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Carbohydrate Research 328 (2000) 343–354

CARBOHYDRATE
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Determination of substituents distribution in carboxymethylpullulans by NMR spectroscopy

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Received 22 December 1999; accepted 12 April 2000

Abstract

The distribution of carboxymethyl substituents in the α-(1 → 6)-linked maltotriosyl repeating units of a carboxymethylpullulan (CMP) series was investigated by high resolution NMR spectroscopy on very short oligomers ($\overline{\text{DP}}\text{n} = 1.2–1.5$) obtained by acid hydrolysis. A series of 2D NMR experiments on parent pullulan, hydrolysed pullulan and CMP was used to assign the proton and carbon chemical shifts of CMP acid hydrolysates. The degree of substitution (DS) and the relative distribution of $–CH_2COONa$ groups at OH-2, OH-3, OH-4 and OH-6 of glucose residues (DS_i) were determined from ^1H NMR measurements. From a set of CMP samples, widely different in degree of substitution, it was observed that the substitution at C-2 is predominant and decreases according to the order C-2 > C-3 > C-6 > C-4. Taking into account the availability of each OH group in the parent pullulan, an order of relative reactivity of hydroxyl groups is defined according to the relation: $R_i = DS_i/n_i$, where n_i is the number of free OH groups in a maltotriose unit (MTU) for a given site C-i, the reactivity order was found to be OH-2 > OH-4 > OH-6 > OH-3. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Pullulan; Carboxymethylpullulan; Polysaccharide derivatives; Hydrolysis; ^1H and ^{13}C NMR structural study

1. Introduction

A variety of pullulan derivatives has been prepared to examine their potential application in the food, cosmetics, pharmaceutical and industrial fields [1–6]. Recently, we synthesized a series of hydrophobically-associating polysaccharides derived from pullulan [7,8]. These were obtained by grafting alkylated or perfluoroalkylated long chain amines onto some carboxylic acid groups of carboxymethylpullulan. We have shown that these polymers exhibit a viscosifying behaviour in aqueous solution depending on structural parameters such as the length and content of hydrophobic groups and the density in carboxylate groups and polymer concentration amoung others.

Pullulan is a linear water-soluble polysaccharide having a maltotriosyl repeating unit (MTU) containing two α-(1 → 4) linkages and one α-(1 → 6) linkage (cf. Scheme 1). The three anhydroglucose units (AGU) in the maltotriose unit are designated A, B and C. Carboxymethylation is performed by etherification of hydroxyl groups of pullulan with sodium chloroacetate under controlled conditions [7,9]. The reaction conditions were chosen so that the resulting polymer has an

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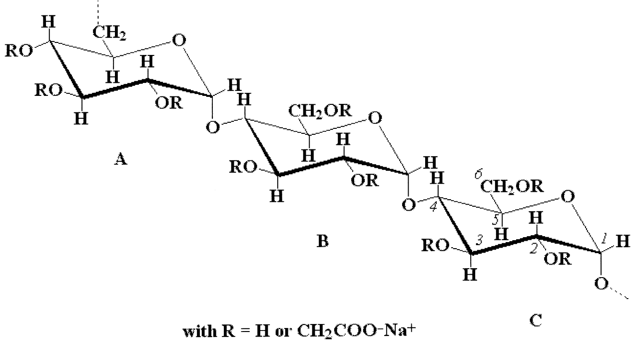
PII: S0008-6215(00)00120-8

average degree of substitution (DS_{AGU}) of hydroxyl groups in the range 0.6–1.2 per anhydroglucose unit.

As the maltotriosyl unit contains nine hydroxyl groups, the partial substitution may occur at all the hydroxylated reaction sites C-2, C-3, C-4 and C-6 resulting in a non-uniform distribution of carboxymethyl substituents. The knowledge of this distribution is of great importance in understanding structure–properties relationships.

The DS is usually determined by analytical techniques; which result only in average DS values without providing information on the distribution of the $-CH_2COONa$ substituent between the different hydroxyl groups of the MTU. NMR spectroscopy is presently one of the effective methods to characterize the structure of polymers [10], but its application to polysaccharides is still limited. This results from the high viscosity of aqueous polymer solutions and the heterogeneity of their structures [11,12]. Owing to the low mobility of these molecules, broadening of ^1H and ^{13}C NMR signals and poor signal-to-noise ratios are generally observed. In the case of carboxymethylcellulose (CMC), this problem was solved by degrading the polymer to very short oligomers [11,12]; ^1H and ^{13}C NMR techniques [13–16] applied to CMC hydrolysates provided detailed structural information including the distribution of carboxymethyl substituent within the glucose unit.

The aim of this work was to determine the distribution pattern of the carboxymethyl substituent between the C-2, C-3, C-4 and C-6 sites available in the maltotriosyl unit. Structural characterization was performed by NMR spectroscopy on hydrolysed CMP samples. Two methods of hydrolysis were compared: enzymatic and acid hydrolysis.



Scheme 1. Structures of pullulan (R = H) and carboxymethylpullulan (R = CH_2–COONa or H).

In this paper, we report a comparative study of pullulan, hydrolysed pullulan and CMP using a series of 2D NMR techniques, including H–H COSY and H–H TOCSY and, in particular, field-gradient HMBC and HSQC techniques which allowed us to assign all relevant peaks. A quantitative proton NMR procedure to provide both the total (DS) and partial (DS_i onto C-i sites) degrees of substitution of selected CMC samples is presented in this study.

2. Experimental

Samples.—Pullulan ($\overline{M}w = 220{,}000$ Da, $\overline{M}n = 120{,}000$ Da) was from Hayashibara Biochemical Laboratory (Okayama, Japan). Sodium chloroacetate, sodium hydroxide and isopropyl alcohol were analytical-grade commercial products and were used without purification. α-Amylase [bacterial from *Bacillus licheniform*, type XII-A, 500–1000 units per mg protein (Biuret)], β-Amylase [crude from barley, type II-B, 20–80 units per mg protein (Biuret)] and Pullulanase (from *Klebsiella pneumoniae*, 10–30 units per mg protein) were products of Sigma (Saint Quentin Fallavier, France). Deuterium oxide, 99.90% D and 18 M D_2SO_4 in D_2O, were purchased from Euriso-Top Company.

