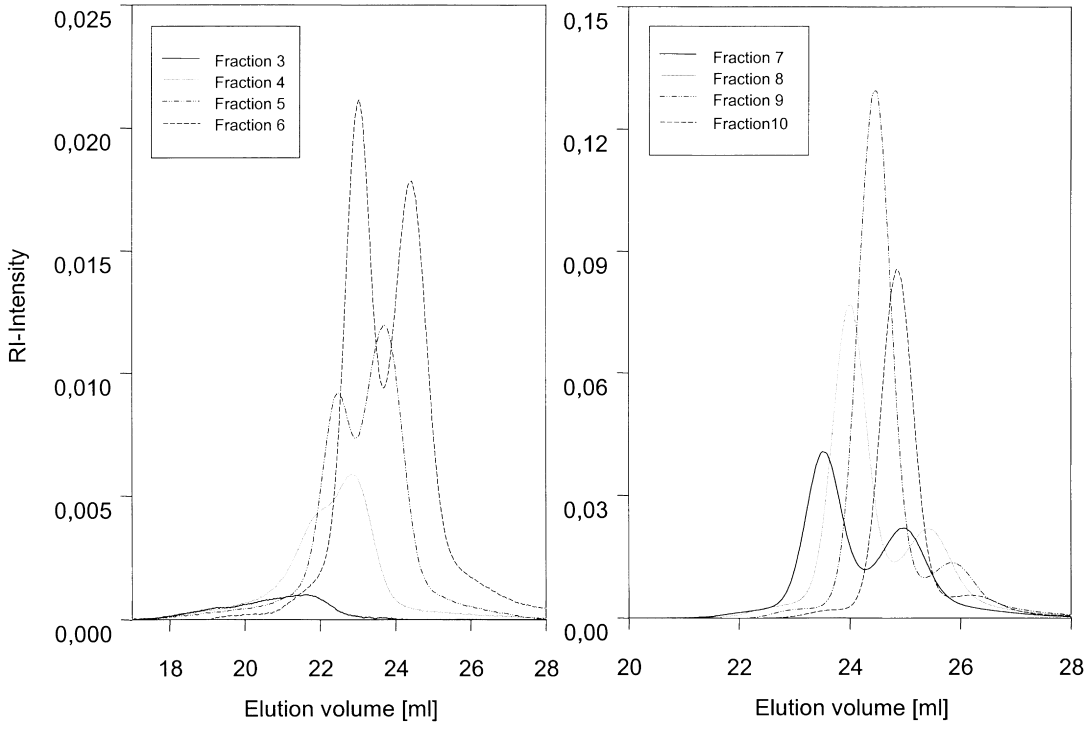

Fig. 3. Analytical SEC analysis of individual fractions of CMC (DS 0.6) after EG fragmentation and preparative SEC.

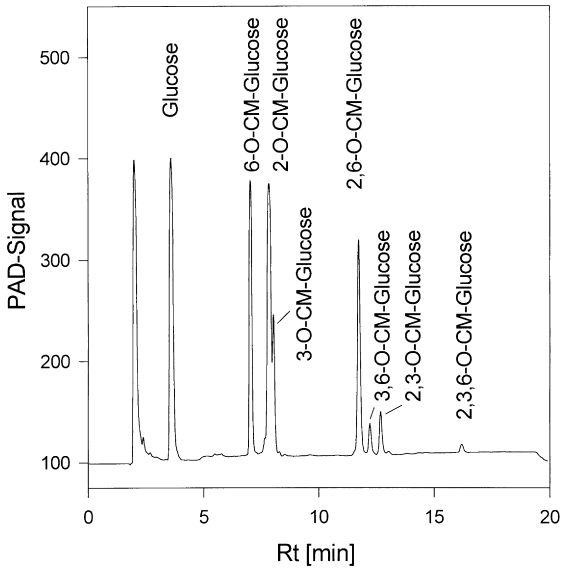

Fig. 4. AEC-PAD separation of an acidic hydrolysate of DS 1.2 CMC.

