Starch Derivatives of High Degree of Functionalization 5. Stepwise Carboxymethylation of Amylose*

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The stepwise reaction of amylose with monochloroacetic acid carried out heterogeneously in methanol—water medium and sodium hydroxide gives carboxymethylamylose (CMA) with high degree of substitution (DS) up to 2.35. The stepwise increase of total DS_{CM} of CMA decreases gradually with increasing DS_{CM} of the starting polymer. For the determination of the functionalization pattern of the CMA both HPLC and ^{1}H NMR spectroscopy after complete depolymerization were applied. These analyses revealed a statistical content of the different repeating units with regard to 2-, 3-, 6-mono-, 2,3-, 2,6-, 3,6-di-, 2,3,6-tri-, and 2,3,4,6-tetra-*O*-carboxymethylglucose as well as glucose and a distribution d of the carboxymethyl functions within the repeating units in the order d(O-2) > d(O-6) ≫ d(O-3).

Recently, in the field of starch research our interest is focused on carboxymethylamylose (CMA) of high degree of substitution (DS) since carboxymethylation is a versatile transformation leading to water-soluble polymers and intermediates with various valuable features. The carboxymethylation of starch by reacting the polymer in alkaline solution (40 % aqueous NaOH) with sodium monochloroacetate was first carried out in 1924 [1]. Up to now different studies concerning the carboxymethylation of starch were published aiming at optimized reaction conditions, *i.e.* to increase product yield and reaction efficiency [2—4] as well as to obtain products of high DS up to 1.0 in nonaqueous media [5, 6].

The properties (viscosity of solution, film forming, interaction with cations, and the formation of supramolecular aggregates, *etc.*) are mainly determined by the total DS, *i.e.* the average number of carboxymethyl groups in the polymer [7]. Moreover, the functionalization pattern may influence the properties as well. In the course of the studies on polysaccharides [8] it was shown that the heterogeneous carboxymethylation of starch activated with aqueous sodium hydroxide solution in methanol—water as slurry medium using monochloroacetic acid as etherifying agent leads to CMS with DS of about 0.4 with a reaction efficiency of 60 % independently of the starch type concerning the botanical source [9].

In the present paper the preparation of carboxymethylstarch samples of high DS by multistep carboxymethylation using the commercially important slurry medium methanol—water is reported. The total DS values, the distribution of the functional groups within the repeating anhydroglucose units as well as the mole fractions of the differently functionalized units were determined by means of ^{1}H NMR spectroscopy and HPLC after complete hydrolytic chain degradation. Moreover, the intact polymers were characterized by means of ^{13}C NMR spectroscopy.

EXPERIMENTAL

The starch sample used was amylose KG pea starch (Stauderer & Co., Altenmark, Germany), amylose content 90 %. Technical grade (minimum 97 %) methanol was used. The sodium hydroxide, acetic acid, and monochloroacetic acid (MCA) were purchased from Fluka.

The ^{1}H NMR analyses were carried out as described in Ref. [10]. For this purpose the CMA samples were hydrolyzed with a mixture of D_2SO_4—D_2O (25 vol. %) within 5 h at 90 °C. The samples were cen-

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trifuged if the solutions appeared turbid. The ^{1}H and ^{13}C NMR spectra were acquired on a Bruker AMX 400 spectrometer, in case of ^{13}C NMR at a concentration of 5 % in D_2O at 60 °C. The number of scans was 16 (^{1}H NMR) and in the range from 25000 to 100000 in case of ^{13}C NMR.

