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Liquid chromatography–mass spectrometry analysis of enzyme-hydrolysed carboxymethylcellulose for investigation of enzyme selectivity and substituent pattern

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Abstract

A series of celloendoglucanases: *Bacillus agaradhaerens* Cel 5a, *Humicola insolens* Cel 5a, *H. insolens* Cel 7b, *H. insolens* Cel 45a, *Trichoderma reesei* Cel 7b, and *T. reesei* Cel 45a were used to hydrolyse carboxymethylcellulose (CMC) and the hydrolysis products were investigated with a novel liquid chromatography–mass spectrometry (LC–MS) method. Separation was achieved using a graphitised carbon chromatographic column which allowed the use of electrospay compatible eluents. Analysis of the compounds produced during enzyme hydrolysis of CMC is used to understand enzyme selectivities and substitution pattern of CMC. Conventional high-performance anion-exchange chromatography (HPAEC)–pulsed amperometric detection (PAD), size-exclusion chromatography (SEC)–refractive index (RI) detection, and reducing end analysis are also used to analyse enzyme-hydrolysed CMC. The LC–MS method presented allows for a more detailed investigation of hydrolysis products, which facilitates characterisation of both enzymes and substrates.

Keywords: Carboxymethylcellulose; Cellulose; Enzymes; Endoglucanases; Carbohydrates

1. Introduction

Modified celluloses are finding increasing use in industrial processes, such as the production of foods, pharmaceuticals, paints, etc. Cellulose is modified in order to affect its physical and chemical properties such as viscosity and solubility. It has been established that not only the nature of the substituent but also the substitution pattern along the cellulose backbone play important parts in defining the characteristics of the final product, which in turn determine the applicability of derivatized cellulose. Cellulose consists of a straight chain of polymerised glucose, linked together with β1 → 4 glycosidic bonds and each glucose unit can be modified on the C2, C3 or C6 hydroxyl group (Fig. 1). Subsequently, up to three modifications per glucose unit are possible for mono carboxymethyl substitution. Different modification processes provide varying degrees of substitution (DS) and differences in substituent distribution. Unfortunately, the correlation between the modification processes and the substituent distribution in the product obtained are not fully understood.

The investigation of the substituent pattern of carboxymethylcellulose (CMC) has been the focus of considerable effort [1–6]. A successful approach to determining the substituent pattern of CMC (and other modified polysaccharides) is enzymatic hydrolysis followed by various chromatographic analysis techniques [4–10]. This approach relies on the fact that polysaccharide-hydrolysing enzymes are partially hindered by the modification of the constituent glucose units, thus providing selective depolymerisation. Endoglucanases depolymerise CMC by catalysing the hydrolysis of glycosidic bonds within the polymer that link the monomers together, subsequently producing shorter chain molecules. However, depending on the structure of the active site of the different endoglucanases, the structural requirements on the polysaccharide will differ, which in turn, will lead to different selectivities. By analysing the hydrolysis

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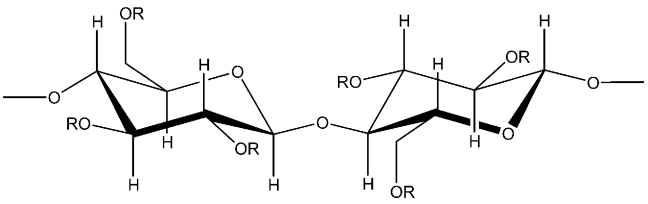

Fig. 1. Structure of CMC. A repeating unit of carboxymethylcellulose, R = H or CH_3COO^-.

products from one or several endoglucanases with different selectivities, it is possible to elucidate information on the substituent pattern of CMC as well as to draw conclusions about enzyme specificity. To date, there are no standard methods for structural analysis of modified cellulose via enzymatic hydrolysis. Enzyme specificities are not fully understood, rendering interpretation of hydrolysis data difficult. It is therefore of interest to correlate the enzyme-hydrolysis pattern both with respect to substrate structure and enzyme specificity.

Conventional analysis techniques for the investigation of modification patterns and cellulase specificities include size-exclusion chromatography (SEC) with refractive index (RI) detection [4,11–13], high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) [1,3,4,14] and reducing end analysis [6,15]. These techniques provide information about the molar mass distribution of the hydrolysates, the degree of depolymerisation that has taken place, and the amount of liberated unmodified sugars [6]. This information is used to understand how efficiently an enzyme hydrolyses a substrate. The information they provide is unspecific with regard to the modified products. In this work, all of these techniques have been applied both for the purpose of confirming that hydrolysis has taken place and for comparison with the liquid chromatography–mass spectrometry (LC–MS) analysis.