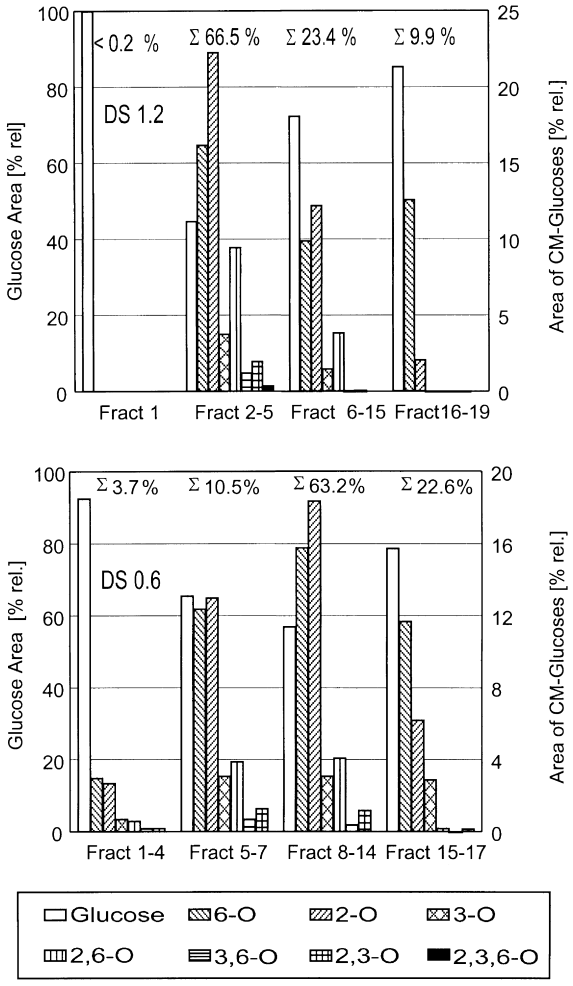

Fig. 5. AEC-PAD analysis of the CMCs (DS 0.6 and 1.2) after EG fragmentation, preparative SEC and acid hydrolysis of the individual fractions.

fraction 1 had a molar mass of 122 600 g/mol and an intrinsic viscosity of 248 ml/g. A continuous decrease in molar mass and intrinsic viscosity was measured for all fractions up to and including fraction 4 (32 800 g/mol and 24.6 ml/g). The viscosity signal of the subsequent fractions could not be included in this study due to insufficient signal intensities of the detector. The molar masses indicated for these fractions refer to a calibration using dextran standards. In some cases elution curves exhibit some shoulders, which are due to several fractions having been recombined. However, the chromatograms demonstrate that preparative SEC, as would be expected, functions according to the size exclusion principle. Effects based on loading mechanisms can only be proposed for the low molar mass fractions.

No analytical SEC chromatograms could be obtained from the material in fractions 1 and 2 of the DS 0.6 CMC sample. The CMC fragments eluted first from the preparative SEC were rather low in DS and could not be redissolved after freeze drying. One possible cause for this may lie in the inactivity of endoglucanase towards crystalline material. The material may have been less accessible to endoglucanase fragmentation due to the presence of morphological cellulose structures (aggregates), which did not take part in the carboxymethylation step. Another reason for this phenomenon could be the presence of gels/associates, which might have been formed during derivatisation, or in the course of the enzymatic fragmentation, from fragments having a lower DS and reduced water solubility. Fractions 3–7 have a lower than average DS. These fractions are distinguished by pronounced shoulders and a bimodal elution curve, both of which are strong indicators of problems in the preparative SEC. A constant improvement of the preparative SEC is demonstrated by the more highly substituted fractions 8–10 and beyond (Fig. 3). The other fractions not shown here have elution curves which are analogous to those from the DS 1.2 CMC sample. Due to the low molar masses and a limited amount of fractionated material it was not possible to base any calculations on the viscosity.

3.5. Anion exchange chromatography of individual fractions

Each fraction from the preparative SEC was hydrolysed with perchloric acid to characterise and to compare the fractions. The eight monomeric units, namely glucose, 2-*O*-, 3-*O*-, 6-*O*-, 2,3-*O*-, 2,6-*O*-, 3,6-*O*-, and 2,3,6-*O*-carboxymethylglucose were separated by anion exchange chromatography in NaOH medium, combined with amperometric detection (AEC-PAD). Fig. 4 is an example of a separation of a DS 1.2 CMC hydrolysate. On average each glucose unit should be substituted by a carboxymethyl substituent. The presence of glucose, di-substituted and even tri-substituted building blocks is a strong indication of the wide variation in the substitution of this CMC sample.

Preliminary evaluations of AEC-PAD separations

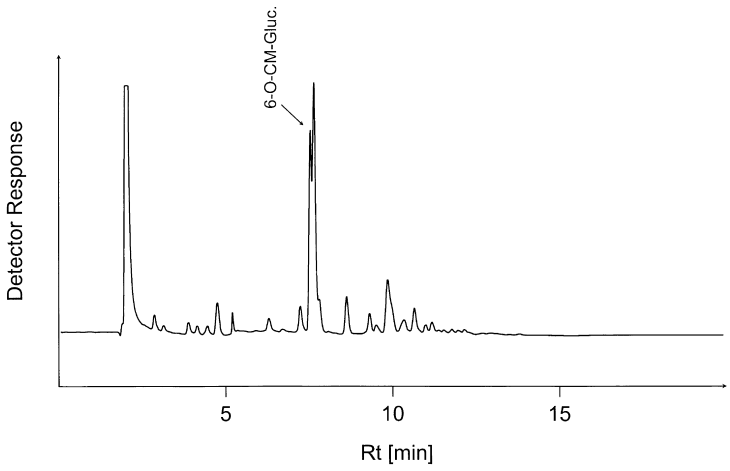

Fig. 6. AEC-PAD analysis of the fractions 16–18 without a prior hydrolysis step.

