ELSEVIER

Carbohydrate Polymers 46 (2001) 227–233

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Determination of the degree of polymerisation of carboxymethyl cellulose by size exclusion chromatography

M. Melander*, T. Vuorinen

Laboratory of Forest Products Chemistry, Helsinki University of Technology, P.O. Box 6300, FIN-02015 HUT, Espoo, Finland

Received 13 July 2000; accepted 14 September 2000

Abstract

A size exclusion chromatographic (SEC) system was developed in order to investigate the molecular weight distribution of depolymerisation products obtained by enzymatic degradation of carboxymethyl cellulose (CMC). The SEC system, which relies on a Superose 12 HR 10/30 column and an eluent containing sodium hydroxide (0.05 M), enabled an effective separation of CMC fragments. Cellulase was used for the degradation of CMC. Degradation was followed as a function of incubation time and cellulase dosage by viscosity measurements.

The CMC hydrolysates separated by SEC were detected as total organic carbon (TOC) and reducing sugars. The degree of polymerisation (DP) of the hydrolysates was then determined by dividing the number of anhydroglucose units by the reducing sugar concentrations. The DP of the CMC fragments obtained after enzymatic hydrolysis ranged from 100 to 1, compared with a DP_v of 170 for untreated CMC samples. The results obtained for a series of CMC fragments agreed with the DP values of standards of known molecular weight. The method is an absolute method; no external calibration was needed to calibrate the Superose 12 HR 10/30 column.

Keywords: Carboxymethyl cellulose; Cellulase; Degree of polymerisation; Enzymes; Reducing sugars; Size exclusion chromatography; Total organic carbon

1. Introduction

Carboxymethyl cellulose is the most widely used cellulose ether today, with applications in the detergent, food, oil exploration, paper, textile, pharmaceutical and paint industries. Because of the different demands on the CMC in these industries, it is important to know the molecular weight distribution (MWD). The SEC method continues to be very useful for MWD analysis of CMC as well as other water-soluble polymers. Numerous articles have been published on aqueous SEC of CMC (Eremeeva & Bykova, 1998; Rinaudo, Danhelka, & Milas, 1993).

One drawback of the SEC method is that it requires column calibration with materials of known molecular weight in order to determine the MWD of polymers. This is generally done with use of a universal calibration procedure, which employs Mark-Houwink coefficients derived from the polymers under investigation and the polymers used in the calibration (Nilsson & Nilsson, 1974). The usual standards for the calibration of SEC columns are globular proteins and dextrans (Hamacher & Sahm, 1985; Horner, Puls, Saake, Klor, & Thielking, 1999; Vuorinen & Alén, 1999). A relevant point in regard to dextrans is that they are more flexible than cellulose and may not accurately represent the hydrodynamic behaviour of cellulose (Wu, 1995).

Degree of polymerisation and M_w are typically determined by light scattering, sedimentation or vapour pressure osmosis (viscosity) techniques (Klemm, Philipp, Heinze, Heinze, & Wagenknecht, 1998).

The aim of our work was to develop a chromatographic method to determine the degree of polymerisation of carboxymethyl cellulose after enzymatic degradation with commercial cellulase. A size exclusion chromatographic system with continuous total organic carbon and reducing sugar detection was developed for the purpose.

2. Materials and methods

2.1. Carboxymethyl cellulose

The commercial and water soluble sodium carboxymethyl cellulose (NaCMC) used in the experiments was Finnfix BW-2 (Metsa Specialty Chemicals Oy) with a degree of substitution (DS) of 0.57. The NaCMC content was about 76% (dry basis), containing after acid metanolysis 13.9% glucose and

* Corresponding author. Tel.: +358-9-451-4231; fax: +358-9-451-4259.
E-mail address: minna.melander@hut.fi (M. Melander).

