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Characterization of Enzymatic Degradation Products of Carboxymethyl Cellulose by Gel Chromatography

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SUMMARY

In order to investigate the molecular weight distribution of depolymerization products obtained by enzymatic degradation of carboxymethyl cellulose (CMC), a high resolution size exclusion chromatographic (SEC) system was developed.

The SEC system using Fractogel® TSK-HW and an eluent containing sodium sulfate and sodium acetate enables an effective separation of the anionic cleavage products to be carried out. The experimental set-up was equipped with a sensitive detection system based on the post-column reaction of carbohydrates with orcinol.

The elution patterns of enzymatic depolymerization products obtained from CMC with different degrees of substitution make it feasible to infer parts of the sequence and the distribution of carboxymethyl groups.

INTRODUCTION

The physico-chemical properties of cellulose ethers, e.g. of sodium carboxymethyl cellulose, are dependent on the degree of polymerization (DP), the degree of substitution (DS) and the substituent distribution pattern. Although it is important for both users and producers to know something about the fine structure at the molecular level, relatively little work has been done in this field (Bhattacharjee & Perlin, 1971).

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The analytical methods mainly used for determining the substitution pattern of water-soluble cellulose derivatives such as CMC are based on measuring the change in viscosity and the amount of glucose and reducing low-molecular sugars formed by cellulolytic breakdown of the polymer chains (Eriksson & Hollmark, 1969; Lindner *et al.*, 1983). From data of this type only indirect conclusions about the length of cleaved polymer chains and the relative amount of the derivatized cellulose fragments can be drawn.

The aim of this paper was to develop a chromatographic method to separate enzymatic degradation products of CMC on the basis of their molecular size. The high resolution size exclusion chromatographic system presented in this work enables an effective separation of fragments formed by enzymatic hydrolysis of CMC and makes the investigation of the molecular weight distribution of different ionic cellulose ethers and their enzymatic degradation products feasible.

EXPERIMENTAL

Materials

The carboxymethyl celluloses used in these studies had similar degrees of polymerization (DP ~ 400–500) whereas the degrees of substitution (DS) were 0·47, 0·79, 1·09 and 1·34 (Hoechst AG, Frankfurt, Federal Republic of Germany).

The cellulolytic enzyme preparation of *Trichoderma viride* (QM9414) was obtained by ultrafiltration (Amicon, UM10) and lyophilization of the culture filtrate. The protein content of the enzyme preparation was 52%, measured by the Lowry procedure with bovine serum albumin as standard (Lowry *et al.*, 1951).

Enzymatic hydrolysis

The enzymatic digestion was performed in buffer medium at 50°C (sodium acetate, 50 mmol litre^{-1}, pH 4·8). To 10 ml of CMC solution (10 g CMC litre^{-1}) 100 μl of cellulase solution (5 mg lyophilisate ml^{-1}) was added and gently mixed for about 17 h. After the appropriate incubation time, the enzyme was denatured by heating the solution at 100°C for 10 min.

Size exclusion chromatography (SEC)

The hydrolysate obtained from enzymatic degradation of CMC was subjected to a chromatographic system using Fractogel® TSK HW-55(S) (E. Merck, Darmstadt, Federal Republic of Germany). The chromatographic set-up consisted of two water-jacketed glass columns (2·5 × 100 cm) and a short pre-column (2·5 × 30 cm; LATEK, Heidelberg, Federal Republic of Germany) connected in series by PTFE tubing. Columns were thermostated at 40°C and packed using the conventional slurry-packing technique. After equilibration, the total length of the gel bed was 210 cm, with a void volume (V_0) of 330 ml. The eluting solvent contained sodium sulfate (50 mmol litre^{-1}), sodium acetate (50 mmol litre^{-1}), pH 5, and sodium azide (0·02%). The flow rate was 0·9 or 1·2 ml min^{-1}. The columns were calibrated with dextran fractions of average molecular weight 5×10^5, 7×10^4, 10^4 g mol^{-1}(Pharmacia AB) maltoheptaose, maltopentaose, maltotetraose, cellobiose and glucose.