Mass spectral analysis is suitable for determining the products in enzyme-depolymerised CMC as it is possible to calculate the mass of all possible hydrolysis products due to the fact that all of the possible monomeric constituents are known. Furthermore, the carboxymethyl moiety is acidic, thus facilitating the formation of negative ions. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) MS studies of enzyme-degraded CMC has been presented [6] and LC–MS analysis of enzyme-depolymerised modified starch have previously been proposed [9,16], however, to the best of our knowledge no LC–MS analysis has been described for CMC. In previous articles, LC–MS of modified carbohydrates is achieved with HPAEC at high NaOH concentrations, necessitating an on-line desalting step prior to mass spectral analysis. In this work, we employed a graphitised carbon column instead, allowing typical LC–MS eluents (water–acetonitrile) to be used thus removing the need for desalting.

2. Experimental

2.1. Substrates and chemicals

CMC used was purchased from Aldrich (catalog no. 419311, lot no. 03508DU, Sigma–Aldrich, St. Louis, MO, USA) and had an average M_r of 250,000 Da and a degree of substitution of 0.7. All CMC used was from the same batch. Ammonium hydroxide, sodium acetate, sodium hydroxide, dinitrosalicyclic acid and Rochelle salt were purchased from Merck (Darmstadt, Germany). All carbohydrate standards (glucose, cellobiose, cellotriose, cellotetraose and cellopentaose) were purchased from Merck. Ammonium acetate was purchased from Acros (Geel, Belgium). The water used in all experiments was purified in a Milli-Q system, Millipore (Bedford, MA, USA).

2.2. Enzyme hydrolysis

The following endoglucanases were used: *Trichoderma reesei* Cel 7b (Tr Cel 7b) (purified from a culture filtrate of *T. reesei* QM 9414 [17]), *T. reesei* Cel 45a core (Tr Cel 45a) (purified according to Karlsson et al. [18]), *Humicola insolens* Cel 5a (Hi Cel 5a), *H. insolens* Cel 7b (Hi Cel 7b), *H. insolens* Cel 45a (Hi Cel 45a) (all *H. insolens* endoglucanases were purified according to Schou et al. [19]) and *Bacillus agaradhaerens* Cel 5a (Ba Cel 5a) (provided by NovoZymes, Bagsvaerd, Denmark). The enzymes were used to degrade CMC in the following manner: CMC was dissolved in 50 mM ammonium acetate at pH 5.0 and after addition of 1 μM enzyme solution the hydrolysis was allowed to proceed for 72 h at room temperature, allowing the reaction to reach completion [6]. The hydrolysates were stored at +4 °C prior to analysis. Unless mentioned otherwise, the hydrolysates were prepared with 10 g/l CMC solutions.

2.3. Size-exclusion chromatography

The SEC system consisted of an isocratic pump (LKB, Bromma, Sweden), an TSK $GMPW_{XL}$ gel column, 7.8 mm i.d. × 300 mm (TosoHaas, Stuttgart, Germany), and an RI detector (ERMA, Tokyo, Japan). The sample was introduced using a six-port injection valve using a 100 μl injection loop. The mobile phase consisted of a 200 mM NH_4OAc aqueous buffer (pH 5, 40 °C) and was pumped at 0.6 ml/min. Chromatographic data were collected using Turbochrom (Perkin-Elmer, San Jose, CA, USA).

2.4. Oligosaccharide analysis

Soluble sugars, glucose, cellobiose, cellotriose, cellotetraose and cellopentaose, were analysed with a HPLC system with an HPAEC Carbopac PA100 column and pulsed amperometric detection (Dionex, Sunnyvale, CA, USA). The detector used the following detection waveform: $E_1 = 0.1$ V ($t_d = 0.20$ s, $t_1 = 0.20$ s), $E_2 = -2.0$ V

(t_2 = 0.01 s), E_3 = 0.6 V (t_3 = 0.01 s), E_4 = −0.1 V (t_4 = 0.06 s) versus Ag|$AgCl_{(sat)}$ reference electrode and a gold working electrode. The oligosaccharides were eluted with isocratic 100 mM sodium hydroxide and a gradient of sodium acetate from 0 to 300 mM for 25 min (10 μl injection loop). Glucose, cellobiose, cellotriose, cellotetraose and cellopentaose standards were used to calibrate the system (concentration intervals: glucose, cellobiose 1–100 μg/l otherwise 1–10 μg/l; $r^2 > 0.999$ for all calibration curves). All samples were diluted to 1/10 concentration prior to injection.

2.5. Reducing end analysis

Dinitrosalicylic acid (DNS) method was used to determine reducing ends [20,21]. A DNS reagent solution was prepared by dissolving 40 g of NaOH pellets and 25 g of 2-hydroxy-3,5-dinitrobenzoic acid in 0.5 l of Millipore water each. The two solutions were combined and the resulting solution was diluted with 1.75 l of Millipore water. The solution was held at 35 °C while 755 g of Rochelle salt ($C_4H_4KNaO_6{\cdot}4H_2O$) were added in small portions. Finally, the solution was diluted to 2.5 l. The amount of reducing ends in a hydrolysate was determined by adding 0.75 ml of DNS reagent to 0.5 ml of hydrolysate (diluted 10 times). The mixture was heated to 100 °C in a water bath for 5 min. After cooling the absorbance at 540 nm was measured using a Shimadzu UV-2401PC spectrophotometer (Kyoto, Japan). Calibration was achieved using glucose standards (concentration interval: 0–1 mM).