At the HPLC measurement CMA samples were hydrolyzed with perchloric acid. 0.1 g of the sample was dispersed in 2 cm^3 of $HClO_4$ (70 %) and after 10 min at room temperature diluted with 18 cm^3 of distilled water. This mixture was kept at 100 °C for 16 h. The solution obtained was carefully neutralized with 2 M-KOH and kept at 4 °C for 1 h to guarantee a nearly complete precipitation of $KClO_4$. The salt was filtered off and washed three times with distilled water. The volume of the solution obtained was reduced to approximately 3 cm^3 and diluted with distilled water to give exactly 5 cm^3 of sample. 50 mm^3 of the sample were injected into two coupled Aminex HPX 87 H columns (BioRad Laboratories) for separation using an autosampler. The column temperature was constant at 65 °C (column oven). As mobile phase 0.005 M sulfuric acid at a flow rate of 0.5 cm^3 min^{-1} was used. The detection of components was carried out by both a refraction index detector (RI) and a chiral detector. The chromatographic data were evaluated by means of the JASCO HPLC-software "Borwin" [11].

Carboxymethylation of Amylose

425 g of air-dry (water content 11 %, determined by TGA, dry matter 378.25 g, 2.31 mol) pea starch in 750 cm^3 of methanol was stirred vigorously, while 210 cm^3 of 45 mass % aqueous sodium hydroxide was added during 15 min at room temperature. Stirring was continued for another 1.5 h at 40 °C, and 150 g (1.59 mol) of monochloroacetic acid were added during a period of 15 min. The mixture was allowed to react for 6 h at 40 °C. After carboxymethylation the mixture was filtered, suspended in 76 mass % aqueous methanol and neutralized with acetic acid. The product was collected by filtration, washed three times with 76 vol. % aqueous methanol, pure methanol (99.9 %) and dried at 110 °C in vacuum (sample CMA *I*).

Degree of substitution: 0.37 (determined by means of HPLC after complete chain degradation).

The subsequent carboxymethylation steps were run in a similar procedure (samples CMA *II*—*X*). Values of the degree of substitution (determined by means of HPLC and ^{1}H NMR spectroscopy) are given in Table 1.

RESULTS AND DISCUSSION

To reach an even activation of the whole starting pea starch (90 % amylose), a treatment with 45 % aqueous NaOH for 90 min at 40 °C in methanol slurry was carried out [8, 9]. The technical process of carboxymethylation of starch is carried out using methanol as organic liquid. Therefore, we studied the stepwise carboxymethylation in methanol—water although other slurry media like ethanol or propan-2-ol may yield products of higher DS_{CM}. In addition, the sodium hydroxide initiates the reaction with monochloroacetic acid (MCA), which was carried out for 6 h at 40 °C. The conditions used guarantee that the reaction mixture can be mixed during the whole course of reaction and that no gelation occurs. Applying a mole ratio of 0.7 mol MCA per mol glucose unit (GU), the carboxymethyl amylose (CMA) sample *I* with a degree of substitution (DS_{CM}) of 0.37 was obtained. Then sample *I* was activated with aqueous NaOH and allowed to react with 0.7 mol MCA per mol modified GU under exactly the same conditions to give CMA sample *II* (DS_{CM} = 0.72, Table 1). This kind of subsequent carboxymethylation was applied 9 times in total using the CMA as starting material, which was obtained in the previous step. Sample *X* possesses a DS_{CM} of 2.35. The DS_{CM} values were determined by means of HPLC using the completely depolymerized samples. The results show that the stepwise increase of the DS_{CM} values of CMA samples *I*—*X* decreases gradually with increasing DS_{CM} of the starting CMA (Table 1). To use monochloroacetic acid instead of sodium monochloroacetate is a key step in the technical procedure.

To determine the functionalization pattern we used the developed HPLC method [8, 10]. This efficient procedure yields the overall DS_{CM} as well as the mole fractions of the 5 main repeating units neglecting the distribution of the functional groups within the repeating units, *i.e.* unmodified glucose (Glc), 2-, 3-, 6-mono-*O*-carboxymethyl glucose (mono-*O*-CMGlc), 2,3-, 2,6-, 3,6-di-, 2,3,6-tri-, and 2,3,4,6-tetra-*O*-CMGlc after a complete acidic depolymerization using perchloric acid. The chromatograms obtained show baseline separated 5 peaks characteristic of these units if RI-detection is applied. The peak areas were used to calculate the mole fractions of the main repeating units. Further peaks assigned to inorganic salts, diglycolic and glycolic acids were observed. The additional detection of signals by the chiral detector is suitable to control the completeness of hydrolysis, because oligomeric components elute at the degree of polymerization (DP) of 4 together with the salt components in front of the CMGlc fractions. Moreover, it is helpful for the peak assignment in the chromatogram with regard to the chiral sugars.