Sodium salt of carboxymethylpullulans.—Carboxymethylpullulan was prepared by reaction of alkali-pullulan with sodium chloroacetate in a 2:1 isopropanol–water mixture according to Ref. [7].

The samples were designated as follows: Na-CMP(X) refers to a sodium salt of carboxymethylpullulan with a degree of substitution $DS_{AGU} = X$ defined as the number of carboxymethyl groups per anhydroglucose unit. It was determined by Eyler's titration [17] using a conductimetric Tacussel type CD 810. For these investigations, a set of Na-CMPs with various DS from 0.6 to 1.2 were synthesized by varying the molar ratio of chloroacetate to AGU.

Enzymatic hydrolysis.—Na-CMP (0.3 g) was dissolved in 10 mL of 0.01 M sodium acetate buffer pH 4.8 (β-amylase), pH 5.5 (pullulanase) or 0.01 M potassium phosphate buffer pH 6.9 (α-amylase). The enzymatic digestion was performed by incubating the buffered polymer solution with 7 U $mL^{-1}$of the respective enzyme. The mixtures were kept at 35 °C. At fixed time intervals, 1 mL of each solution was taken and placed in a boiling water bath for 10 min to stop the reaction. Then, 100 μL of the supernatant filtered through a 0.45 μm Millex-HV type membrane were injected onto a size-exclusion chromatography system (SEC with two TSK G4000PW and G6000PW columns) equipped with a multi-angle laser light scattering photometer (MALLS-Wyatt Dawn-F photometer) and a differential refractive index detector (Shimadzu RID-6A). The sample was eluted by 0.1 M $LiNO_3$ at a flow rate of 0.6 mL min^{-1}. The ASTRA V-4.0 software package was used to calculate the molecular weight ($\overline{M}$n). The refractive index increment of pullulan and Na-CMP solutions were, respectively, 0.146 and 0.16 mL g^{-1} [7].

Acid hydrolysis.—Hydrolysis was achieved by heating 1 g of pullulan or Na-CMP(0.5) in 20 mL of 2.4 M perchloric acid at 65 °C. The degradation kinetics were followed by varying the reaction time from 1 to 8 h. After hydrolysis, the resulting solution was cooled in an ice-bath, neutralised with potassium hydroxide and the precipitate of potassium perchlorate was removed by filtration. The water was then removed in a rotatory evaporator and the solid residue dried under diminished pressure for 12 h at 50 °C. For SEC/MALLS analysis, 0.1 mL of each sample ($c = 3-5$ mg mL^{-1}) dissolved in 0.1 M $LiNO_3$ was filtered through a 0.45 μm Millex-HV type membrane before injection.

NMR spectroscopy.—^1H and ^{13}C NMR measurements were performed at 35 °C with two a Bruker AVANCE DMX 500 and a Bruker AVANCE DMX 600 equipped, respectively, with a QXI (^1H, ^{13}C, ^{15}N, ^{31}P) and TXI (^1H, ^{13}C, ^{15}N) 5 mm probe. Approximately 30 mg of sample was directly dissolved in 0.5 mL of D_2O in the NMR tube; 0.1 mL of D_2SO_4 was added into the tube in order to shift the residual HOD signal upfield (8 ppm). All NMR spectra were referenced to sodium 4,4-dimethyl-4-silapentane-1-1-sulfonate (DSS).

^1H NMR spectra were recorded with a 9.62 Hz spectral window digitized with 32 K points. The 1D ^{13}C spectra were recorded between 0 and 200 ppm using a 30,300 Hz spectral window digitized into 64 K points. Broad band proton decoupling was employed. HSQC spectra were measured using 512 increments of 16 scans each of 2048 complex points, with spectral widths in f_1 and f_2 of 22,637 and 5000 Hz, respectively, and a relaxation delay of 2 s. The HMBC spectra used similar conditions but with 24 transients per increment, and Δ delay of 40 and 80 ms. The HSQC spectra were displayed in phase sensitive mode and the HMBC in absolute value. All two-dimensional NMR data matrices were zero-filled in the f_1 dimension. Prior to Fourier transformation, a phase shifted sine-bell filter function was applied. Processing of NMR data was performed on a SGI Indigo2, using the Bruker program Xwinnmr 2.1 and Aurelia 2.0. method. The inverse-gated decoupling method with a relative delay of 40 s was used to suppress NOE and thus allow correct quantitative evaluation of the ^{13}C NMR spectra. The deconvolution of ^1H spectra of CMP was executed using the XEDPLOT-XWIN NMR Bruker's program.

3. Results and discussion

The choice of degradation procedure of the carboxymethyl pullulans.—Two methods of hydrolysis were tested, i.e., enzymatic and acid, i.e., to obtain solutions of low viscosity whose NMR spectra could be interpreted.

Enzymatic hydrolysis.—Na-CMP(0.8) was incubated with three different types of enzymes known to attack pullulan [18,19]. α-Amylase, an endo-enzyme, splits the α-(1 → 4) linkages while β-amylase, assigned as an exo-enzyme, hydrolyses α-(1 → 4) bonds from the non-reducing end groups. Pullulanase hydrolyses the α-(1 → 6) links [19] to produce mainly maltotriose. To distinguish between hydrolytic and enzymatic reactions, the degra-