2.6. LC–MS analysis

The chromatographic system consisted of a HP 1100 system equipped with a hypercarb graphitised carbon black analytical column, 100 mm × 2.1 mm, 5 μm (Chromtech, Hägersten, Sweden) using a flow rate of 0.3 ml/min and an injection volume of 20 μl. Separation was performed by gradient elution using (A) Millipore water and (B) acetonitrile both containing 5 mM ammonium acetate with the pH adjusted to 9 by ammonium hydroxide. The gradient elution was as follows: from 0 to 5 min an isocratic region at 5% B, from 5 to 15 min a linear gradient from 5 to 95% B followed by an isocratic region of 95% B until 35 min. Prior to injection the samples were filtered using M_r 5000 Da cut-off Millipore Ultrafree centrifuge filtration tubes (Millipore). Four injections were analysed per sample. The LC system was connected to an Esquire-LC ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) operated in negative ion mode. The mass spectrometer was set to scan between 100 and 1400 *m/z*. Nitrogen was used as a drying gas and was pumped at 7 l/min with a temperature of 350 °C. Nitrogen was also used as a nebuliser gas and was kept at 30 psi (1 psi = 6894.76 Pa). The following voltages were used: nebuliser 4000 V, end cap 3500 V and capillary exit 80 V.

3. Results and discussion

3.1. Size-exclusion chromatography

Size-exclusion chromatography (also known as gel permeation chromatography) is often used to characterise the molar mass distribution of polymers, both natural and synthetic [22–25]. SEC has previously been used in the investigation of hydrolysates of CMC [4,6,11]. After enzyme hydrolysis, the shift in molar mass distribution provides information on how successfully an enzyme has depolymerised the sample. Although useful, SEC offers little information about the chemical properties of the formed products. When investigating enzyme selectivities, it is valuable to know not only the molecular weight of a product but also the degree of substitution. Also, it is not practical to use SEC to monitor single compounds, especially in the low mass range. Thus in this work, SEC was used only to confirm that enzymatic hydrolysis had taken place (Fig. 2). The clear shift in retention between unhydrolysed and hydrolysed CMC clearly demonstrates that the samples have been degraded. Although all the peaks for hydrolysed CMC overlap, it is possible to observe differences in hydrolytic efficiency. As the most retained sample is the most hydrolysed, the enzymes hydrolytic efficiency is as follows: Hi Cel 5a > Ba Cel 5a > Tr Cel 7b > Hi Cel 7b > Hi Cel 45a > Tr Cel 45a. These findings are in agreement with the reducing ends analysis (Table 1) with the exception of Ba Cel 5a and Tr Cel 7b, which were in reverse order. It is possible to improve the SEC in order to investigate differences between the hydrolysates, however that is beyond the scope of this work.

3.2. Reducing ends and soluble sugars

The productions of reducing ends and soluble underivitised sugars after hydrolysis were determined (Table 1). The number of reducing ends liberated from the substrate can be used as a measure of an enzyme's hydrolytic efficiency as one can calculate to what degree the substrate has been depolymerised. Ba Cel 5a, Hi Cel 5a, Hi Cel 7b and Tr Cel 7b yielded 12–14% depolymerisation, whereas Hi Cel 45a and Tr Cel 45a only yielded 7% depolymerisation. The production of soluble sugars can also be used as a measure of hydrolytic efficiency. In Table 1, the production of soluble sugars (glucose equivalent) generally follows the production of reducing ends. It is noteworthy that the distribution of which sugars are liberated varies between enzymes even when the hydrolytic efficiency is comparable. For example, Hi Cel 5a and Hi Cel 7b are similar in their ability to hydrolyse CMC, but Hi Cel 7b only produces glucose (in detectable amounts) whereas Hi Cel 5a liberates glucose, cellobiose and cellotriose. Tr Cel 45a yielded the most diverse mixture of soluble sugars while showing poor hydrolytic efficiency. This information, in itself, is useful in the elucidation of enzyme specificities. Being

