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Characterization of cellulose derivatives and their migration behavior in capillary electrophoresis

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Abstract

Uncharged chemically modified cellulose derivatives were mobilized in electric fields through the adsorption of charged and hydrophobic surfactant molecules. The steep and sudden onset in electrophoretic mobility was characteristic of a cooperative binding process and a migration model based on thermodynamic equilibria is presented along with experimentally determined association constants and aggregation numbers. Due to an uneven modification along the polymer chains, separation of different groupings within a sample mixture could be obtained and differently substituted celluloses were selectively distinguished. To discriminate between different preparations derivatized with the same substituent groups, enzymatic or acid hydrolysis was applied utilizing various enzymes with different origin as well as acids like hydrochloric, trifluoroacetic and perchloric acid. The methodology presented here might serve as a general approach for the assessment of cellulose derivative structures and their relation to technical properties desired in the food, medical and pharmaceutical industries.

Keywords: Cellulose derivatives; Capillary electrophoresis; Cooperative binding; Enzymatic and acid hydrolysis

1. Introduction

Cellulose derivatives and other water-soluble polysaccharides play a significant role in many technical, industrial and medical applications [1] as colloidal stabilizers, emulsifiers and flow controllers, and are also used in the food and coatings industry as well as in oil-well drilling. These properties and the non-toxic nature of polysaccharides have made them serve as part of regulating systems in pharmaceutical formulations to control release rates of active substances [1]. Many drugs are of an amphiphilic nature and, hence, the general physicochemical problem of amphiphile/polymer interaction has to be addressed.

Cellulose, a renewable resource, is the world's most abundant polymer consisting of β-(1→4) linked glucopyranosyl units. Due to inter- and intramolecular hydrogen bonding, primarily by the C-6 and C-3 hydroxyls of the repeating monosaccharides, the polymer is basically insoluble in most solvents. By chemical substitution of the hydroxyl groups, cellulose ether and ester derivatives are formed which make cellulose less crystalline and more water-soluble. The solubility is essentially proportional to the degree of substitution

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(DS) and solvating power of the substituent groups [2] and to a lesser extent dependent on the molecular weight of the polymer. Solution properties include the formation of gels and becoming opaque above a well-defined temperature, the cloud point [3], where phase separation occurs. Hence, a knowledge of the substitution pattern as well as the distribution along the cellulose backbone is essential for understanding of the structure–property relationships. During isolation of the cellulose and chemical modification of the hydroxyl groups, heterogeneities often arise due to incomplete substitution, side-reactions, depolymerization, oxidation etc [4]. This heterogeneity has been confirmed experimentally by gas chromatography fast-atom-bombardment mass spectrometry (GC–FAB–MS) on both an oligomer and monomer level [5]. The substitution, furthermore, imparts a greater stability to enzymatic hydrolysis, especially by the presence of bulky groups positioned at C-2 [6] which also is the most reactive of the hydroxyl groups.

Various cellulose derivatives exhibit diverse physicochemical characteristics but even batch-to-batch variations have been observed in pharmaceutical applications [7]. A variance in performance could influence drug formulation and release of active substance(s) in a biological system which, ultimately, could alter the therapeutic effect of the product. To ensure quality and avoid costly fall in production, fast and reliable screening methods are needed and capillary electrophoresis is a favourable option [8,9]. First, the cellulose molecules are converted to fluorescent derivatives through the covalent coupling to a fluorophore at the reducing end and then injected into a capillary electrophoresis system equipped with laser-induced fluorescence detection which offers the high sensitivity needed. Since most chemically modified celluloses lack protolytic groups, one exception being the carboxymethylated derivatives, they are immobile in an electric field. The remaining and underivatized hydroxyl groups are too few or not fully accessible because of steric reasons for successful complexation with borate which would render the molecules mobile as anionic complexes. The charge-to-friction ratio achieved through the coupling to a multiply charged derivatizing agent is suitable for the analysis of hydrolyzed samples, i.e., polysaccharide mapping, but rather inadequate for the intact polymer chains of molecular weights reaching up to several million Daltons. For these kinds of solutes, charged surfactants which bind to the polymer have been employed resulting in a high electrophoretic mobility [10].