The detection of the carbohydrates relied on the post-column reaction with orcinol/sulfuric acid and photometric detection at 420 nm. The automated analyzing system (John & Dellweg, 1973) was assembled from modular components: a peristaltic pump (type PLG, Desaga, Heidelberg), a 15 m reaction glass coil in a 95°C heating bath (Haake N2B) and a photometer and recorder. Glass coils, fittings and acid resistant pump tubings were purchased from Technicon Corporation. The samples containing 10 mg CMC-fragments were applied via a sample loop size of 1 ml.

RESULTS

The high resolution SEC-system using Fractogel TSK HW-55(S) and an eluent containing the electrolyte components sodium sulfate and sodium acetate allowed an effective chromatographic separation of CMC fragments obtained by enzymatic degradation. Whereas dissociation of aggregated depolymerization products of carboxymethyl cellulose (CMC) occurred in the electrolyte solvent, anomalously high molecular weight distributions could be observed if distilled water was used as the eluent.

The molecular weight calibration shown in Fig. 1 appears to be linear for carbohydrates in the range 200 to about 2×10^5 g mol^{-1}. To

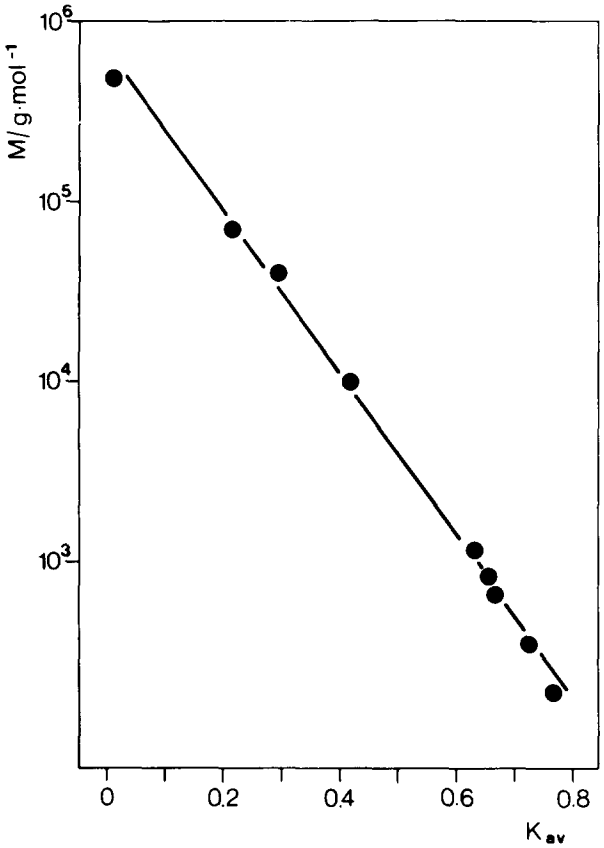

Fig. 1. Calibrating plot for determination of the molecular weight of carbohydrates in the range of ~200-2 x 10^5 g mol^{-1}: gel, Fractogel® TSK HW-55(S); gel bed, 2020 x 25 mm; column temperature, 40°C; flow rate, 0·9 ml min^{-1}.

demonstrate the effectiveness of the separation, the enzymatic degradation of CMC (DS 0·47) was followed using gel chromatography to characterize the depolymerization products. The elution pattern of the degraded CMC with a DS of 0·47 (Fig. 2, solid line) shows that the fragments obtained after enzymatic hydrolysis have a molecular weight ranging from about 200 g mol^{-1} (glucose) to about 1·8 X 10^3 g mol^{-1} (substituted oligomers). Fragments with a higher degree of polymerization could only be observed in low amounts. As expected from the decrease in specific solution viscosity during enzyme digestion experi-