0144-8617/01/$ - see front matter
PII: S0144-8617(00)00303-9

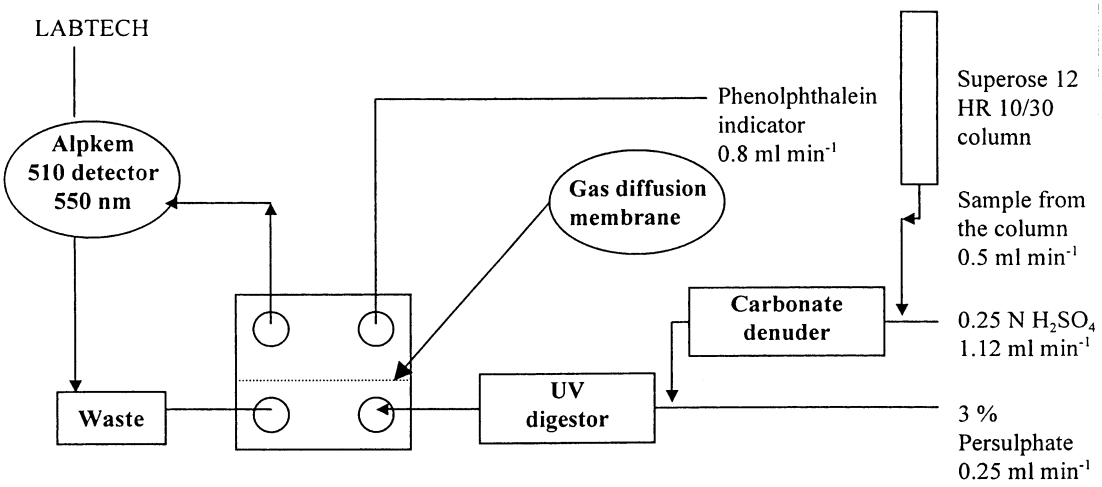

Fig. 1. Flow diagram for determining the molecular weight distribution and TOC of CMC fragments.

6.7% xylose (Holmbom & Örså, 1993). The samples of NaCMC were dissolved in water and filtered through a 0.2 μm filter before SEC analysis.

2.2. Enzymatic hydrolysis of CMC

Low molecular weight salt in the sodium carboxymethyl cellulose solution used in the experiments was removed by dialysis (Thomas Scientific®, pore size 6000–8000 Da) before the enzymatic hydrolysis. The purified CMC solution (0.3% determined by the phenol–sulphuric acid method) was then hydrolysed with commercial *Trichoderma longibrachiatum* cellulase (Econase®CE, Röhm Enzyme Finland Oy). The cellulase activity (15.000 ECU/g) was determined by a method developed by Bailey and Nevalainen (1981). The enzymatic hydrolysis was carried out at 0.3% consistency at 50°C for 3 and 24 h. The pH of the samples was 5.5. Both low (74 nkat/g of the dry weight of the sample) and high (1477 nkat/g of the dry weight of the sample) enzyme dosages were used in the treatments. After an appropriate incubation time, the enzyme was denatured by heating the CMC samples at 100°C for 15 min.

The concentration of CMC in the solution before and after the enzymatic treatment was determined by phenol–sulphuric acid assay (Dubois, 1956).

2.3. Viscometry

The decrease in the degree of polymerisation before and after the enzymatic treatment was followed by viscosity measurements. Dynamic viscosity determination was carried out with a capillary viscometer AVS 350 at 25 ± 0.1°C.

2.4. Size exclusion chromatography with TOC detection

The molecular weight distributions of the CMC hydrolysates, filtered through a 0.2 μm filter, were determined by SEC on a Superose 12 HR 10/30 column (Pharmacia Biotech, Sweden), made from 12% highly cross-linked porous agarose (Andersson, Carlsson, Hagel, & Pernemalm, 1985). Sodium hydroxide (0.05 M) was used as eluent at a rate of 0.5 ml/min. The injection volume was 200 μl. The samples emerging from the column were acidified with sulphuric acid to convert inorganic carbon to carbon dioxide, which was then removed by membrane gas diffusion. The samples, now free of carbon dioxide were then