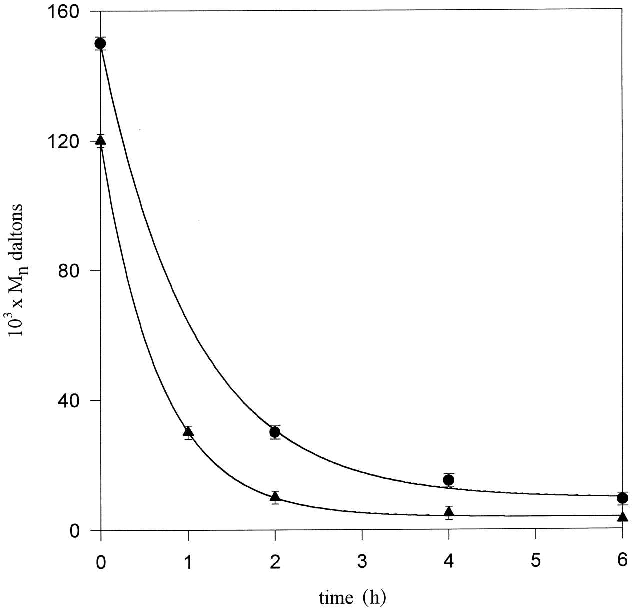


Fig. 1. Plots of number average molecular weight ($\overline{Mn}$) against acid hydrolysis time for pullulan (▲) and Na-CMP(0.8) (●).

dation of carboxymethylpullulan in the absence of enzyme was studied: hydrolysis was negligible. Degradation was followed by SEC/MALLS measurements. α-Amylase and pullulanase were found to be strongly inhibited by carboxymethylpullulan since no reduction in molecular weight was detected after 15 days of incubation.

The chromatographic profiles of Na-CMP(0.8) hydrolysed by β-amylase shifted to higher elution volumes with hydrolysis time indicating that the polymer was slowly degraded by the enzyme. The molecular weight $\overline{Mn}$ decreases from 15×10^5 to $20 \pm 2 \times 10^3$ Da after 13 days of incubation. Therefore, the partial carboxymethylation of hydroxyl groups makes pullulan resistant to enzymatic attack. Similar results were obtained with other modified pullulans [18,20] and the authors observed that the rate of hydrolysis decreases as the degree of chemical modification increases [18].

Acid hydrolysis.—From studies of carboxymethylcellulose, hydrolysis in different concentrated acidic media yields substituted dimers or monomers with no effect on the carboxymethyl groups [13–15]. Because of the sensitivity of pullulan toward strong acids, the hydrolysis of our samples was conducted in 2.4 M perchloric acid at 65 °C, the reaction time ranging from 1 to 8 h. Fig. 1 shows the degradation curves of pullulan and Na-CMP(0.8) obtained from SEC/MALLS measurements. The value of $\overline{Mn}$ decreases with reaction time and reaches, after 6 h of hydrolysis, a value of 3 and $8 \pm 2 \times 10^3$ Da for pullulan and CMP, respectively. Because of the very poor light scattering by samples degraded for 8 h, it was not possible to determine a $\overline{Mn}$ value with accuracy. Nevertheless, the comparison between the two methods indicates that acid degradation is faster and leads to shorter polymeric chains.

The acid hydrolysates were analysed by NMR spectroscopy. Fig. 2 shows the ^1H spectra of untreated and acid-treated pullulan and Na-CMP(0.8) samples. Comparison of the spectra indicates improved resolution for the degraded samples, especially for CMPs.

As shown in Fig. 2(a), the ^1H spectrum for pullulan is characterized by three peaks resolved from the envelope of peaks resonating between 3.3 and 4 ppm [21]. The signals at 5.30, 5.28 and 4.86 ppm were assigned to the three anomeric protons of the anhydroglucose units noted A, B and C in Scheme 1. Comparison of the peak areas revealed that pullulan has α-(1 → 4) and α-(1 → 6) linkages in a ratio of 2:1 as would be expected for a α-(1 → 6) maltotriose repeating unit.

In the case of the degraded pullulan (Fig. 2(b)), two new sets of signals detected at 5.18 and 4.61 ppm represent, respectively, the α and β anomeric protons in the terminal reducing glucose residues. They appear as multiplets (two overlapping doublets with the same coupling constant) indicating that at least two types of oligomers result from the cleavage of the glycosidic links in pullulan. The intensities of these signals give the relative proportion of α and β forms in equilibrium in aqueous medium. For this sample, 38% was in the α and 62% in the β anomeric conformation, in good agreement with the literature [22,23].

After hydrolysis, the signal of the three anomeric protons internal to the polymer chain became less intense, particularly those belonging to rings involved in the α-(1 → 4) linkages. The integration of these peaks gave a ratio close to 0.5 between the α-(1 → 4) and α-(1 → 6) linkages instead of 2 before hydrolysis. Therefore, the acid hydrolysis cleaves preferentially the α-(1→4) linkages in pullulan. Besides, the degree of polymerisation ($\overline{\mathrm{DPn}}$), i.e., the average number of anhydroglucose units in the polymeric chain, can be quantitatively determined from the ratio of