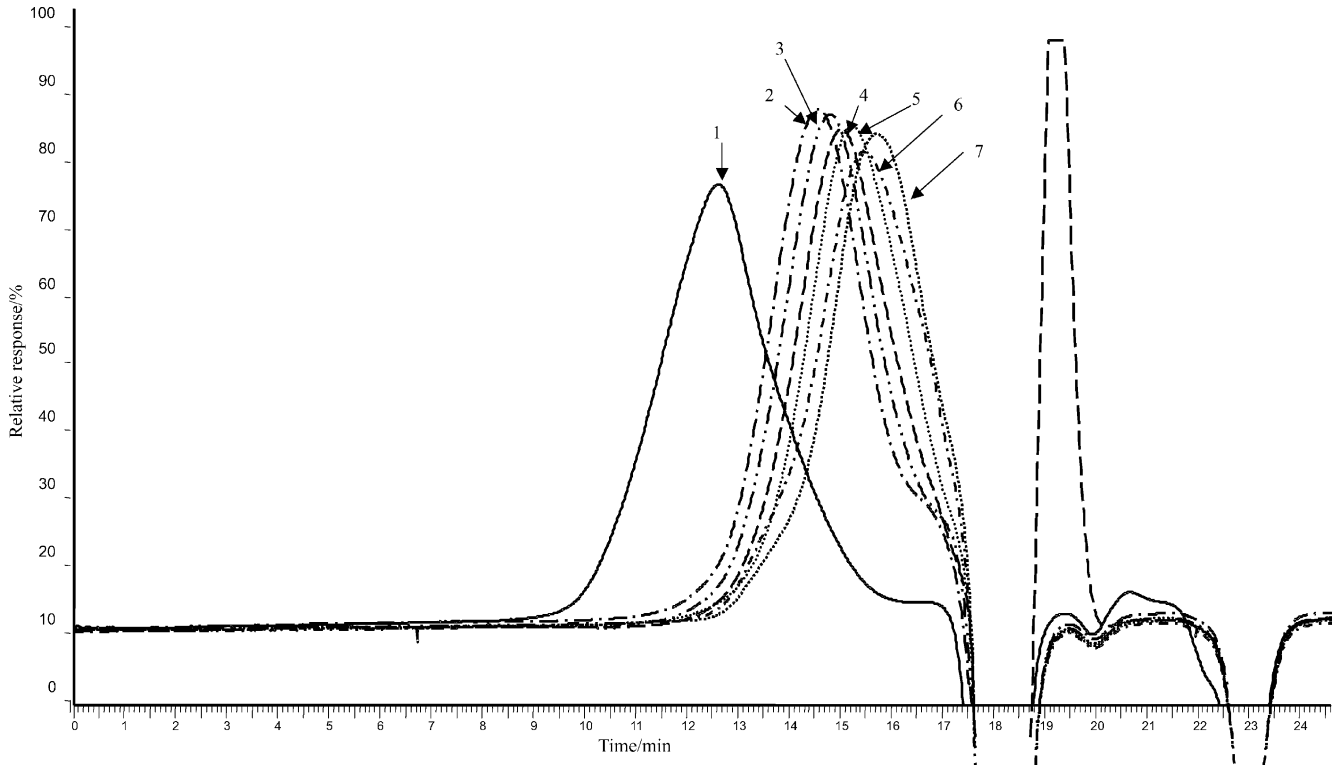

Fig. 2. SEC RI chromatograms for unhydrolysed CMC (ten times dilution) and enzyme hydrolysed CMC (five times dilution). The traces are: (1) unhydrolysed CMC; (2) Tr Cel 45a-; (3) Hi Cel 45a-; (4) Hi Cel 7b-; (5) Tr Cel 7b-; (6) Ba Cel 5a-; (7) Hi Cel 5a-hydrolysed CMC.

able to investigate the modified sugars liberated in enzyme hydrolysis would further facilitate this type of investigation.

Fig. 3 shows typical HPAEC–PAD chromatograms of CMC enzyme hydrolysates, used to determine soluble sugars. The major peaks are identified as being from underivitised cello-oligosaccharides by the use of external standards. However, several unidentified peaks can be observed especially in the 15–20 min region. It is reasonable to assume that the unidentified peaks are modified cello-oligosaccharides as PAD is sensitive towards compounds that readily undergo electrochemical oxidation. Although the response is affected when the hydroxyl groups are converted into carboxymethyl groups, the modified cello-oligosaccharides should be expected to undergo electrochemical oxidation. There are some noticeable differences in the peaks in this region both with respect to size and retention times. It would therefore be desirable to use this information when analysing the hydrolysates. However, due to the lack of standards it is not possible to identify all the observed peaks. Furthermore, due to the acidic nature of the carboxymethyl moiety many hydrolysis products are not eluted from the anion-exchange column. Large cellulose molecules with a number of carboxymethyl moieties carry such a high charge that it is not possible to elute them using normal HPEAC eluents. This is confirmed by the fact that when the column is regenerated after CMC hydrolysate analysis, a large peak is eluted by regeneration solution. Coupling the instrument to an electrospray ionisation (ESI)-MS system can circumvent the limitation of needing standards as mass spectrometry offers identification by molecular mass. However, HPAEC eluents (typically NaOH and NaOAc in aqueous solution) are not readily compatible with ESI-MS. The chemical noise that high salt concentrations cause, necessitate the use of an on-line desalting unit [9,26], which in turn, causes band broadening.