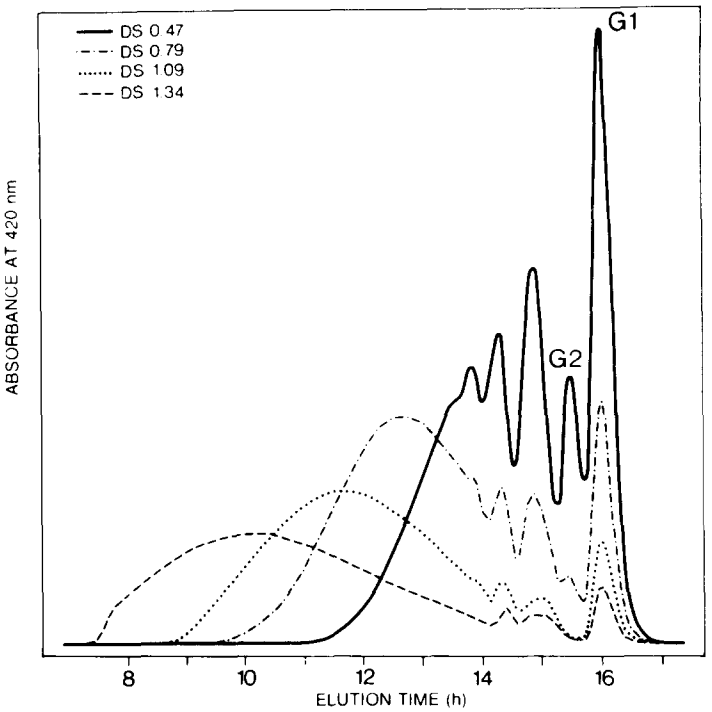

Fig. 2. Elution pattern for CMC fragments obtained after complete enzymatical degradation of carboxymethyl cellulose with different degree of substitution (DS). Flow rate, 0·9 ml min^{-1}.

ments, the fragment distribution of enzymatically degraded carboxymethyl cellulose is dependent on the DS of the substrate. SEC of degraded CMC substrates with different DS shows clearly that the proportion of oligomeric products essentially decreases on increasing the degree of substitution (see Fig. 2). The decrease in the average elution time indicated that the polymer chain cleavage by the enzyme is less effective at higher DS values, leaving fragments with a higher degree of polymerization. The change in the molecular weight distribution of CMC fragments in the course of enzymatic hydrolysis of CMC (DS 0·47) is shown in Fig. 3. The elution time of the original CMC is about 6 h. However, the average molecular weight of the polymer cannot be estimated from its elution time, because the hydrodynamic volume of the anionic CMC is certainly not equal to dextran of

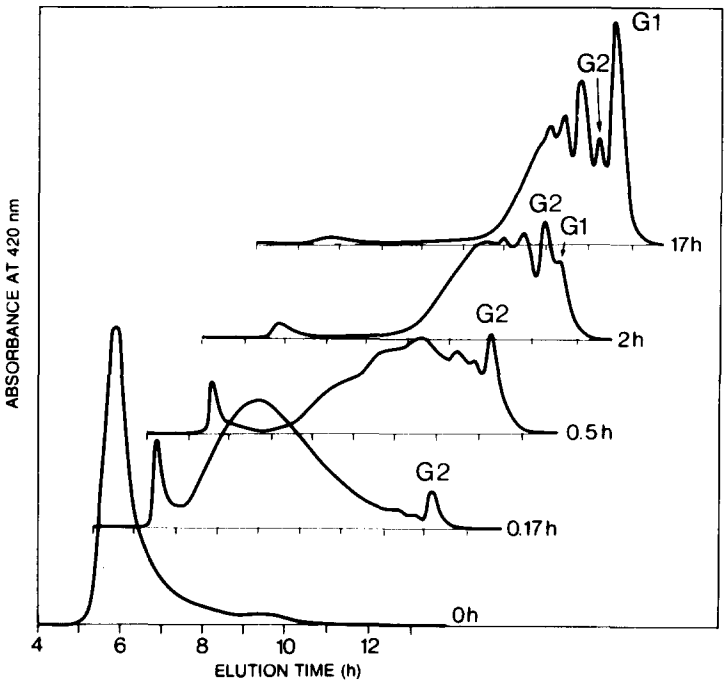

Fig. 3. Elution pattern for carboxymethyl cellulose (DS 0·47, 0 h) and CMC depolymerization products obtained in the course of enzymatic degradation after 0·17, 0·5, 2 and 17 h incubation with cellulase complex (*Trichoderma viride*) at 50°C. Flow rate, 1·2 ml min^{-1}.

molecular weight $\sim 2 \times 10^5$ g mol^{-1} as would be predicted from the elution time. The form of the elution curve does not reflect the real molecular weight distribution of the polymer. The elution pattern obtained after an incubation time of 10 min (0·17 h) at 50°C shows that the polymer cleavage occurred rapidly but the amount of small oligomeric fragments obtained is very low. In addition to the disaccharide fraction (G2) there are small amounts of tri- and tetra-saccharides ($t_R = 11 \ldots 12$ h) detectable, whereas glucose seemed only to be formed in negligible amounts. It is remarkable that the change in specific viscosity during the first 0·17 h of digestion is nearly identical to the value obtained after complete enzymatic degradation.