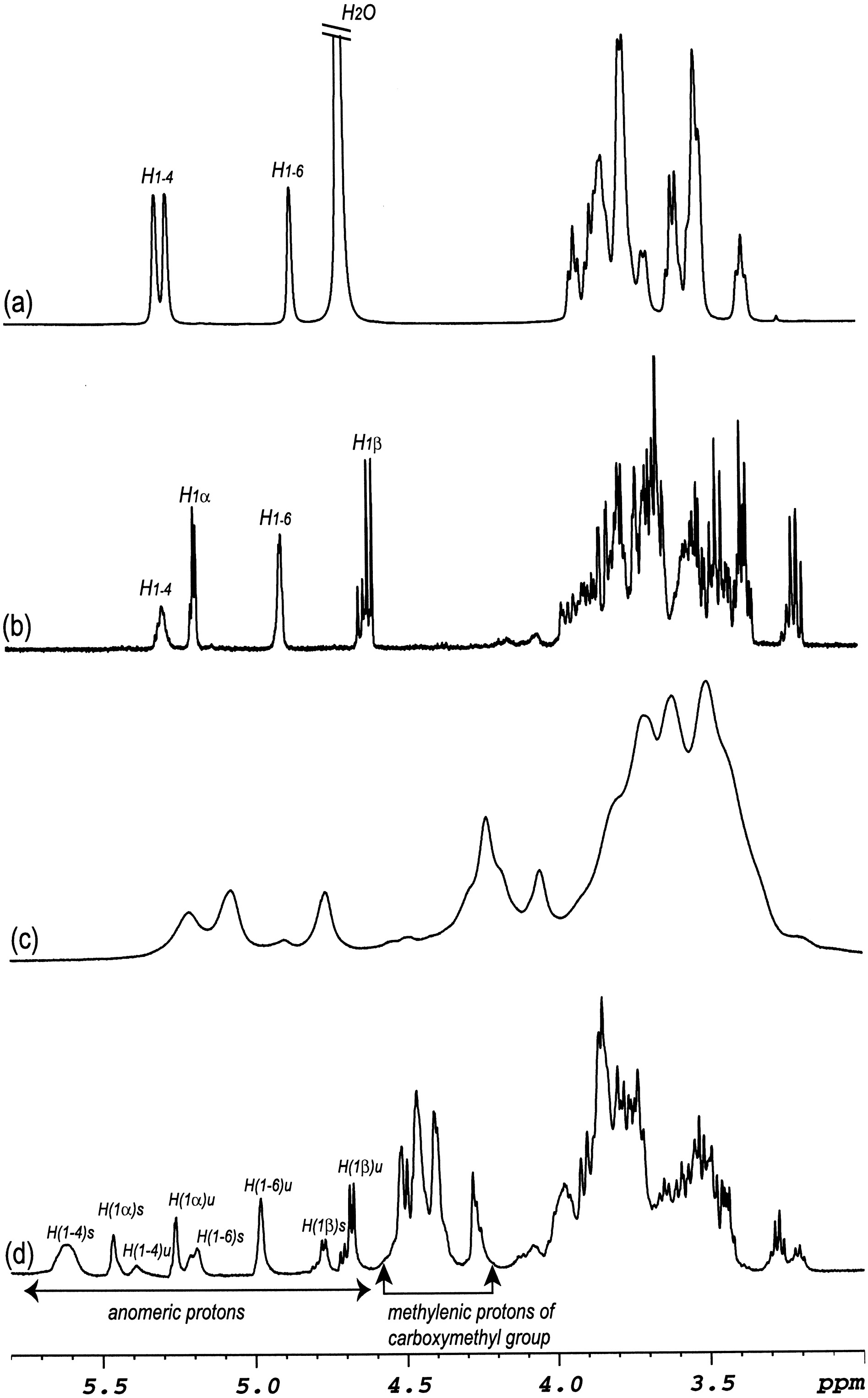


Fig. 2. Proton NMR spectra (500 MHz). (a, c) pullulan (D_2O) and Na-CMP(0.8) ($D_2O + D_2SO_4$); (b, d) acid hydrolysed pullulan and Na-CMP(0.8) ($D_2O + D_2SO_4$). Resonances arising from the anomeric protons at the α-(1→4) and α-(1→6) linkages and the α and β reducing terminal end are indicated. **u** and **s** refer to unsubstituted and substituted unit at C-2.

Table 1
^{1}H and ^{13}C chemical shifts (ppm) of pullulan in deuterated water at 298 K [a]

AG unit	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
A	5.28	3.52 [b]	3.62	3.38	3.84	3.70	3.85
B	5.30	3.54 [b]	3.88	3.52	3.77	3.82	3.82
C	4.86	3.52	3.93	3.60	3.77	3.77	3.77
	C-1	C-2	C-3	C-4	C-5	C-6	C-6
A	100.6	72.1 [c]	73.4	69.8	71.9 [c,d]	66.8	66.8
B	100.1	72.0 [c]	73.7	78.1 [c,d]	71.7	61.1 [c,d]	61.1 [c,d]
C	98.3	71.5	73.8	77.7 [c,d]	70.7 [c,d]	60.7 [c,c]	60.7 [c,c]

[a] Reference: DSS, 0 ppm for ^{1}H and -3.0 ppm for ^{13}C NMR spectra.

[b] Reversed in relation to Ref. [21].

[c] Reversed in relation to Ref. [28].

[d] Reversed in relation to Refs. [21] and [28].

the intensities of peaks belonging to all the anomeric protons over those belonging to α and β anomeric protons. A value of $\overline{\mathrm{DPn}} \sim 1.4$ was found. The same calculation from signals corresponding to the linked and the free reducing end C-1 in the quantitative ^{13}C NMR measurement gave a similar value for $\overline{\mathrm{DPn}}$(~ 1.6).

As seen in Fig. 2(d), partial carboxymethylation of pullulan gives a much more crowded NMR spectrum particularly in the spectral region between 4 and 5.7 ppm. The substitution of hydroxyl groups caused a splitting of anomeric protons signals. Similar observations were reported for hydrolysed carboxymethylcellulose [13]. Most significantly, the resonance from methylene protons of the carboxymethyl substituent were completely resolved from those of other protons. They appeared as a set of peaks in the 4.2–4.6 ppm spectral region and correspond to the four substitution sites namely O-2, O-3, O-4 and O-6. The good resolution of these resonance in the NMR spectrum gives good experimental support that it is possible to determine carboxymethyl distribution pattern of the CMP polymers by NMR structural analysis. In this example, an approximate $\overline{\mathrm{DPn}}$ of 3.4 was determined after 8 h of degradation: continuing hydrolysis for 14 h increased the efficiency of degradation to give a $\overline{\mathrm{DPn}}$ of 1.4. This corresponds approximately to a mixture of dimers and monomers.

NMR spectral analysis

Pullulan and acid-hydrolysed pullulan. The assignment of the individual resonances in the ^{1}H and ^{13}C spectra of pullulan was made on the basis of the results of ^{1}H–^{1}H homonuclear scalar correlation (COSY [24] and TOCSY [25]) and ^{1}H–^{13}C heteronuclear correlation (HMBC [26], HSQC [27]) spectroscopy and published data [21,28].