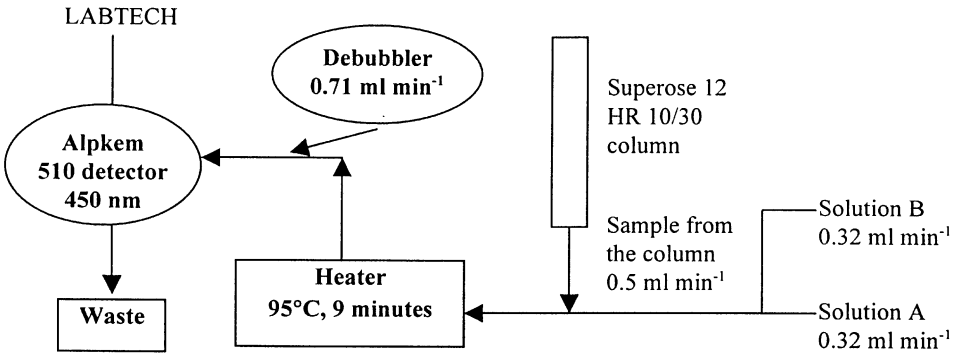

Fig. 2. Flow diagram for determining molecular weight distribution with the continuous detection of reducing sugars.

Table 1
Enzymatic treatments of CMC and their viscosities (capillary viscometer)

Enzymatic treatment	Viscosity (mPa, 25°C)
CMC	4.06
CMC, dialysed	3.67
1477 nkat/g, 24 h, 50°C	0.94
74 nkat/g, 24 h, 50°C	1.01
1477 nkat/g, 3 h, 50°C	0.93
74 nkat/g, 3 h, 50°C	1.05

mixed with a persulphate-solution ($K_2S_2O_8$) and subjected to ultra-violet radiation digestion. During the digestion, organic carbon was converted to CO_2 (Nordin, 1983). The generated CO_2 was diffused through a silicon membrane into a weakly buffered phenolphthalein indicator containing buffer (Na_2CO_3, $NaHCO_3$), phenolphthalein ($C_{20}H_{14}O_4$, methyl alcohol) and detergent (Triton X-100, isopropyl alcohol) solutions. The decrease in the colour intensity of the indicator, which is proportional to the carbon concentration, was monitored continuously with an Alpkem 510 detector at 550 nm (Mitikka-Eklund, Halttunen, Melander, Ruuttunen, & Vuorinen, 1999; Suurnäkki, Li, Buchert, Tenkanen, Viikari, Vuorinen & Odberg, 1997). The data were collected with a LABTECH program. The flow diagram for this system is illustrated in Fig. 1.

2.5. Size exclusion chromatography with determination of reducing sugars

The reducing sugars were determined by the neocuproine assay described by Chaplin (1986). The filtrate (200 μl) containing CMC was eluted through a Superose 12 HR 10/30 column (Pharmacia Biotech, Sweden) with 0.05 M NaOH at a rate of 0.5 ml/min. The reagents A (Na_2CO_3, glycine, $CuSO_4{\cdot}5H_2O$, water) and B (neocuproine·HCl and water + Triton X-100 and isopropyl, 1:1) and the samples from the column were eluted into the flow analyser and heated for about 9 min at 95°C. Reducing sugars of the samples were monitored continuously with an Alpkem Flow Solution analyser at 450 nm. The data were collected with a LABTECH program. A flow diagram of the system is shown in Fig. 2.

3. Results and discussion

The SEC system using the column Superose 12 HR 10/30 and an eluent containing sodium hydroxide allowed an effective chromatographic separation of CMC fragments obtained by enzymatic degradation. Earlier work has shown that cleavage of the polymer chain with enzymes is less effective at higher degree of substitution, leaving fragments with a higher degree of polymerisation (Gelman, 1982; Hamacher & Sahm, 1985; Horner et al., 1999). Eremeeva and Bykova (1998) have shown that MWD analysis of CMC is preferably carried out under alkaline rather than acidic conditions because there are no undesirable effects and samples with higher M_w can be analysed.

The degradation of the molecule by enzymatic hydrolysis was followed by dynamic viscosity measurement. The viscosities before and after the enzyme treatment are