Compared with low molecular weight fragments, such as cellobiose (G2), the rate of glucose formation is rather slow. Although the small

glucose peak was covered by the cellobiose peak during the first incubation period (0·5 h), it was distinguishable from G2 after an incubation time of 1-2 h; the glucose fraction reached its maximum after about 17 h. The amount of glucose enzymatically produced under these conditions is about 21% of the total carbohydrate content detected by orcinol/sulfuric acid. The G1- and G2-fractions seem not to be substituted because their retention times are identical to the standards of glucose and cellobiose, respectively. All the other cleavage products observed are superimposed by carboxymethyl cellooligomers of as yet unknown molecular weight and structure.

DISCUSSION

A wide range of applications has been reported for size exclusion chromatography but so far there are only a few examples of the gel chromatographic characterization of water-soluble anionic polysaccharides and the separation of their enzymatic degradation products (Lambert *et al.*, 1982; Sutherland, 1984). The main problem with SEC-characterization of polyelectrolytes such as carboxymethyl cellulose and the separation of their enzymatic degradation products is the selection of an appropriate eluent. The nature of the buffer and the ionic strength are essential factors in avoiding unwanted interaction with the stationary phase material.

An effective separation of CMC fragments, obtained by enzymatic degradation of CM-cellulose, can be achieved using Fractogel® TSK HW-55 (S) as stationary phase and an eluent containing the electrolyte components sodium sulfate and sodium acetate. Although the carboxymethyl group is partially protonated at pH 5, there is no indication that in the presence of the buffer components (Na_2SO_4/Na-acetate) the elution rates of the CMC fragments are influenced by the gel matrix. More recent analyses carried out at pH 7·6 showed no significant difference compared with the elution pattern detected at pH 5. The TSK HW-55 gel is applicable over a wide molecular weight range but a more efficient separation of oligomeric fragments with a molecular weight $< 2000\ g\ mol^{-1}$ can be obtained using, e.g., Fractogel TSK HW-40(S) (Hamacher, unpublished results). This stationary phase is certainly not suitable for getting a real distribution of partially degraded CMC chains. The column packing material is a mechanically

and chemically stable hydrophilic vinyl polymer with an excellent resolution and an extended column life because the gel does not support bacterial growth. The on-line carbohydrate detection using orcinol/sulfuric acid was not influenced by the electrolyte components, but there was an uncertainty concerning the detection of substituted glucose units. The peak area below the extinction curve decreased with increasing degree of substitution, in spite of a constant number of monomeric units applied to the column (see Fig. 2). The glucose detection with orcinol/sulfuric acid is obviously dependent on the degree of substitution and presumably on the substitution pattern of the glucose molecules. The influence of the distribution of carboxymethyl groups on the orcinol/sulfuric acid reaction is not yet known.

The molecular weight of oligomeric CMC fragments with $M < 500$ g mol^{-1} correlates with their retention time, but the molecular weight distribution of higher molecular depolymerization products cannot be estimated using the calibration curve (see Fig. 1) because the hydrodynamic volume of the substituted CMC depolymerization products are, of course, not equal to the maltodextrins, cellooligomers and dextran standards.

The chromatographic procedure presented enables a practicable means of separating the enzymatic CMC fragments in preparative amounts and makes it possible, in combination with other analytical methods, to predict parts of the polymer sequence and the distribution of the carboxymethyl groups. By means of a structural analysis of the separated oligomers it is possible to infer the number of adjacent, unsubstituted glucose units in the polymer which are necessary to obtain an enzymatic cleavage by endo-glucanases.

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