The results of HPLC experiments summarized in Table 1 reveal that the mole fraction of Glc decreases continuously with increasing DS_{CM} as expected. At the DS_{CM} of 1.95 (CMA *VII*) only traces of glucose were found. Up to the reaction step 3, the mole fraction of mono-*O*-CMGlc increases to 0.629 (CMA *III*, DS_{CM} = 1.07) and subsequently decreases to a value of 0.104 (CMA *X*, DS_{CM} = 2.35). As a consequence, the

Table 1. Results of the Determination of the Substituent Pattern of Carboxymethylamylose (CMA) Using HPLC and ^{1}H NMR Spectroscopy after Depolymerization of the Polymers and the Comparison of the HPLC Data with Statistic Calculations Using the Spurlin Model

CMA	HPLC[a]						^{1}H NMR[b]			
	DS[c]	Mole fraction					DS[d]	Partial DS at position		
		Glc	Mono-*O*-CMGlc	Di-*O*-CMGlc	Tri-*O*-CMGlc	Tetra-*O*-CMGlc		2	3	6
I	0.37	0.650	0.330	0.019	–	–	nd	nd	nd	nd
II	0.72	0.365	0.549	0.087	–	–	nd	nd	nd	nd
III	1.07	0.155 (0.194)[e]	0.629 (0.585)	0.203 (0.204)	0.012 (0.017)	–	1.04	0.724	0.113	0.207
IV	1.33	0.072 (0.088)	0.565 (0.508)	0.325 (0.356)	0.038 (0.047)	–	1.36	0.833	0.149	0.381
V	1.55	0.036 (0.038)	0.456 (0.397)	0.428 (0.477)	0.080 (0.088)	–	1.61	0.900	0.184	0.530
VI	1.77	0.017 (0.037)	0.338 (0.299)	0.505 (0.515)	0.140 (0.149)	–	1.78	0.852	0.265	0.659
VII	1.95	0.009 (0.014)	0.248 (0.210)	0.526 (0.573)	0.213 (0.203)	0.004 –	1.97	0.922	0.294	0.750
VIII	2.09	0.006 (0.012)	0.192 (0.181)	0.515 (0.558)	0.281 (0.250)	0.006 –	2.05	0.921	0.350	0.775
IX	2.22	0.003	0.141	0.492	0.356	0.007	2.10	0.908	0.389	0.801
X	2.35	0.001	0.104	0.449	0.434	0.011	2.18	0.879	0.461	0.840

a) HPLC measurements after complete hydrolytic depolymerization; *b*) ^{1}H NMR measurements after depolymerization; *c*) degree of substitution (DS) = $c_{\mathrm{mono}} + 2c_{\mathrm{di}} + 3c_{\mathrm{tri}} + 4c_{\mathrm{tetra}}$; *d*) DS = $\sum \mathrm{O}_i$ (i = 2, 3, 6); *e*) calculated values (see the text) are given in brackets; nd – not determined.

amount of the mole fraction of di-*O*-CMGlc becomes significant at a $\mathrm{DS_{CM}}$ of 1.07 (CMA *III*), increases further to 0.526 (CMA *VII*, $\mathrm{DS_{CM}} = 1.95$) and finally decreases slightly to the step 10. The tri-*O*-CMGlc can be detected after reaction step 3 (CMA *III*, $\mathrm{DS_{CM}} = 1.07$) and increases to a maximum of 0.434 (CMA *X*) as a result of subsequent carboxymethylation steps. The mole fraction of 2,3,4,6-tetra-*O*-CMGlc is less than 1 % in any case and results from the low amount of end groups of amylose and the small amount (10 %) of branched amylopectin present in the starch material [8]. The observations discussed could be predicted on the basis of the structural features and the resulting reactivity.