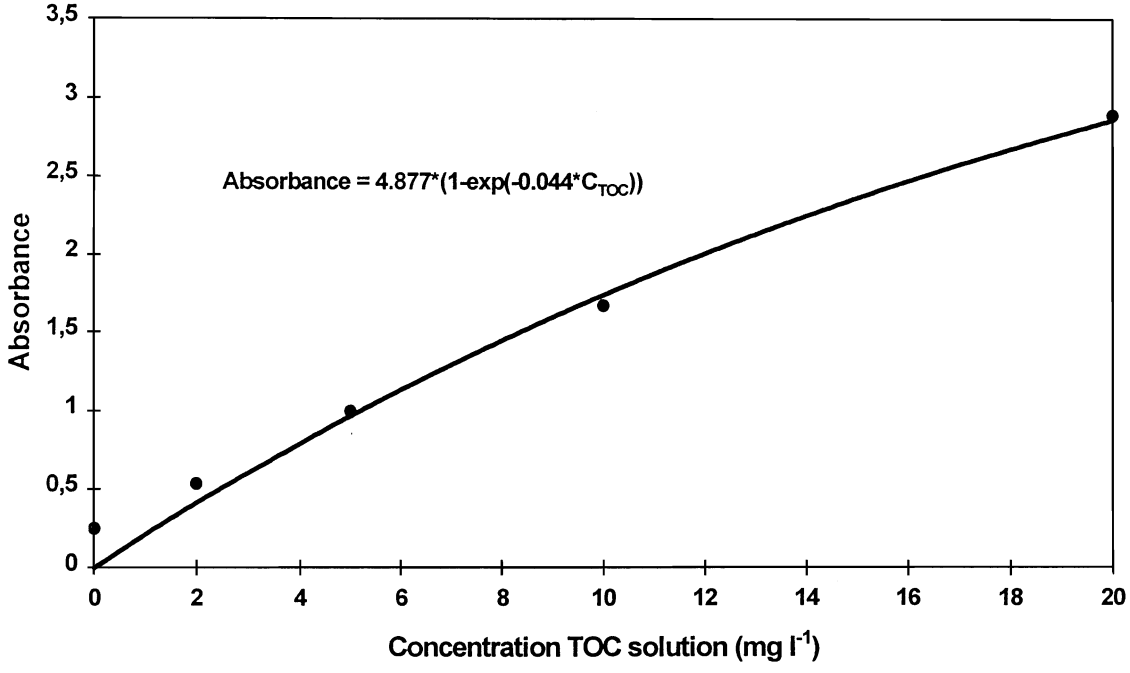

Fig. 3. SEC calibration curve derived with a TOC solution as standard; column Superose 12 HR 10/30, eluent NaOH and flow rate 0.5 ml/min. Correlation coefficient 0.981 for absorbance = $4.877(1 - \exp(-0.044C_{TOC}))$.