This paper reports on the analysis and separation of different cellulose derivatives. The migration of intact polymer chains in the presence of charged amphiphiles is described by a simple cooperative binding model and calculated isotherms will be presented. Polysaccharide mapping using enzymatic as well as acid hydrolysis enabled distinction between different brands of the same cellulose derivative.

2. Experimental

Chemicals.—All chemicals used in this study were received from Sigma (St. Louis, MO), except for sodium cyanoborohydride ammonium persulfate (Aldrich, Milwaukee, WI). Hydroxypropyl celluloses with average molecular weights of 100,000 and 1,000,000 (HPC 100,000, 1,000,000), hydroxypropyl methyl cellulose (HPMC) 4659 cps as a 2% water solution, and carboxymethyl celluloses (CMC) were commercially available samples (Aldrich, Milwaukee, WI). The degree of substitution for the CMCs were 0.7, 0.9, and 1.2, respectively, and all of them with an average molecular weight of 250,000. The ethyl hydroxyethyl cellulose (EHEC) derivatives DVT 96016 and E411 G were generously supplied by Akzo Nobel AB (Stenungssund, Sweden) and with a monomer degree of ethylene oxide substitution of 0.9 and 2.3 and an ethyl group subtitution of 1.9 and 0.9, respectively. The degree of polymerization was 800 and 1250. Hydroxyethyl cellulose (HEC), HPMC 6 cps and 10,000 cps (as 2% water solutions and with an average molecular weight of 15,00 and 150,000, respectively), HPC LF and CMC 12M31P were kind gifts from Astra Hässle (Mölndal, Sweden). The linear polyacrylamide of an average molecular weight of 0.7–1 million Daltons and utilized as a sieving polymer solution in capillary electrophoretic separations was purchased from Polysciences, Inc. (Warrington, PA).

Column Preparation.—Various lengths of fused silica capillaries (Polymicro Technologies, Phoenix, AZ; 50 μm I.D. and 185 μm O.D.) were used as separation columns. They were coated with a layer of linear polyacrylamide according to a slightly modified version of the Hjertén method [11]: first,

the new capillary was treated with 0.1 M NaOH for 1 h and then rinsed with 20 mM acetic acid followed by methanol. The silica wall was activated by coupling of γ-methacryloxypropyltrimethoxy silane (10 μL dissolved in 1 mL dichloromethane containing 0.02 M acetic acid) to the inner surface during a 60-min treatment performed under nitrogen pressure. The capillary was then rinsed with methanol and water (solvent compatibility). Subsequently, a 4% (w/w) acrylamide solution, containing 1 mL/mL of N,N,N′,N′,-tetramethylethylenediamine(TEMED)and 1 mg/mL ammonium persulfate, was passed through the capillary under nitrogen pressure for 30 min. Finally, the capillary was rinsed with water and dried under a stream of nitrogen for 10 min. In-between runs, the capillary was rinsed with the separation medium for 30 s.

Sample derivatization. The polysaccharide samples were converted into fluorescent derivatives through reductive amination [9] with 20 mM 1-aminopyrene-3,6,8-trisulfonic acid, APTS (Lambda Fluoreszenztechnologie, Graz, Austria), in 10% acetic acid and 0.1 M sodium cyanoborohydride at 60 °C for 2 h in 100 μL glassvials, diluted 100 times in 2.5 mM sodium azide and stored at 4 °C. The samples were injected hydrodynamically except for buffer media containing 4% linear polyacrylamide where electrokinetic injection at 50 V/cm and with a duration of approximately 1 s was utilized.

Apparatus.—The home-made instrumental setup for capillary electrophoresis/laser-induced fluorescence detection consisted of a high-voltage power supply (Spellman High Voltage Electronics, Plainview, NY) capable of delivering 0–40 kV. On-column fluorescence detection was accomplished with an argon ion laser (ILT Model 5425ASL-00, Ion Laser Technology, Salt Lake City, UT) as excitation source, operating at 488 nm. The emitted fluorescence was collected at 90° by a microscope objective, measured by a photomultiplier tube at 515 nm after laser line filtering (FWHM = 10 nm), and finally visualized by an analog recorder.