To obtain further information on the structural features, the experimental results were compared with statistical calculations of the mole fractions according to *Spurlin* using the results of ^{1}H NMR analysis (see below) [13, 14]. The results of calculation of the mole fractions are given in Table 1 (values in brackets). For samples *IX* and *X* the statistics was not calculated because the differences between DS determined by means of HPLC and NMR, respectively, are too high. It is obvious that the experimental data fit the Spurlin statistics sufficiently. Consequently, it can be concluded that a statistic amount of the different repeating units appears, which is an important result with regard to the determination of structure—property relationships.

^{1}H NMR spectroscopy is a rapid and convenient method for the determination of the functionalization pattern within the repeating unit. It is carried out after depolymerization of the CMA with 25 vol. % D_2SO_4. Fig. 1 shows a typical spectrum of the hydrolyzed CMA sample *V* and the peak assignment. The results obtained with samples *III*—*X* are summarized in Table 1. The partial $\mathrm{DS_{CM}}$ (x_i) values were calculated according to eqn (*1*) where *A* represents

$$x_i = [\tfrac{1}{2}A(\text{Methylene protons at position O-}i)]/ \\ /[A(\text{H-1}\alpha,\ \text{O-2s}) + [A(\text{H-1}\alpha,\ \text{O-2u}) + \\ + A(\text{H-1}\beta,\ \text{O-2s}) + A(\text{H-1}\beta,\ \text{O-2u})]^{-1} \qquad (1)$$

$$\mathrm{DS} = \sum_i x_i$$

the peak area, O the oxygen atom at the position i (i = 2, 3, 6), H-1 the hydrogen atom at the anomeric C, α, β the configuration of glucose, s is used for substituted, u for unsubstituted positions [15]. A functionalization at position 4 was not included since the corresponding signal does not appear. Only traces of tetra-*O*-CMGlc were found by HPLC measurements as already discussed. Even in case of sample *X* with a high $\mathrm{DS_{CM}}$ of 2.35 the mole fraction of tetra-*O*-functionalized units is 0.01 only. Consequently, the partial $\mathrm{DS_{CM}}$ in the position O-4 is 0.002. This is below the detection limit of the NMR method [16].