Table 1
Soluble sugars and reducing ends

	Glucose (mM)	Cellobiose (mM)	Cellotriose (mM)	Cellotetraose (mM)	Cellopentaose (mM)	Glucose equivalents (mM)	Reducing ends (mM)	Depolymerised (%)
Ba Cel 5a	0.61	1.24	0.02	nd	nd	3.14	6.6	13.3
Hi Cel 45a	0.02	1.15	0.21	nd	nd	2.95	4.3	7.0
Hi Cel 5a	2.71	0.54	0.01	nd	nd	3.81	6.9	14.1
Hi Cel 7b	3.56	nd	nd	nd	nd	3.56	6.0	12,2
Tr Cel 45a	0.48	0.42	0.11	0.05	0.01	1.92	3.5	7.1
Tr Cel 7b	3.5	2.3	nd	nd	nd	3.51	7.0	14.1

The amount of soluble underivitised sugars produced during enzyme hydrolysis as well as the amount of reducing ends and the degree of degradation.

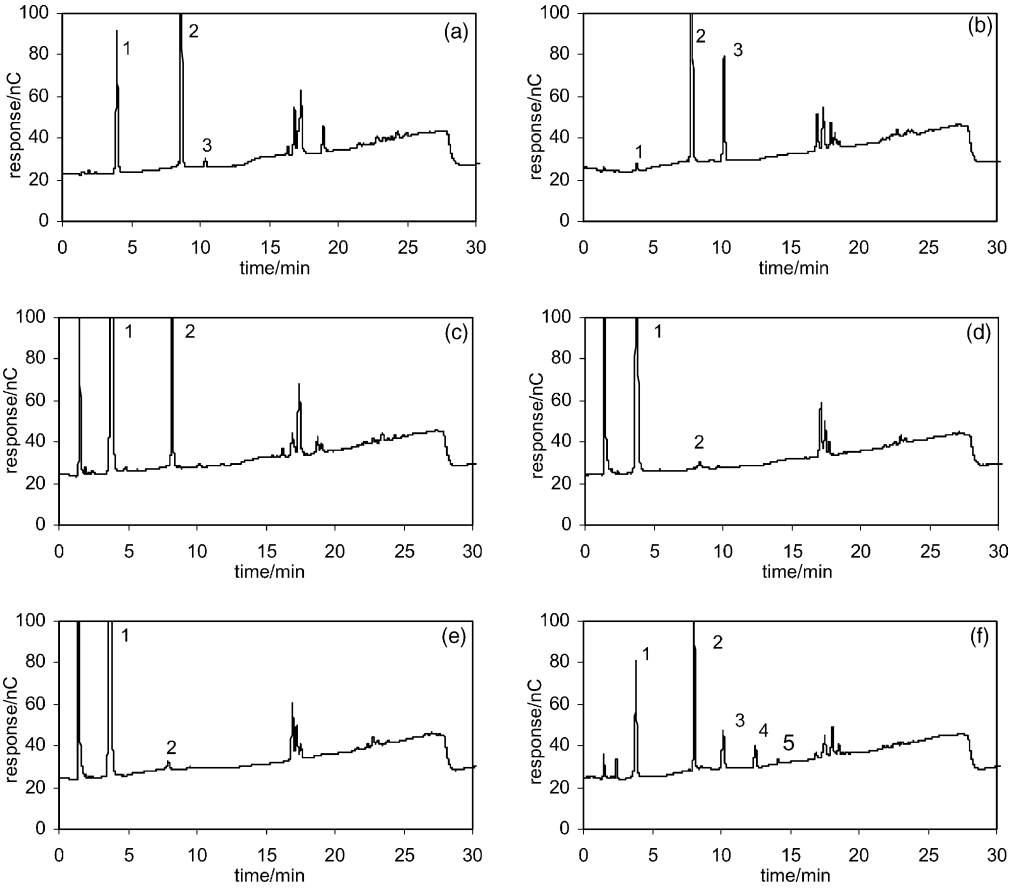

Fig. 3. HPAEC–PAD chromatograms for CMC enzyme hydrolysates. The hydrolysates were produced using: (a) Ba Cel 5a; (b) Hi Cel 45a; (c) Hi Cel 5a; (d) Hi Cel 7b; (e) Tr Cel 45a; and (f) Tr Cel 7b. The major peaks were identified as: (1) glucose; (2) cellobiose; (3) cellotriose; (4) cellotetraose; and (5) cellopentaose.

The fact that many analytes of interest remain trapped on the column is a more serious problem. Aside from the fact that information about the sample is lost, it is detrimental to the performance of the column.