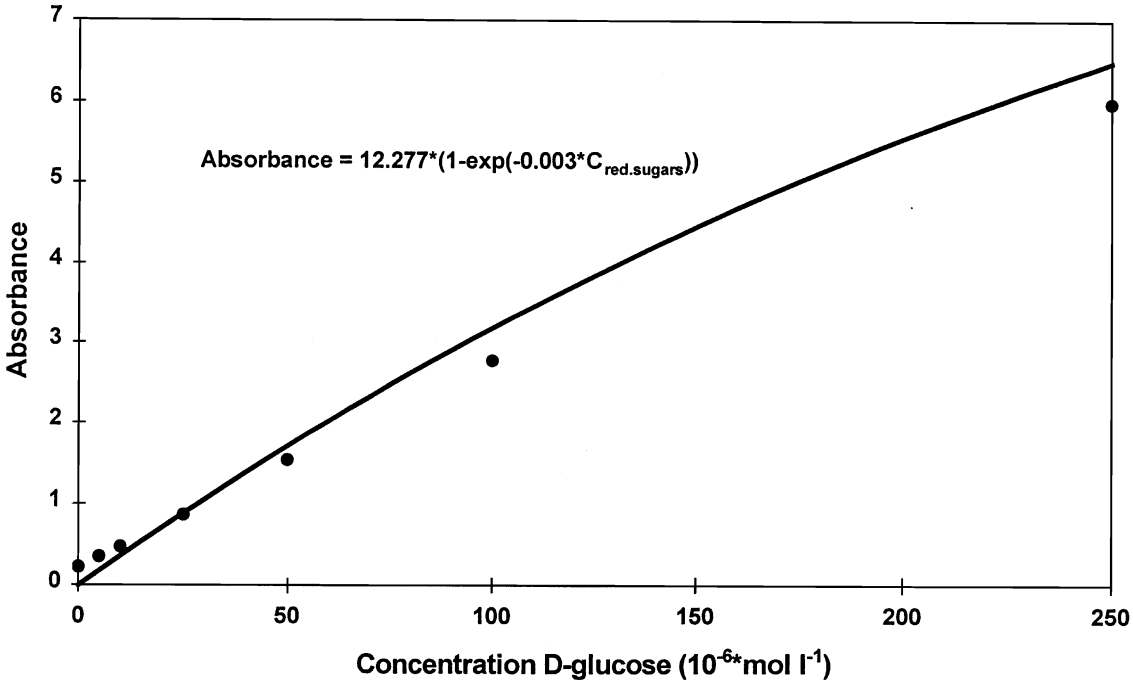

Fig. 4. SEC calibration curve prepared with D-glucose standard; column Superose 12 HR 10/30, eluent NaOH and flow rate 0.5 ml/min. Correlation coefficient 0.995 for absorbance $= 12.277(1 - \exp(-0.003C_{\text{red.sugar}}))$.

presented in Table 1. As can be seen, the enzymatic treatment split the linkages between the glucose units, which led to a decrease in viscosity.

The SEC system with TOC detection illustrated in Fig. 1 was calibrated with a TOC solution containing potassium hydrogen phthalate ($C_6H_5KO_4$), sulphuric acid (H_2SO_4) and deonized water. The calibration curve (Fig. 3) was expressed by Eq. (1), calculated by fitting the calibration data to a non-linear regression on SYSTAT software (Systat Inc.). The TOC concentrations of the CMC samples were

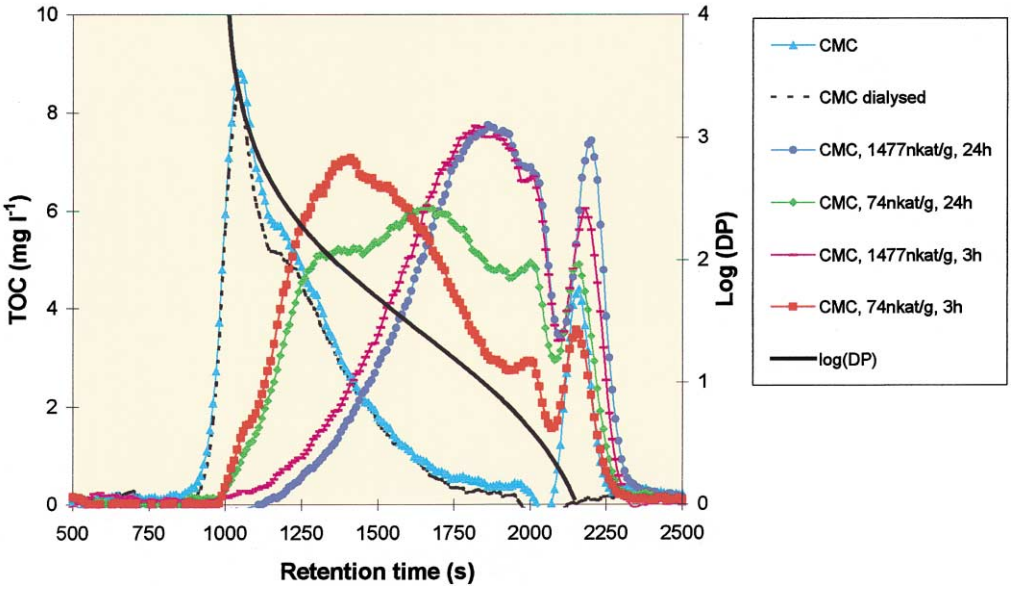

Fig. 5. Plot of TOC concentrations versus retention time for CMC fragments eluted (0.05 M NaOH) through the Superose 12 HR 10/30 column. The retention time for the glucose peak (DP 1) is at about 2200 s and for cellopentaose (DP 5) at about 2060 s (data not shown). The solid line denotes to the calculated selectivity curve of the column.