Enzymatic hydrolysis.—150 μL of a 1% cellulose solution was incubated with 20 IU of four cellulase (EC 3.2.1.4 from *Penicillium funiculosum*, *Aspergillus niger*, *Trichoderma reesei*, *Trichoderma viride*) and one hemicellulase preparation (*Aspergillus niger*), respectively, at pH 4.85 (50 mM acetic acid and 25 mM ammonia) and 37 °C for a duration of 24 h. The reaction was stopped by a combination of ultrasonification for 5 min followed by immediate derivatization. The samples were then stored at −20 °C before appropriate dilution and injection into a separation system. No remaining enzymatic activity could be detected in the electropherograms as judged from the invariable separation pattern obtained.

Acid hydrolysis. 150 μL of a 1% cellulose solution was incubated 16 h at 100 °C in a 0.1 M solution of hydrochloric, trifluoroacetic or perchloric acid, respectively, followed by derivatization with APTS.

3. Results and discussion

Native polymer chains.—The electrophoretic mobilities, μ_{ep}, for the three HPCs versus the concentration of sodium dodecylsulphate (SDS) and for HPC 100,000 versus the sodium decylsulphate concentration are shown in Fig. 1. The electrophoretic migration of uncharged cellulose polymer chains is induced by the binding of anionic surfactant molecules along the polymer backbone. The migration curves are characterized by a steep and sudden onset of μ_{ep} at 1.0 mM SDS and 20 mM decylsulphate. As the concentration at which the surfactants start to bind to the celluloses is lower than the critical micelle concentration (cmc) it has been referred to as the critical aggregation

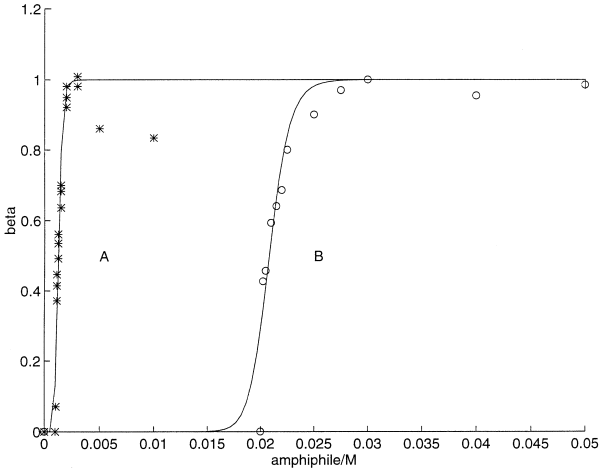

Fig. 1. Electrophoretic mobility for the three hydroxy propyl celluloses (HPCs) versus the concentration of sodium dodecylsulphate (curve A) and HPC 100, 000 versus sodium decylsulphate concentration (curve B). The electrolyte buffer was 50 mM citric acid and 50 mM tris at pH 3.95. The solid line represent least-squares non-linear fitting of the binding model given by eq (1). β is the coverage expressed as the relative mobility, μ_{ep}/μ_{max}.

concentration (cac) [12–14. An increase in buffer concentration to 75 mM citric acid/tris did not change the onset of migration (data not shown). This indicates an absence of micelles as cmc is expected to decrease with ionic strength. In agreement with published results [12–14 the present findings indicate a binding of monomeric charged detergent molecules to the cellulose chains. No differences in migration behavior between the three HPCs could be observed within the experimental error. The importance of hydrophobic interaction in the cooperative binding is shown by the higher concentrations of surfactant needed for migration in the case of the 10-carbon chain decylsulphate.