The assignment of all proton and carbon signals in the maltotriosyl unit has been completed, and the data are collected in Table 1. Absolute chemical shifts are slightly different of those reported by Mc Intyre et al. [21] and Arnosti et al. [28], which is probably due to differences in samples temperature, concentration and reference standards. Nevertheless, the comparison between our assignments and those of the precedent authors led to noticeable discrepancies: reversal of H-2 resonance positions in glucose residues A and B, reversal of C-4 resonance positions in glucose residues B and C, C-5 in glucose residues A and C and finally C-6 in glucose residues B and C. These differences arise from the assignment of the H-3 protons. The β-effect expected for H-3 protons in rings B and C — due to the α-(1 → 4) linkages — allowed us to assign unambiguously the resonances at 3.88 and 3.93 ppm to these two protons; the signal at 3.62 ppm is thus assigned to the H-3 proton of ring A.

The structure of pullulan acid hydrolysates ($\overline{\mathrm{DPn}} \sim 1.4$) was determined by 1D and 2D ^{1}H and ^{13}C NMR experiments. NMR investigations identified with certainty the resulting molecules as being mainly α- and β-glucose residues [29] and α- and β-isomaltose units [28].

Acid hydrolysed carboxymethylpullulans.— The following structural analysis for CMP samples was conducted with the sample having the lowest DS (DS ~ 0.58). We assumed that, in this case, the monosubstitution took place predominantly. Moreover, the degradation process was optimized in order to simplify the NMR study.

The ^{1}H spectra of CMP(0.58) after hydrolysis for 14–48 h are displayed in Fig. 3. In Fig. 3(a), by comparison of the spectrum of degraded pullulan, the doublets at 5.26 and 5.46 ppm were assigned to H-1 of α anomers while the ones at 4.68 and 4.77 ppm were attributed to H-1 of the β anomers. For the latter one, minor overlapping signals are observed assigned to dimers residue. The doubleta which appear at 5.38–5.61 ppm and 4.96–5.18 ppm were assigned to H-1 of glucose residues involved, respectively, in (1→4) and (1→6) linkages of disaccharide structures. By comparison with the ^{1}H NMR spectrum of degraded pullulan, the peaks at 4.77, 5.18, 5.46 and 5.61 ppm arise from H-1 of residues in which O-2 is carboxymethylated. Peaks arising from anomeric protons of glucosyl units substituted or unsubstituted at O-2 were designated **s** and **u**, respectively, in Fig. 2(d) and Fig. 3(a).

When the degradation time increased from 14 to 48 h, signals corresponding to H-1 involved in the (1→4) and (1→6) bonds, and the small doublets located close to H-1β peaks disappear, while the methylenic proton resonances of the carboxymethyl groups remained unchanged. The $\overline{\text{DPn}}$ decreased from 1.4 to approximately 1 without loss of

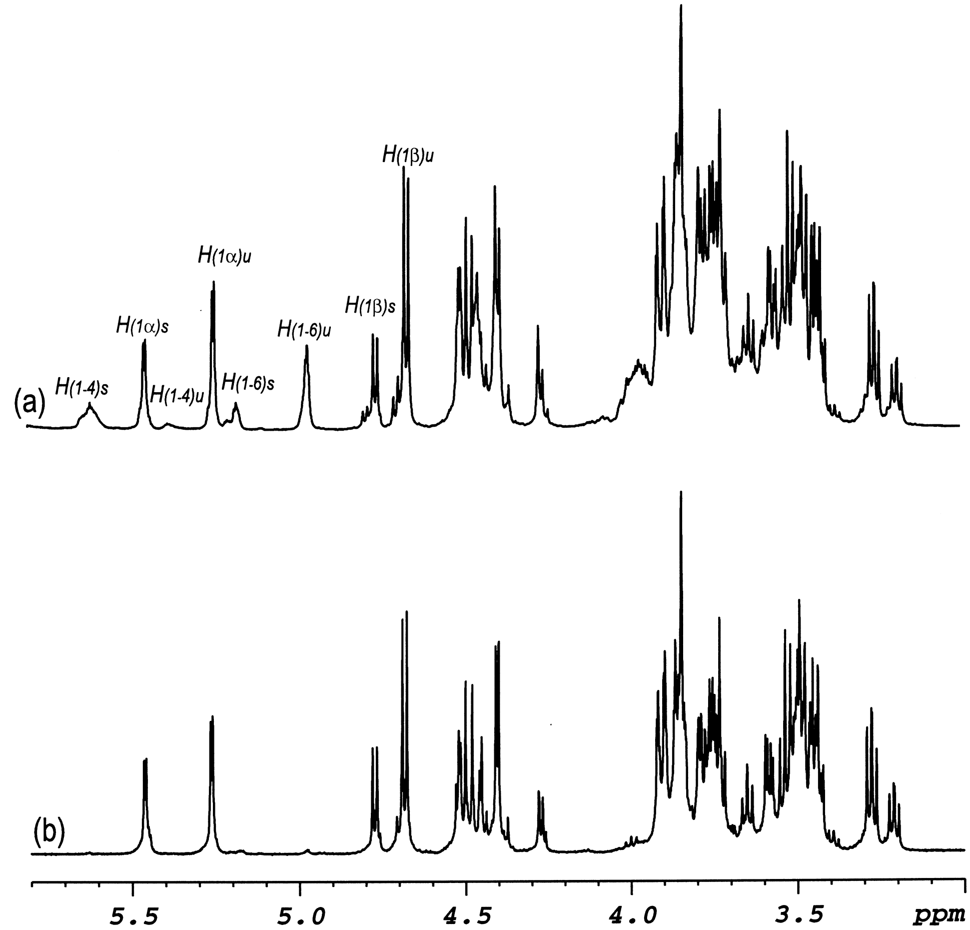


Fig. 3. Proton NMR spectra (600 MHz) of Na-CMP(0.58) as a function of hydrolysis time: (a) 14 h, and (b) 48 h (in $D_2O + D_2SO_4$).