revealed that neighbouring fractions from preparative SEC gave comparable results. These fractions were therefore combined into one bar chart for greater clarity (Fig. 5). The proportion of these combined fractions was calculated from the RI signal of the preparative separation; the values are given at the top of the bar chart. The principal proportion (63.2%) of the DS 0.6 CMC was found in fractions 8–14. For the DS 1.2 sample the main proportion (66.5%) was concentrated in the high-molar-mass fractions 2–5. The fractions representing the medium separation range of the column (fractions 6–15) were only made of 23.4% of the initial mass. The low-molar-mass fraction of the DS 0.6 CMC consisted of 22.6% of the initial material. Due to the reduced accessibility of the DS 1.2 CMC for endoglucanase the low-molar-mass proportion of this sample amounted to only 9.9%.

In both CMC samples more than 60% of the material had an above-average DS (fractions 8–14 for the DS 0.6 sample; fractions 2–5 for the DS 1.2 sample).

Surprisingly, the proportion of non-substituted glucose units was somewhat higher in those fractions which were first eluted from the preparative SEC column compared with the following fractions. The quantity of this material derived from the DS 1.2 CMC was lower when compared with that from the DS 0.6 CMC. The latter CMC had a minor proportion of substituted glucose units, not only in fractions 1–4, but also in the subsequent fractions 5–7. The results from analytical SEC point to the presence of aggregated and gel-like particles in those fractions that were not separated in accordance with regular SEC principles. However, it was not possible to clarify whether these fractions were cut out of the polymer chain or whether this material was present in the CMC samples from the very beginning.

The proportion of glucose and the seven individual glucose derivatives shown in Fig. 5 have been calculated using the response factor for glucose due to the lack of reference material. The absolute values for the individual carboxymethylated glucoses may differ from those obtained in the calculation presented here. One possible approach would have been to use the response factors given by Kragten et al. (1992) based on combined AEC-PAD and NMR investigations. Results showed, however, that the response factors could not simply be transferred from one AEC-PAD system to the other. This is the reason why we started preparative work on isolating the corresponding glucose derivatives to be used for calibration purposes.

Although an absolute determination of the individual components of CMC was not possible, it was demonstrated that 6-*O*-carboxymethylglucose and 2-*O*-carboxymethylglucose were the major elements in all fractions. 3-*O*-carboxymethylglucose was only found in small quantities. Within the class of di-substituted modules 2,6-*O*-carboxymethylglucose predominated. 2,3,6-*O*-Carboxymethylglucose could only be detected in fractions 2–5 of the more highly substituted CMC. An analysis of the individual SEC fractions of the enzymatically fragmented CMCs was able to show that the DS was steadily reduced as the chain length decreased. This was especially true for the DS 1.2 sample. In fractions 2–5 all possible building blocks could be detected, whereas fractions 16–18 consisted only of 6-*O*- and 2-*O*-carboxymethylglucose in addition to the non-substituted unit. These fractions were also directly analysed by AEC-PAD without a prior hydrolysis step. 6-*O*-carboxymethylcellulose was detected as a direct degradation product of the endoglucanase action (Fig. 6). This finding is a clear indication that endoglucanase hydrolysis may not be limited to positions with two or more non-substituted modules. Apparently the enzyme may tolerate carboxymethylated glucose units provided that they are substituted in position 6. This results supports the findings of Kondo and Nojiri (1994), who were able to show that 6-*O*-methylcellulose was degraded by the cellulase of *Trichoderma reesei* whereas 2,3-di-*O*-methylcellulose was not.

4. Conclusion

A method for the detailed analysis of CMC has been developed, which includes an enzymatic fragmentation, a preparative separation according to molar mass, a further conversion of the fragments by acid hydrolysis and a final analysis step by anion exchange chromatography. Due to the fact that endoglucanase action is restricted to the lesser substituted regions, this enzyme constitutes a specific tool for characterising the substituents along the polymer chain. It was demonstrated that two conventionally prepared CMC samples included regions of high and of low substitution. As the density of substitution increased, the efficiency of the enzyme became more limited. Consequently, the DS 1.2 CMC was considerably less fragmented than the DS 0.6 CMC. Nevertheless low-molar-mass degradation products could also be detected in the DS 1.2 hydrolysate.

On the basis of the results from the analytical SEC and the AEC-PAD analysis of the individual fractions, it can be concluded that aggregated or gel-like particles were eluted in the void volume of the preparative SEC column.

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