The steep electrophoretic mobility curves display the characteristics of a cooperative binding process and can be phenomenologically described according to Hill's equation [15,16]

$$\beta = K^N[\text{surfacant}]^N/(1 + K^N[\text{surfacant}]^N) \quad (1)$$

where β is the coverage expressed as μ_{ep}/μ_{max}, K the binding constant for one surfactant molecule to a binding site on a polymer chain and N is the aggregation number. The electrophoretic mobility is proportional to the number of surfactant molecules bonded to the cellulose, i.e., it mirrors the binding isotherm. The migration velocity reaches a maximum, μ_{max}, when all sites are occupied and its magnitude will be determined by the number of binding sites and the aggregation number. Theoretical isotherms or mobility response curves versus surfactant concentration are presented in Fig. 2 and illustrate the effects of K and N on mobility. The aggregation onset is primarily governed by the magnitude of the binding constant, whereas N determines the slope of the binding isotherm (the model simplifies to an ordinary Langmuir equation for $N = 1$). For large N values, an almost instantaneous saturation of the binding sites occur. The solid lines in Fig. 1 were obtained by least squares non-linear fitting of eq (1) to the data and the resulting K and N values were: 782; 8 and 48; 22, respectively. An N-value of 9–10 in a 3 mM SDS and 0.20% EHEC system was recently reported [17]. The experimental results indicate that a critical β value limits the cooperative character of the binding. For the higher surfactant concentrations, the formation of micelles competes with the binding process and either stabilizes or decreases the β values. This can be seen from the decline in

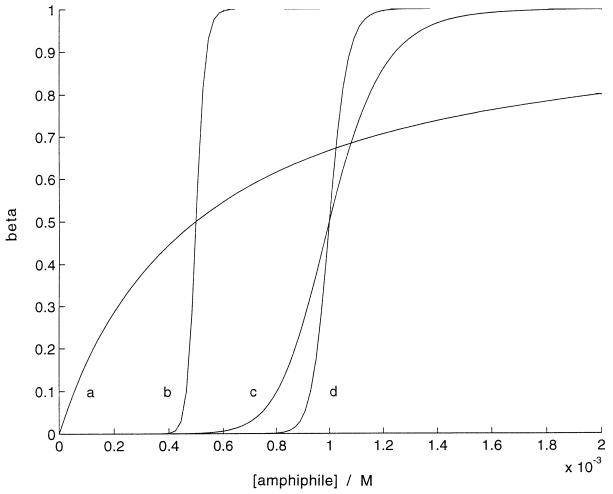

Fig. 2. Theoretical binding isotherms using eq (1). The values used were: (a) $K = 1000$, $N = 1$; (b) $K = 2000$, $N = 30$; (c) $K = 1000$, $N = 10$; and (d) $K = 1000$, $N = 30$.

mobility at SDS concentrations higher than 2 mM. A minor effect from the increase in ionic strength upon addition of SDS is expected to be present as well. A critical value in mobility (or β) could, furthermore, be related to the fact that chemically modified celluloses have a very expanded structure in aqueous solution and when surfactants are added a change in solvation occurs resulting in a bulkier and less expanded chain [18].

The derivatization of alkali cellulose with propylene oxide is known to result in a uniform HPC structure [19]. For HEC samples, on the other hand, extensive chaining reactions take place and, additionally, a substantial fraction of the Glc units remain unsubstituted resulting in highly heterogeneous chain structures. Such differences were detected in capillary electrophoretic separations of HPC and HEC samples in the presence of 10 mM SDS (Fig. 3(a) and (b)) where numerous peaks could be resolved for the latter. (The first four sharp peaks originate from excess reagent and by-products formed during the derivatization. The electrophoretic mobilities were calculated from the migration times of the broad peak maxima emerging at 4–5 min.) It is reasonable to assume that a high degree of chaining was beneficial for the amphiphile adsorption and induced a high electrophoretic mobility whereas a low DS of the Glc moieties weakened this interaction resulting in a decreased charge-to-friction ratio and, hence, mobility.

For celluloses containing chargeable groups, such as CMCs, resolute electrophoretic mobility is

not a problem due to the dissociative property of carboxylic acid moieties placed along the cellulose backbone. A suitable degree of dissociation and hence migration velocity could be achieved by variation of pH as demonstrated in Fig. 4(a). The apparent pKa values for carboxymethylated celluloses with DS of 1.2, 0.9, and 0.7 were 3.1, 3.2 and 3.2, respectively, as evaluated at half of the maximum mobility. In this limited DS range, a linear relationship between the mobility and degree of substitution was obtained which can be used for electrophoretic determination of the DS for unknown samples. At higher charge densities, however, a deviation from this behaviour might be expected due to counter ion condensation. The separation selectivity, defined as the ratio in electrophoretic mobilities, were highest around the pKa values and leveled off thereafter (data not shown). A separation of the DS 1.2 and 0.7 samples is shown in Fig. 4(b).