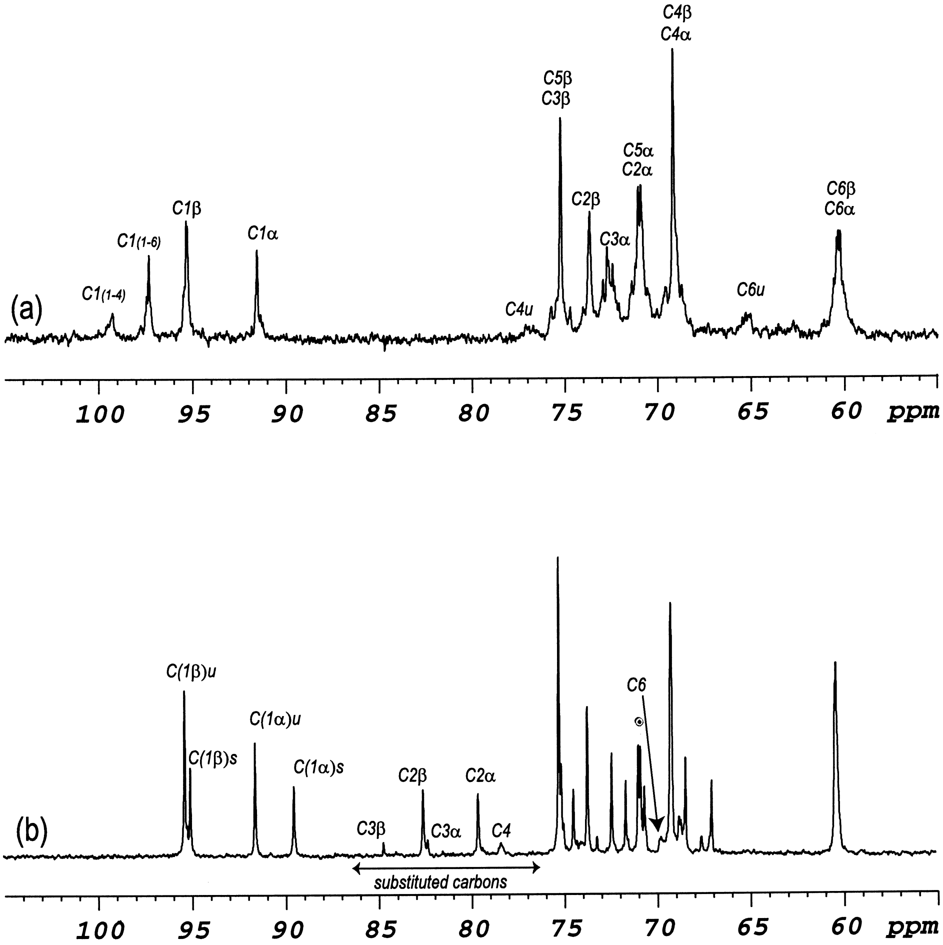


Fig. 4. Comparison of ^{13}C NMR spectra in $D_2O + D_2SO_4$ of (a) pullulan and (b) Na-CMP(0.58) acid hydrolysates.

–O–CH_2–COOH groups. Extended hydrolysis yielded a mixture of substituted and unsubstituted glucose residues.

Another interesting feature was the comparison between the ratio of peak areas for H-1αs and H-1βs ($\alpha/\beta = 1$) and that for H-1αu and H-1βu ($\alpha/\beta = 0.33$). Carboxymethylation at OH-2 favours the α form.

A comparison of the ^{13}C NMR spectrum of hydrolysed pullulan with that of Fig. 4 shows additional peaks corresponding to: (i) the methylenic carbons of the CM group, and (ii) the substituted carbons in the ring. Perturbation of the chemical shifts of the carbons next to the substituted carbons is also to be expected. The signals for the carbonyl carbon atoms of carboxymethyl groups are found at about 180 ppm, outside the spectral region of interest.

The ^{13}C NMR spectrum was assigned on the basis of to the expected effects of O-substitution on ^{13}C chemical shifts: large (8–10 ppm) downfield shifts for the substituted carbons (69.38, 78.04, 79.32, 82.01, 82.3 and 84.43 ppm), and smaller (1–2 ppm) upfield shifts for the carbons next to the substituted ones (66–75 ppm) [30]. In addition, signals from the –CH_2 carbon atoms of the CM substituent are apparent between 66 and 69 ppm.

The 2D COSY, TOCSY and HSQC (Fig. 5(a)) experiments allowed the ^{13}C NMR spectrum to be assigned and those belonging to the differently substituted carbon atoms (C-2, C-3, C-4 and C-6) are shown in Fig. 4. The HMBC experiment allowed the assignment of methylenic protons in the 4.1–4.6 ppm region as seen in Fig. 5(b). The ^{13}C and ^{1}H assignments for all the monosaccharides derivatives are summarized in Table 2. These data confirm that the Glc residues in NaCMP(0.58) are mainly monosubstituted or unsubstituted.

Determination of the degree of substitution.—Several authors [12,14,15] have quantified the DS of CMC samples by integrating areas of ^{13}C signals. Gautier et al. [15] and Nehls et al. [12] calculated the partial DS$_i$ from the areas of signals from substituted and