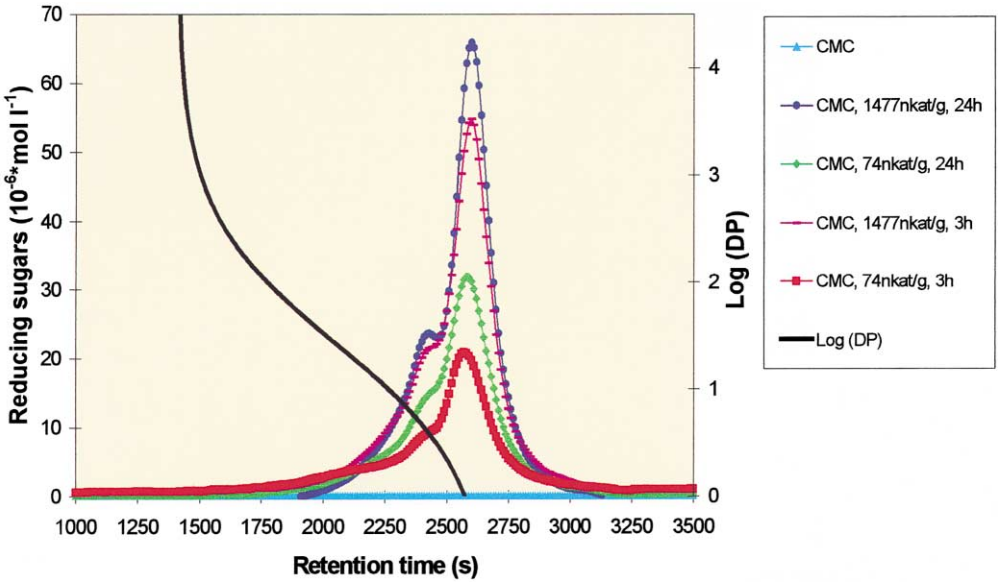

Fig. 6. Plot of reducing sugars versus retention time for CMC fragments eluted through the Superose 12 HR 10/30 column. The peak of glucose (DP 1) is at about 2620 s and for cellopentaose (DP 5) at about 2480 s (data not shown). The solid line signifies to the selectivity curve of the column.

then calculated with this equation.

$$C_{\mathrm{TOC}} = \ln(4.877(4.877 - \text{absorbance})^{-1})(0.044)^{-1} \quad (1)$$

The reducing sugar concentrations of the CMC samples were estimated with the calibration Eq. (2). D-Glucose was used as calibration solution (Fig. 4) and the equation was calculated with SYSTAT software (Systat Inc.).

$$C_{\mathrm{red.sugar}} = \ln(12.277(12.277 - \text{absorbance})^{-1})(0.003)^{-1} \quad (2)$$

Fig. 5 shows how the sodium in the commercial NaCMC solution is effectively removed by dialysis. Furthermore, it can be seen from the selectivity curve (Eq. (5)) that the allowed exclusion limit of the column is exceeded for large molecular weight CMC fragments with a retention time close to 1000 s. In other words the DP value of the untreated CMC sample could not be defined, even if the second major peak at 2.2 (retention time 1200 s) gave a DP value of about 160, which corresponded quite well with the DP_v of 170, which was reported by Mitikka-Eklund et al., 1999. The CMC fragments (DS 0.57) obtained after enzymatic hydrolysis had a DP ranging from about 100 to 1. Larger enzyme dosages and longer incubation times resulted in smaller molecular weights. When the enzymatic dosage was 1477 nkat/g and the hydrolysis was carried out