Hydrolyzed polymer chains.—An alternative approach for the analytical assessment of physicochemical property differences and structural irregularities of a polymer chain/mixture is hydrolysis, either by enzymatic or chemical means. A great number of cellulases have been isolated and several are commercially available. Generally, these preparations consist of a mixture of components displaying exo as well as endo enzymatic activities [2], i.e., they cleave from the non-reducing end or randomly along the polymer backbone. Hence, 1% solutions of HPMC 10,000 were incubated with a large excess of one hemicellulase and four cellulase preparations of different origin, according to standard procedures (see Experimental). The electropherograms were evaluated by observing differences in the degree of hydrolysis,

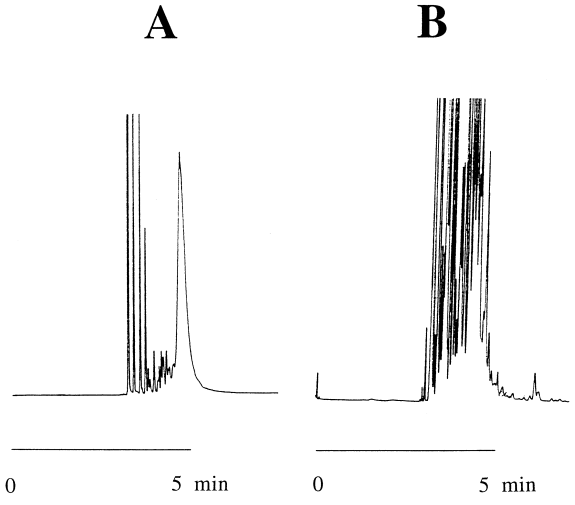

Fig. 3. Capillary electrophoretic separation of the (A) HPC LF and (B) HEC samples, respectively. The electrolyte buffer was 25 mM citric acid, 32.5 mM tris, 10 mM sodium acetate and 10 mM SDS. The electric field strength was −350 V/cm using 25 cm of effective capillary length.