Generally speaking, HPAEC–PAD is a useful and widely applicable instrument for the analysis of carbohydrates and is one of the most important techniques nowadays for the investigation of carbohydrates [27,28]. The drawbacks mentioned above show that there is a need for alternative methods for carbohydrate analysis.

3.3. LC–MS analysis

The graphitised carbon black column offers some advantages compared to the more commonly used HPAEC column. Primarily, it enables the analysis of an-ionically modified cellulose hydrolysis products. There is also much less chemical noise as the eluents are more compatible with electrospray mass spectrometry. As there is no need for desalting, the band broadening associated with on-line desalting is avoided. On the down side, the retention mechanisms of GCB columns are poorly understood, making it difficult to predict the retention times and order. Also, we found it was necessary to filter the hydrolysates in order to avoid column fouling. Enzymes interacting with the column packing material probably caused the fouling.

The *m*/*z* was calculated for all possible hydrolysis products with a degree of polymerisation of up to 8. The following formula was used to calculated the *m*/*z* values for the modified hydrolysis products (Table 2):

$$\frac{m}{z} = \frac{180.06 + 162.05\,(n-1) + 58.01\,s}{s}$$

where n is the degree of polymerisation and s is the number of substitutions. The value 180.06 is the mass of a single glucose unit. Each additional glucose unit increases the mass with 162.05 mass units and each substitution contributes with 58.01 mass units. As each DP can carry up to three substituents, s cannot exceed $3n$. Although each carboxymethylated hydrolysis product can be ionised to varying degrees, only fully ionised products were observed, probably due to the alkalinity of the eluents (i.e. each carboxymethyl group contributed with one negative charge). Consequently, modified products were monitored as having the same charge as their number of modifications. Unmodified hydrolysis products were monitored as $M - 1$ (i.e. one deprotonated hydroxyl group). Chromatograms for each *m*/*z* of interest were produced and all observed peaks were integrated and thus a

Table 2
Monitored *m*/*z* values

Mod	dp							
	1	2	3	4	5	6	7	8
0	179.1	341.1	503.2	665.2	827.3	989.3	1151.4	1313.4
1	237.1	399.1	561.2	723.2	885.3	1047.3	1209.4	1371.4
2	147.0	228.1	309.1	390.1	471.1	552.2	633.2	714.2
3	117.0	171.0	225.1	279.1	333.1	387.1	441.1	495.1
4		142.5	183.0	223.6	264.1	304.6	345.1	385.6
5		125.4	157.8	190.2	222.7	255.1	287.5	319.9
6		114.0	141.0	168.0	195.1	222.1	249.1	276.1
7			129.0	152.2	175.3	198.5	221.6	244.8
8			120.0	140.3	160.5	180.8	201.1	221.3
9			113.0	131.0	149.0	167.0	185.0	203.1
10				123.6	139.8	156.0	172.2	188.4
11				117.6	132.3	147.0	161.8	176.5
12				112.5	126.0	139.5	153.0	166.5
13					120.7	133.2	145.7	158.1
14					116.2	127.7	139.3	150.9
15					112.2	123.0	133.8	144.6
16						118.9	129.0	139.2
17						115.3	124.8	134.3
18						112.0	121.0	130.0
19							117.7	126.2
20							114.6	122.7
21							111.9	119.6
22								116.8
23								114.2
24								111.8

All possible hydrolysis product with a degree of polymerisation (dp) ≤8 were monitored. Each dp can carry up to three modifications (mod).

Table 3
Detected hydrolysis products

Mod	dp							
	1	2	3	4	5	6	7	8
0	abcef	abcef	a					
1	abcdf	abcdf	bcdef	abcdef				
2	df	bcdef					a	
3	ac	a	a	abcdf		a		
4		abcfe		a	a	a	abcfe	
5					ac	bcf		
6			a		bcdef	e	abcdef	
7								
8					abcfe	bcdef	c	
9			abcef					
10								a
11							abe	bdf
12								
13								
14								
15								
16						a		
17							a	
18								
19								
20								
21								
22								ac
23								
24								

Products detected in CMC hydrolysates using the following enzymes: a = Ba Cel 5a, b = Hi Cel 45a, c = Hi Cel 5a, d = Hi Cel 7b, e = Tr Cel 45a, and f = Tr Cel 7b.