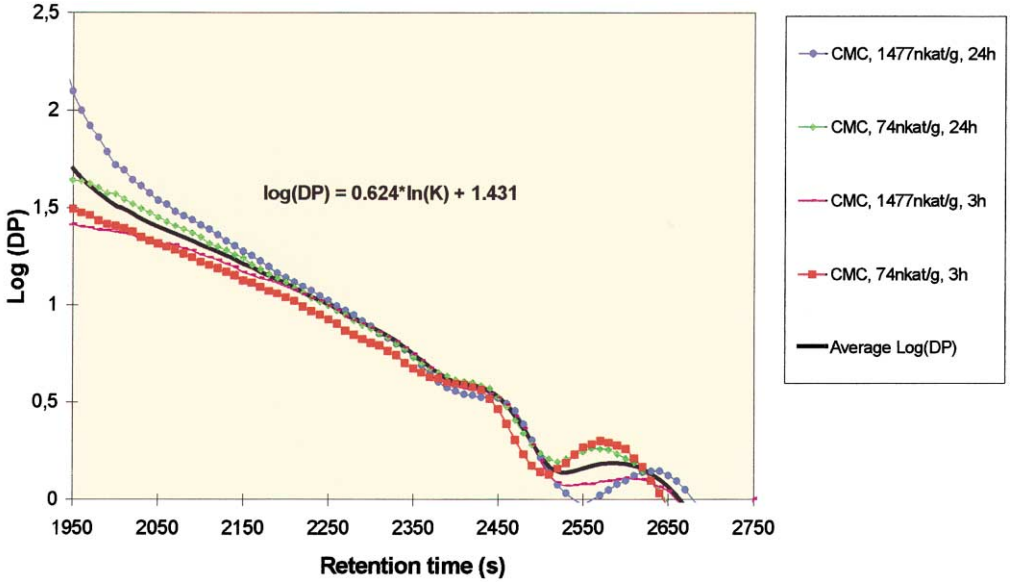

Fig. 7. The average log (DP) calculated from the experimental results. Correlation coefficient 0.993 for log (DP) = $0.624 \ln[(t_t - t)(t - t_0)^{-1}] + 1.431$.

for 24 h, we can assume that the CMC degradation was nearly complete. Fig. 5 shows that the glucose peak with retention time of 2100–2300 s is stronger for the more efficient enzyme treatment.

Fig. 6 shows the molecular weight distributions and reducing sugars for CMC treated with cellulase under different conditions. As can be seen, degradation was greater for enzyme dosage 1477 nkat/g sample than for the dosage 74 nkat/g sample and more reducing sugar was obtained. The decrease in sensitivity with increased molecular weight is also clear. The major peaks of the reducing sugars have the same retention time as glucose at 2620 s, suggesting that the DP must be about 1. The untreated CMC sample gave no response to reducing sugars suggesting that in this case there were no degradation of non-reducing sugar units, even if it is a well-known phenomenon that the chemicals used for the determination of reducing sugars may degrade oligosaccharides.

The approximate DP values of the CMC fragments were calculated with the following equations. First the number of anhydroglucose units was calculated by Eq. (3) and then the DP values were estimated by dividing the anhydroglucose units with the concentration of reducing sugar in Eq. (4). Before the DP values were calculated the retention times of the TOC spectra were corrected to the same retention times as for the reducing sugars by running glucose with both methods (difference 420 s).

$$C_{\text{anhydr.glc.}} = C_{\text{TOC}}(M_{\text{C}}(6 + 2\text{DS}))^{-1} \tag{3}$$

$$\text{DP} = C_{\text{anhydr.glc.}}(C_{\text{red.sugar}})^{-1} \tag{4}$$

where M_{C} denotes the atomic weight of carbon and DS the degree of substitution of CMC.

The calculated DP values of the CMC samples are presented in Fig. 7. As can be seen, a reliable curve fitting can be received only at a certain area of the curve when the retention times are between 1950–2500 s (maximum DP about 50). If the retention times go above 2500 and under 1950 s the calculated DP values were not reliable because the allowed sensitivity of the reducing sugar method was exceeded. The selectivity curve for the column (Figs. 5 and 6) follows the Eqs. (6) and (7) calculated by fitting the estimated DP values (Fig. 7) to non-linear regression on SYSTAT software (Andersson et al., 1985; Wu, 1995). The selectivity curve of the column denotes to the range of fractionation by molecular weight or in this case by the degree of polymerisation of the CMC samples.

$$K = (t_{\text{t}} - t)(t - t_0)^{-1} \tag{5}$$

$$\text{Log(DP)} = 0.624 \ln(K) + 1.431 \tag{6}$$

or

$$\text{Log}(M_{\text{W}}) = 0.623 \ln(K) + 3.718 \tag{7}$$

where t_0 and t_{t} correspond to elution times at the void and total volumes of the Superose 12 HR 10/30 column. The standards glucose and cellopentaose fit well the calculated selectivity curve 5 and 6.