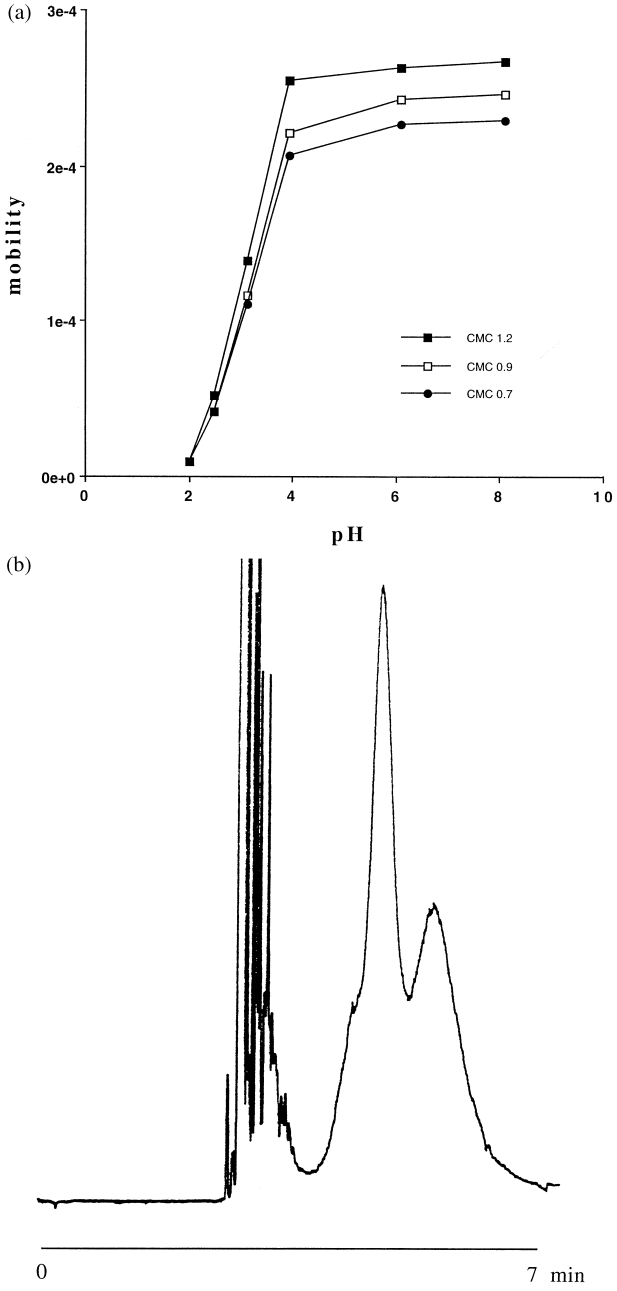

Fig. 4. (a) Electrophoretic mobility of three carboxymethylated celluloses versus pH at a constant ionic strength of 0.03 utilizing citric and phosphate acid and tris. The DS were 0.7, 0.9, and 1.2, respectively. (b) Capillary electrophoretic separation of carboxymethylated celluloses with a DS of 1.2 and 0.7. The buffer was 25 mM citric acid and 25 mM tris at pH 3.95. The effective capillary length was 37.5 cm and run at −500 V/cm (14 μA).

estimation of remaining chain lengths, and variability of the peak shape for a given degree of polymerization (DP). Electropherograms for the two most diverse cases (cellulase from *Aspergillus niger* (AN) and *Trichoderma reesei* (TR)) are displayed in Fig. 5(a) and (b). An almost completely converted HPMC 10,000 into mono- and disaccharide units. The regular pattern obtained with the TR cellulase preparation shows an oligomer distribution of modified Glc units with sequences unaccessible to cleavage by the enzyme(s). For early migrating species, intra-DP dispersion was indicated by partially resolved peaks, which merged with increasing DP as a consequence of the increasing number of possible combinations and appeared merely as broad humps. The extent of peak broadening for a given DP is thus an indirect indication of the original structural variability. TR was used throughout the rest of this study as its inherent constraint towards certain structural modifications would be manifested in an improved separation selectivity. This is exemplified by the electrophoretic discrimination of the two EHEC samples DVT 96016 and E411 G, i.e., Fig. 6(a) and (b). The regular separation pattern of the DVT 96016 preparation indicates a more uniformly substitution of the cellulose chains compared to E411 G which has a higher degree of ethylene oxide substitution and possibly chaining.

Of the many acidic conditions utilized for cellulose hydrolysis [2], 0.1 M solutions of hydrochloric, trifluoroacetic or perchloric acid was employed to gain further information on the intricate composition variance of modified celluloses. Four per cent linear polyacrylamide was included in the separation buffer as a polymer sieving medium to

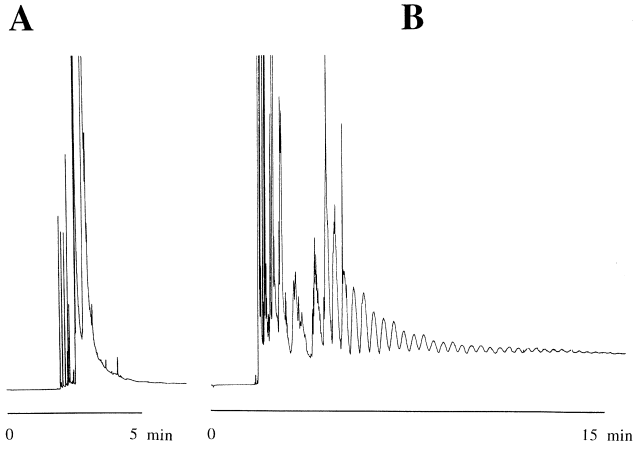

Fig. 5. CE mapping of HPMC 10,000 after enzymatic hydrolysis with cellulase from (A) *Aspergillus niger* and (B) *Trichoderma reesei*. The buffer was 25 mM citric acid and 25 mM tris at pH 3.95. The effective capillary length was 45 cm and run at −450 V/cm (12 μA).