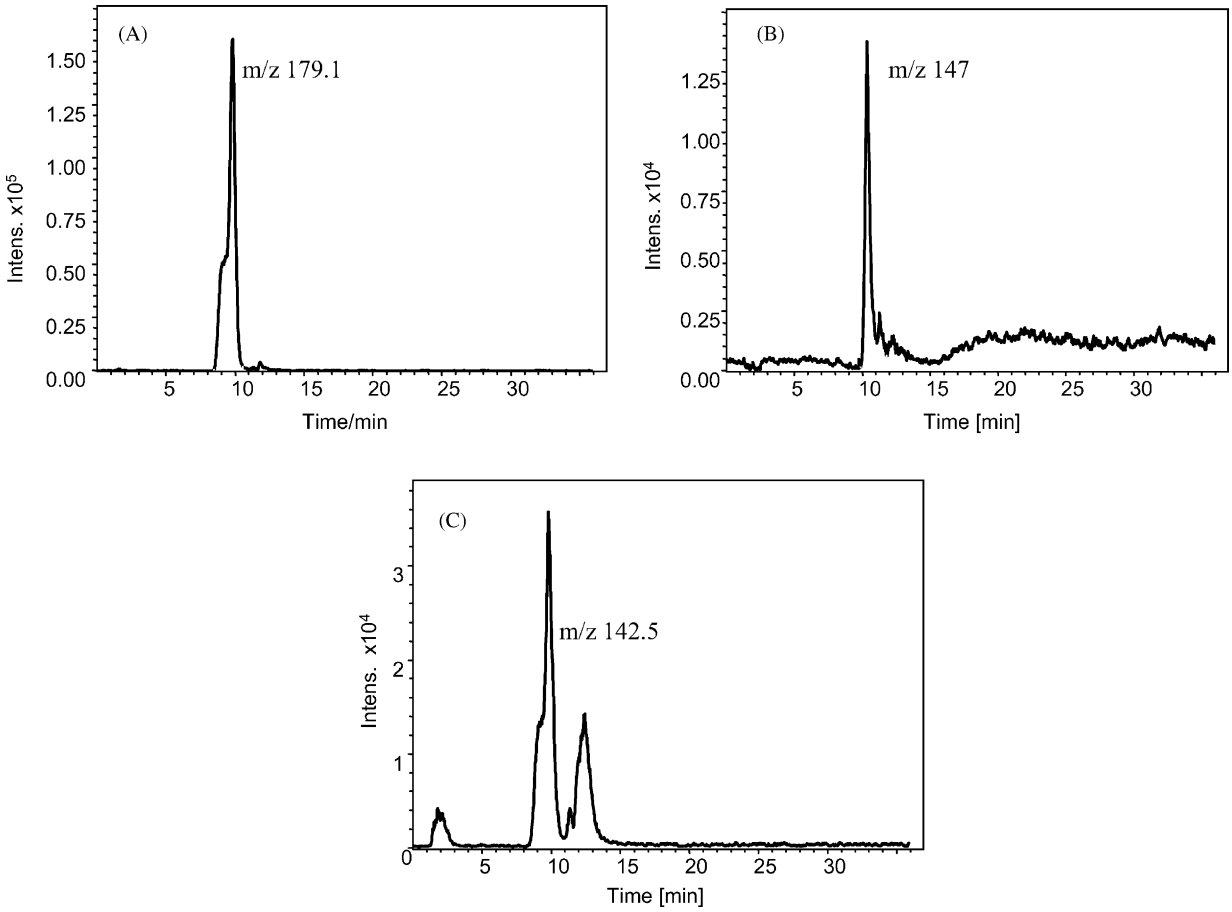

Fig. 4. Typical mass chromatograms for the *m*/*z* values that correspond to: (A) glucose 179.1; (B) di-substituted glucose 147; and (C) tetra-substituted cellobiose 142.5.

total of 116 *m*/*z* were monitored. The default signal width for the chromatograms is ±0.5 *m*/*z*. However, the signal width for the chromatograms was reduced to ±0.2 when the default width could lead to misidentification. Fig. 4 shows typical mass chromatograms for an enzyme hydrolysate. Although it is not possible to determine the concentration of the analytes (as no standards are available) it is clear that the detected compounds are well above the limit of detection. The fact that several peaks are observed in Fig. 4C is not surprising as tetra substituted cellobiose has 30 possible isomers.

In order to simplify the data, the peak areas for each *m*/*z* have been combined. When investigating enzyme selectivity, the ability to form certain products is of more interest than the amount of products formed. In Table 3, the formed products using all six enzymes are shown. In order to determine which compounds were detectable the following criteria were established: the peak heights must be above three times the noise for the mass chromatogram, and the peak had to be higher than 10 000 response units. Each enzyme produced a unique pattern of hydrolysis products. Although only six enzymes were investigated in this study, it may be possible to use the hydrolysis pattern as a fingerprint.