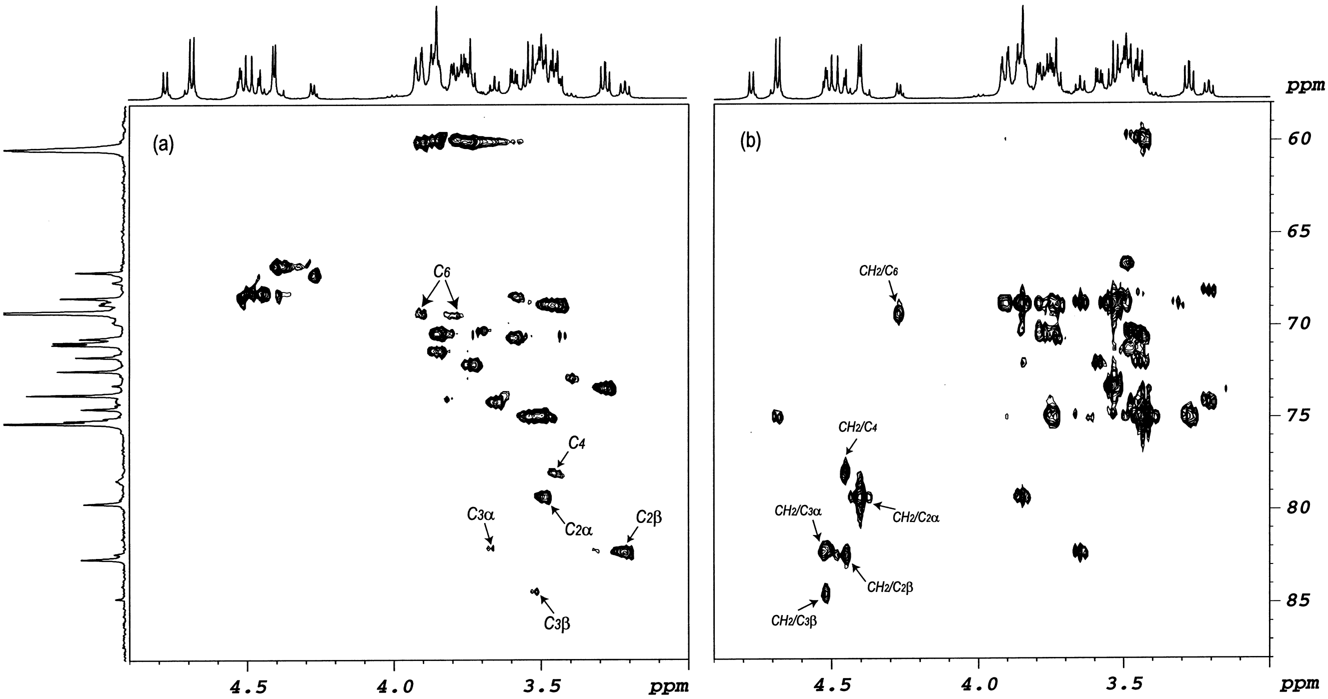


Fig. 5. Sections of the 600 MHz HSQC and HMBC maps of acid-hydrolysed Na-CMP(0.58) in $D_2O + D_2SO_4$: (a) HSQC, the cross-peaks corresponding to substituted carbons C-i are noted; (b) HMBC, the correlations between the substituted carbons C-i and the methylenic protons of substituent in the CMP hydrolysates.

Table 2
^{1}H and ^{13}C chemical shifts (in ppm) of glucose and the *O*-(carboxymethyl)glucose (*O*-CM-glucose) in acidified aqueous solutions [a]

Compound	C-1	C-2	C-3	C-4	C-5	C-6	–*C*H$_2$	
α-Glucose	91.33	70.70	72.14	68.92	70.60	60.07		
β-Glucose	95.09	73.43	75.00	68.86	75.00	60.15		
2-*O*-CM-α-glucose	89.23	**79.32**	71.41	68.92	70.38	59.99	66.78	
2-*O*-CM-β-glucose	94.81	**82.30**	74.19	68.86	74.87	60.07	68.24	
3-*O*-CM-α-glucose		70.29	**82.07**	68.51	74.75	59.99	68.51	
3-*O*-CM-β-glucose		72.91	**84.43**	68.51	70.29	59.96	68.51	
4-*O*-CM-α and β-Glucose				**78.04**			68.24	
6-*O*-CM-α and β-glucose						**69.38**	67.33	
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	–C*H*$_2$
α-Glucose	5.26	3.59	3.73	3.43	3.85	3.92	3.76	
β-Glucose	4.68	3.28	3.51	3.47	3.56	3.90	3.73	
2-*O*-CM-α-glucose	5.46	3.49	3.85	3.43	3.85	3.85	3.79	4.41
2-*O*-CM-β-glucose	4.77	3.22	3.65	3.47	3.56	3.92	3.76	4.48
3-*O*-CM-α-glucose		3.70	3.68	3.58	3.81			4.52
3-*O*-CM-β-glucose		3.39	3.52	3.58	3.52	3.85	3.79	4.52
4-*O*-CM-α and β-glucose				3.45				4.47
6-*O*-CM-α-glucose						3.98	3.86	4.27
6-*O*-CM-β-glucose						3.90	3.79	4.27

[a] Reference DSS at 0 ppm for ^{1}H NMR spectrum and −3 ppm for ^{13}C NMR spectrum.

Table 3
Substitution degrees (DS_i) and relative reactivities (R_i values in %) of hydroxyl groups in CMP acid hydrolysates

CMPs	DS_{AGU}		DS_i [c]				DS_{MTU} [d] ± 0.1	R_i [e]			
	DS_{tit} [a] ± 0.05	DS_{NMR} [b] ± 0.1	DS_2 ± 0.05	DS_3 ± 0.1	DS_4 ± 0.2	DS_6 ± 0.05		R_2	R_3	R_4	R_6
Na-CMP(0.58)	0.58	0.66	1.18	0.38	0.22	0.22	2.0	39	12.7	22	11
Na-CMP(0.85)	0.85	1.0	1.75	0.51	0.31	0.40	3.0	58.3	17	31	20
Na-CMP(1.05)	1.05	1.1	1.96	0.52	0.37	0.45	3.3	65.3	17.3	37	22.5
Na-CMP(1.20)	1.20	1.3	2.12	0.71	0.49	0.68	3.9	70.7	23.7	49	34

[a] Values obtained from conductimetric acid titration.
[b] Values obtained from NMR measurements.
[c] DS_i = partial DS at C-i position.
[d] DS_{MTU} = DS of maltotriose unit.
[e] $R_i = (DS_i/n_i) \times 100$.

unsubstituted C-atoms at positions 2, 3 and 6, that, together give the total DS. Other authors have used the ^{13}C resonances of the carbonyl carbon atoms of the substituent [30].

We tried to calculate the DS of CMP samples from areas of signals of substituted compared with those of the appropriate unsubstituted C-atom of the AGU. We failed because of overlap between the peaks corresponding to the substituted C-3α at 82.07 ppm and the substituted C-2β 82.30 ppm. Moreover, the weak intensity of substituted C-4, C-3β and C-6 signals does not allow, taking into account the poor signal-to-noise ratio, a quantitative evaluation of area of these peaks. It was therefore not possible to determine the partial DS_i directly from the ^{13}C NMR spectrum.