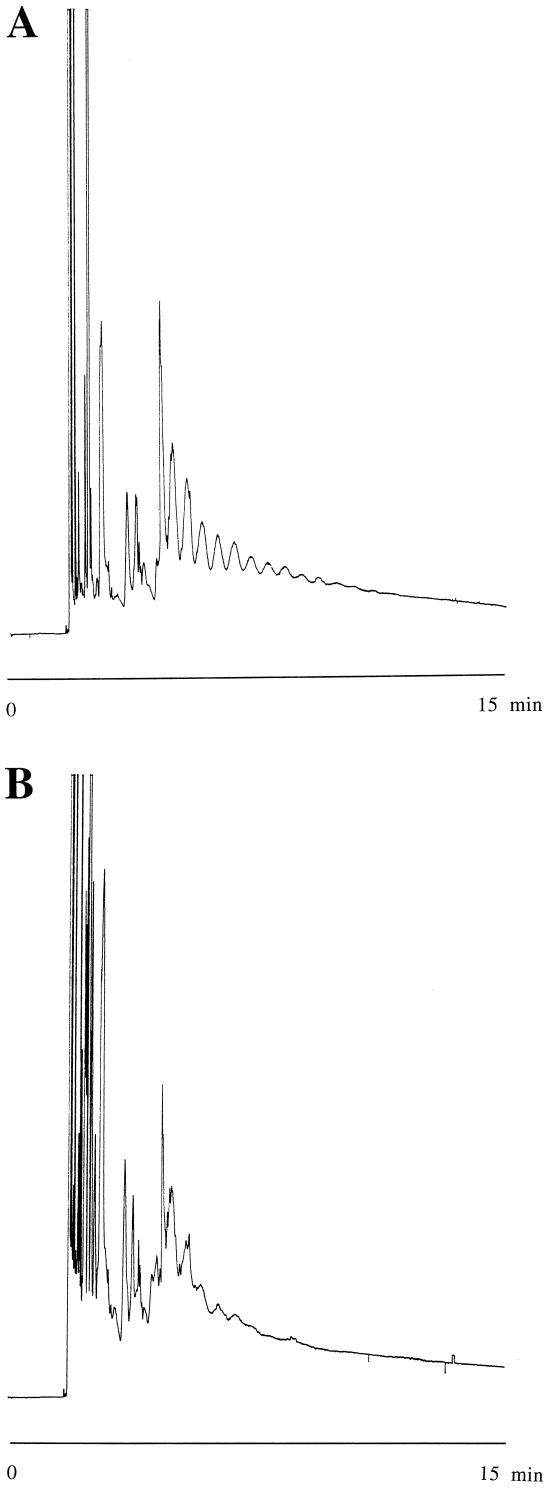

Fig. 6. *Trichoderma reesei* digest of EHEC (A) DVT 96016 and (B) E411 G. Separation conditions as in Fig. 5 except for an effective capillary length of 40 cm.

enhance the differences in electrophoretic mobility (data not shown) with molecular size of HPMC 10,000 fragments formed after 16 h of hydrolysis and perchloric acid was chosen for further studies as it resulted in the most well resolved peaks. Analytical discrimination of all three HPMCs, i.e., 6, 4659 and 10,000, could thus be obtained in a SDS based system as evidenced in Fig. 7(a),(c). HPMC 10,000 deviated the most by the appearance of higher oligomers and the tentative monomers migrating at 3.5 to 4.5 min were better resolved, presumably, indicating a higher degree of substitution. Structural investigation of the enzymatic fragments by capillary liquid chromatography and electrospray–mass spectrometry is currently under way [20]. The samples could also be differentiated in a 4% linear polyacrylamide system too but the pattern were harder to interpret.