The fact that all enzymes except Tr Cel 45a produced modified glucose suggests that these enzymes are able to hydrolyse a glycosidic linkage adjacent to a modified unit. The fact that several heavily modified long chain products are liberated is indicative of a block-wise modification pattern. When comparing Table 3 with Table 1, we can see that the LC–MS method is unsuitable for the analysis of underivitised sugars. In Table 1, glucose is detected in all hydrolysates and cellotriose is detected in four of the hydrolysates. In Table 3, glucose is not detected for the Hi Cel 7b hydrolysate, the enzyme that gave the highest yield of glucose, and cellotriose is only detected in the Ba Cel 5a hydrolysate. The poor sensitivity towards the underivatized cellodextrines is probable due to low ionisation efficiency. A pH of 9 is sufficient to ionise carboxymethyl groups ($pK_a \sim 4.8$), however the deprotonation of hydroxyl groups calls for a much higher pH, which would entail using salts (i.e. NaOH) which, in turn, would call for on-line desalting. Thus, the LC–MS method complements rather than replaces HPEAC–PAD.

In Tables 4 and 5, semi-quantitative hydrolysis data are shown. The samples used in these cases contained 20 g/l CMC (instead of 10 g/l). The only limitations applied on peak quality were that the peak height should exceed three times the noise; absolute peak height was not taken into account. For Tr Cel 7b and Ba Cel 5a the number of

Table 4
Ba Cel 5a hydrolysate products

Mod	dp							
	1	2	3	4	5	6	7	8
0	1.54×10^7	7.46×10^6	2.27×10^4					
1		1.23×10^5	1.44×10^5					
2	7.75×10^4	2.75×10^6		2.48×10^6		1.91×10^7		
3	4.16×10^7		2.46×10^7		4.51×10^6	1.52×10^5	7.02×10^4	1.46×10^7
4			8.29×10^4			2.29×10^7		4.60×10^6
5				1.27×10^7	1.50×10^6	4.21×10^4	1.34×10^7	
6			2.77×10^6	2.04×10^7	2.83×10^5	1.72×10^7		2.38×10^6
7				4.20×10^4	2.85×10^7	1.80×10^7		1.49×10^6
8			6.60×10^6	1.52×10^6		4.50×10^7	6.96×10^4	
9			7.73×10^7			3.32×10^4	3.77×10^5	
10				7.93×10^5				
11								6.59×10^6
12						1.59×10^5		
13					3.46×10^7		7.47×10^6	
14								
15						2.19×10^7		
16								
17								
18								2.98×10^5
19								
20								
21								2.11×10^5
22								1.12×10^6
23								
24								

Peak areas of degradation product formed during enzyme hydrolysis of CMC using Ba Cel 5a. The peak areas listed are the integrated signal intensities for the peaks detected in the *m/z* chromatograms that correspond to each dp, mod combination.

Table 5
Tr Cel 7b hydrolysate products

Mod	dp							
	1	2	3	4	5	6	7	8
0	2.00×10^7	1.78×10^6		2.48×10^4	1.61×10^7			
1	1.16×10^7	1.01×10^7	2.23×10^6	3.42×10^5				
2	9.05×10^5	3.26×10^5	6.57×10^5	5.46×10^4	2.19×10^5	1.08×10^5	1.27×10^5	1.24×10^5
3	1.18×10^6	7.63×10^4	3.50×10^5	4.70×10^6		4.61×10^6	2.39×10^5	
4		2.09×10^6	3.08×10^5		2.18×10^5	1.19×10^7	1.33×10^5	
5					1.70×10^7	4.99×10^6		
6			3.73×10^6		4.45×10^4		7.01×10^5	
7								3.61×10^4
8			6.48×10^3		8.13×10^6	1.79×10^5	9.46×10^6	
9			5.73×10^5					7.38×10^4
10								6.24×10^5
11							1.99×10^7	2.37×10^6
12							8.40×10^4	
13							6.84×10^4	
14								
15						5.28×10^5		
16								
17							5.50×10^5	
18							6.82×10^6	
19								
20								
21								
22								1.18×10^6
23								
24								

Peak areas of degradation product formed during enzyme hydrolysis of CMC using Tr Cel 7b. The peak areas listed are the integrated signal intensities for the peaks detected in the *m/z* chromatograms that correspond to each dp, mod combination.

detectable compounds were 48 and 47, respectively, which is approximately half of the total number of monitored compounds. Only 27 of the compounds were detected in both hydrolysates, 38 compounds were only detected in either one or the other of the hydrolysates. For compounds detected in both hydrolysates, the signal areas of the compounds can differ with up to two decades. This demonstrates that the LC–MS method can be used for semi-quantitative sample-to-sample comparison, as well as for qualitative analysis.

4. Conclusions

The LC–MS method presented offers a tool for qualitative and semi-quantitative analysis of enzyme-degraded carboxymethylcellulose. It offers the possibility to compare degradation products without the need for standards. Although a study of the enzyme activity and substituent pattern is beyond the scope of this work, it is possible to draw some conclusions about both from the data presented. All enzymes have the ability to hydrolyse a glycosidic bond adjacent to a modification (substituted monomers were detected in all hydrolysates) and the carboxymethylcellulose used was substituted in a block wise fashion (highly substituted DP 7 with six modifications was observed in all hydrolysates).

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