4. Conclusions

The usefulness of size exclusion chromatography in the characterisation of CMC was exploited in the development of a new method for the determination of degree of polymerisation of CMC. In the method, CMC fragmented with cellulase is separated according to molecular mass by size exclusion chromatography and TOC and reducing sugars are detected in the CMC fragments. The advantage of the method is that the degree of polymerisation of the CMC fragments can be determined without the need for external calibration of the Superose 12 HR 10/30 column.

Acknowledgements

The authors thank Timo Pääkkönen for his excellent laboratory assistance. The work was financed by the Fibre Engineering programme of TEKES (National Technology Agency, Finland).

References

Andersson, T., Carlsson, M., Hagel, L., & Pernemalm, P-Å. (1985). Agarose-based media for high-resolution gel filtration of biopolymers. *Journal of Chromatography*, *326*, 33–34.

Bailey, M. J., & Nevalainen, K. M. H. (1981). Induction, isolation and testing of stale *Trichderma reesei* mutants with improved production of solubilizing cellulase. *Enzyme and Microbial Technology*, *3*, 153–157.

Chaplin, M. F. (1986). Monosaccharides. In M. F. Chaplin & J. F. Kennedy, *Carbohydrate analysis: a practical approach* (pp. 1–25). Oxford: IRL Press.

Conner, A. H. (1995). Size exclusion chromatography of cellulose and cellulose derivatives. In C. Wu, *Handbook of size exclusion chromatography* (pp. 331–352). New York: Marcel Dekker.

Dubois, M. (1956). Colorimetric method for determination of sugars related substances. *Analytical Chemistry*, *28*, 350–356.

Eremeeva, E. T., & Bykova, O. T. (1998). SEC of mono-carboxymethyl cellulose (CMC) in a wide range of pH; Mark-Houwink constants. *Carbohydrate Polymers*, *36*, 319–326.

Gelman, A. R. (1982). Characterization of carboxymethyl cellulose: distribution of substituent groups along the chain. *Journal of Applied Polymer Science*, *27*, 2957–2964.

Hamacher, K., & Sahm, H. (1985). Characterization of enzymatic degradation products of carboxymethyl cellulose by gel chromatography. *Carbohydrate Polymers*, *5*, 319–327.

Holmbom, B., &Örså, F. (1993). Methods for analysis of dissolved and colloidal wood components in papermaking process waters and effluents. *Seventh International Symposium on wood and pulping chemistry*, Beijing, China, Vol. 2 (pp. 810–817).

Horner, S., Puls, J., Saake, B., Klohr, A. -E., & Thielking, H. (1999). Enzyme-aided characterisation of carboxymethyl cellulose. *Carbohydrate Polymers*, *40*, 1–7.

Klemm, D., Philipp, B., Heinze, T., Heinze, U., & Wagenknecht, W. (1998). *Analytical methods in cellulose chemistry Comprehensive cellulose chemistry* (pp. 167–217). Weinheim: Wiley.

Mitikka-Eklund, M., Halttunen, M., Melander, M., Ruuttunen, K., &Vuorinen, T. (1999). Fiber engineering. *10th International Symposium on Wood and Pulping Chemistry* (pp. 432–439), Yokohama, Japan, Vol. I, Oral presentation.

Nilsson, G., & Nilsson, K. (1974). Molecular-weight distribution determination of clinical dextran by gel permeation chromatography. *Journal of Chromatography*, *101*, 137–153.

Nordin, P. (1983). Monitoring of carbohydrates with periodate in effluents from high-pressure liquid chromatography. *Analytical Biochemistry*, *131*, 492–498.

Rinaudo, M., Danhelka, J., & Milas, M. (1993). A new approach to characterising carboxymethyl celluloses by size exclusion chromatography. *Carbohydrate Polymers*, *21*, 1–5.

Suurnäkki, A., Li, Q-T., Buchert, J., Tenkanen, M., Viikari, L., Vuorinen, T., & Ödberg, L. (1997). Effect of enzymatic removal of xylan and glucomannan on the pore size distribution of kraft fibres. *Holzforschung*, *51* (1), 27–33.

Vuorinen, T., & Alén, R. (1999). Carbohydrates. In E. Sjöström & R. Alén, *Analytical methods in wood chemistry, pulping and papermaking* (pp. 37–71). Berlin: Springer.