In the case of the perchloric acid hydrolyzate of the multiply charged carboxymethylated cellulose, aminodextran, a positively charged polymer, was used as an additive to promote charge and size selective differences between cellulose components [9]. The profile for CMC 12M31P in the presence of 50 μM aminodextran, with a molecular weight of 10,000 and carrying 3.2 positive charges, is exhibited in Fig. 8. The degree of complexation between the aminodextran(s) and the CMC fragments is governed by the ionic strength, charge density and flexibility of the polymer chains. The latter might enable a spatial orchestration for cooperative interaction between the oppositely charged groups. Accordingly, the abundance of peaks and lack of regularity in migration times give an indication of the intricate and complex variation of the structure along a chemically modified polymer chain.

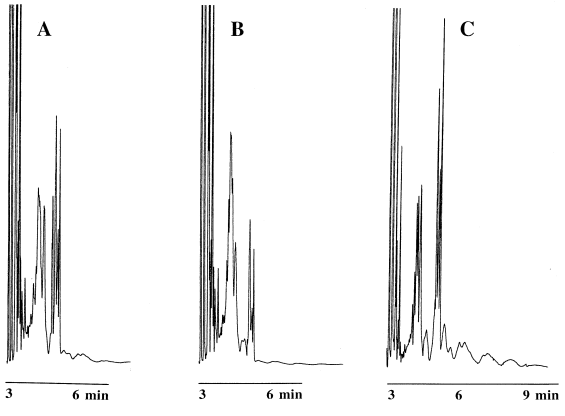

Fig. 7. Capillary electrophoretic discrimination of (A) HPMC 6, (B) 4659 and (C) 10,000 after 16 h of perchloric acid hydrolysis at 100 °C. The separation buffer was 50 mM citric acid, 75 mM tris and 5 mM SDS. The samples were run at −300 V/cm (26 μA) using an effective capillary length of 37.5 cm.

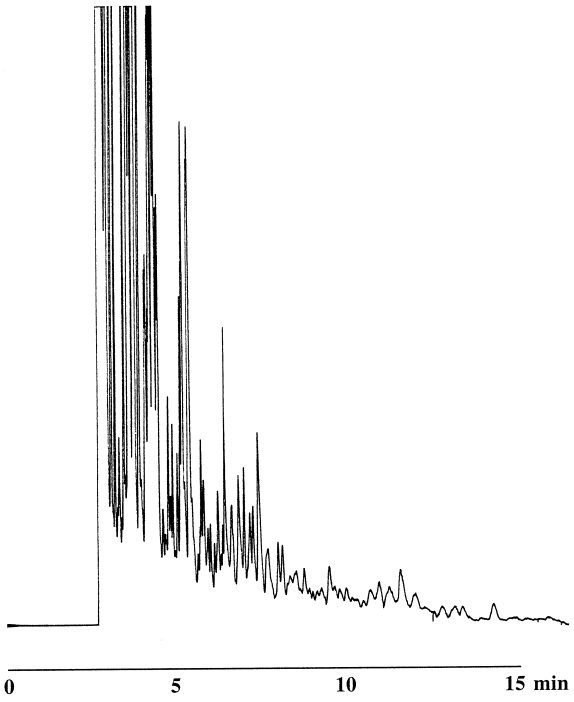

Fig. 8. Oligosaccharide mapping of CMC 12M31P after a *Trichoderma reesei* cellulase hydrolysis. Separation buffer was: 25 mM citric acid and 25 mM tris containing 50 μM aminodextran (average molecular weight of 10,000 and carrying 3.2 positive charges). The effective capillary length was 37.5 cm and run at −500 V/cm (14 μA).

4. Conclusions

Electrophoretic migration of uncharged chemically modified celluloses has been induced through the adsorption of charged surfactants added to the electrolyte buffer. The onset of the cooperative adsorption process could be phenomenologically described by a cooperative Langmuir adsorption model. The adsorption isotherm was found by measurement of the electrophoretic mobility as a function of detergent concentration, thus, enabling the evaluation of the association constant and aggregation number of hydrophobic surfactant molecules complexed to a site on the cellulose chain. Carboxymethylated celluloses of different degrees of substitution were resolved at an optimal pH of 3. In order to discriminate between different kinds of cellulose derivatives carrying the same substituent groups, enzymatic and acid hydrolysis

was performed. A feature of the analytical methodology put to use here is the fast analysis time.

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