The problem was solved by using the spectral region between 4.0 and 6.0 ppm of the ^1H NMR spectrum, which is the richest in analytical information. For this study, a set of four CMP samples having a $\overline{DS}$ ranging from 0.58 to 1.2 were hydrolysed to $\overline{DPn}$ of about 1.2–1.5. A degradation time of several days is required for samples having the highest DS to obtain such low $\overline{DPn}$. Moreover, as seen above, the resolution of the methylene protons of the carboxymethyl groups was sufficiently good after 14 h of hydrolysis to determine the partial DS_i.

From the previous discussion on the spectral analysis of degraded CMP, it is clear that the DS_{AGU} measurement in the NMR procedure is simply the ratio of two spectral areas, A/B, where A is one half of the peak areas corresponding to the methylene protons of carboxymethyl substituents in the 4.1–4.6 ppm region, and B is the peaks areas of all anomeric protons in the 4.6–6.0 ppm region. The data on DS_{AGU} determination of the different samples are listed in Table 3. For comparison, DS_{AGU} values measured by a standard conductimetric acid titration are also included. Although the agreement between values obtained by these techniques is generally good, the NMR results tend to be slightly higher. Similar values of DS_{AGU} were found for all CMP precursors (acid titration) and derived dimers and glucose residues. These results confirm that no changes in the molecule had taken place upon acid hydrolysis other than main chain cleavage.

The distribution of the CM substituents at C-2, C-3, C-4 and C-6 hydroxyl groups may be obtained by integration and comparison of the relevant signals areas. The peaks of methylene protons of CM groups bound to C-2β and C-4 overlap (Fig. 5(b)) and therefore prevent the direct evaluation of the partial degrees of substitution.

As it was shown previously, carboxymethylation of the OH group at C-2 produced a substantial downfield shift (0.2–0.5 ppm) of the H-1 resonance. Accordingly, the individual DS_i value at C-2 may be estimated separately from the integration of anomeric protons of residues substituted at C-2. For example, in the NMR spectrum of Na-CMP(0.58), the ratio between downfield-shifted and all anomeric proton resonances was 0.39. This means that about 39% of OH groups at C-2 are substituted. As CMP pullulan is constituted from maltotriose repeating units, the global DS related to this sequence, designated as DS_{MTU}, is 3 DS_{AGU} and the partial DS_i value at C-2 (namely DS_2) is 1.18.

Of the carboxymethyl protons signals, that corresponding to the substitution at C-6 is differentiated, which allows the determination of DS_6 directly by integration. The difference in chemical shifts of methylene proton peaks of substituents at C-2, C-3 and C-4 are too small to evaluate DS_3 and DS_4. The spectral region corresponding to these signals was deconvoluted using the XEDPLOT-XWIN NMR (Bruker) program until the constructed Lorentzian curves fitted the experimental ones. The partial DS_i value at C-3 was estimated from peak integration employing the deconvoluted spectra and the DS value at C-4 calculated by the following relation: $DS_4 = DS_{MTU} - (DS_2 + DS_3 + DS_6)$. The structural parameters that were determined are summarized in Table 3. As seen in Fig. 6, in the range of $DS_{MTU} \sim 2.0-3.9$ (corresponding to $DS_{AGU} \sim 0.66-1.3$), the partial degrees of substitution vary almost linearly with the global DS. The distribution of the CM substituents, which is independent of DS_{MTU}, decreases according to the order C-2 > C-3 > C-6 > C-4. These results indicate that about 57% of $-CH_2-CO_2Na$ groups are at C-2 but the analysis does not account for the availability of the different hydroxyl groups within the maltotriose unit.

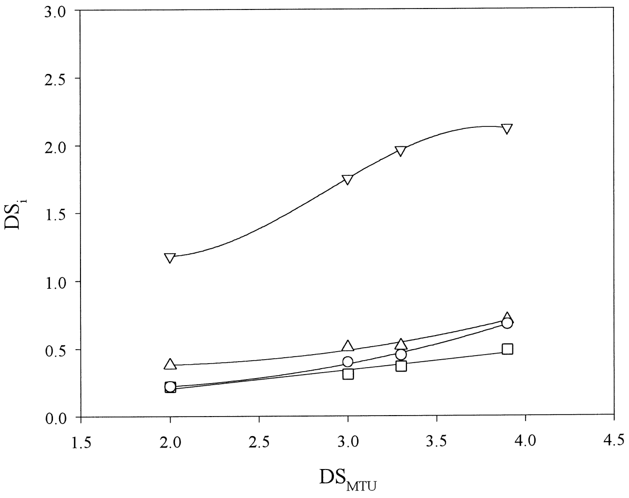


Fig. 6. Partial degree of substitution (DS_i) as a function of total degree of substitution (DS_{MTU}): DS_2 (▽), DS_3 (△), DS_4 (□), DS_6 (○)

For example, the carboxymethylation can occur at these C-3 positions whereas only two at C-6 of units B and C can be substituted. If the availability of each OH group is considered, we can define an order of relative reactivity of hydroxyl groups according to the relation: $R_i = DS_i/n_i$, where n_i is the number of free OH groups in MTU for a given site C-i. Finally, with regard to the R (%) values (Table 3), the reactivity of hydroxyl groups follows the order: OH-2 > OH-4 > OH-6 > OH-3.

In conclusion, the present results show that carboxymethylation takes place initially at C-2 and C-4; and reaction at C-6 and C-3 positions follows. The relative reactivity of the C-2 hydroxyl groups in the maltotriosyl units, observed in the present study fits the results obtained for carboxymethylcellulose and dextran. When carboxymethylation was achieved in heterogeneous basic media with sodium chloroacetate as reagent, the order of reactivity of hydroxyl groups was found to be OH-2 > OH-6 > OH-3 for cellulose [12–16] and OH-2 > OH-4 > OH-3 for dextran [31]. In spite of the more complex repeating unit of pullulan derivatives compared with cellulose, the distribution pattern of carboxymethyl groups in CMP samples is readily determined by means of ^{1}H NMR of acid hydrolysates. Hence, the present results should provide a basis for further structural investigations not only for pullulan derivatives but also for other complex polysaccharides.

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