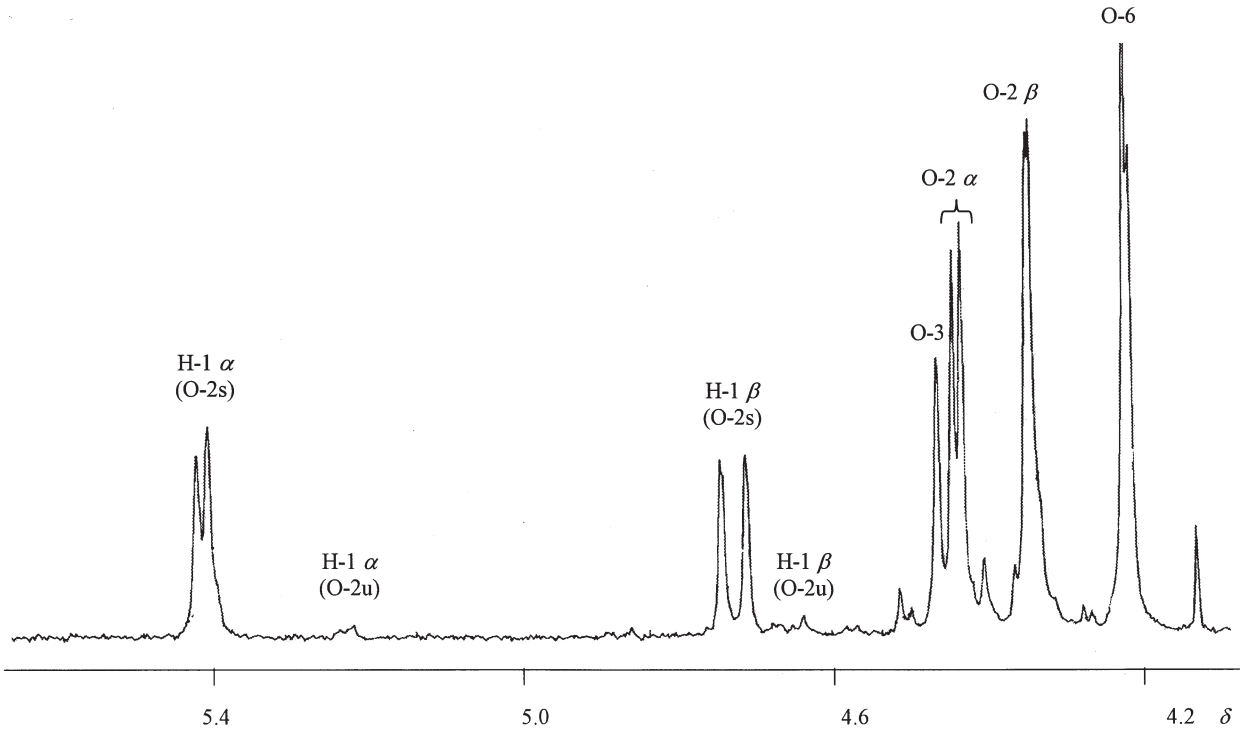

Fig. 1. ^{1}H NMR spectrum of carboxymethylamylose sample *V* after hydrolytic chain degradation in 25 % D_2SO_4.

The partial DS_{CM} of position 2 is comparatively high in any case. The value for carboxymethylation in position 3 is always the lowest one independently of the total DS_{CM}. With increasing total DS_{CM} the values for O-6 functionalization become significantly higher. Concerning the total DS_{CM}, which can be calculated from the ^{1}H NMR data as well, there are differences compared with values obtained from HPLC (Table 1). For the ^{1}H NMR analysis of the partial DS at O-6 it is important to use extensively purified samples since glycolate and diglycolate yield signals between 4.2 and 4.3, *i.e.* in the range of chemical shift of the CH_2 groups in position O-6. In the present work, samples without an additional purification were used in order to exclude changes in sample composition. Therefore, the O-6 values may be too high. By HPLC analysis it was revealed that all samples contain at least traces of glycolate and diglycolate. After some carboxymethylation steps, a degradation of the polymer may be expected.

From these results it appears that the OH groups at C-2 possess a high reactivity and that a carboxymethylation of position 3 is rather difficult under the reaction conditions applied. As a consequence, the synthesis of completely functionalized products cannot be achieved in an efficient manner under the heterogeneous reaction conditions used in this study. In case of carboxymethylcellulose an almost equal functionalization of positions 2 and 6 was found at comparatively low total DS_{CM} values. Moreover, at high concentration of the aqueous NaOH solution (30—40 %), a preferred functionalization at position 6 occurs [12] which is a further difference between the two polyglucans.

A representative ^{13}C NMR spectrum (spectral range from 55 to 105) of the intact CMA *V* with a DS_{CM} = 1.55 measured in D_2O is shown in Fig. 2. The peak for the unmodified C-6 position is visible at $\delta = 60.9$. The signal for the carboxymethylated position 6 cannot be resolved, but appears as a shoulder at $\delta \approx 69.5$. The intensities of these peaks agree with the functionalization at these positions as determined by means of ^{1}H NMR spectroscopy. The signals at δ = 70.1—70.9 are assigned to the methylene carbon atoms of the carboxymethyl substituents. The carbon atoms of the unmodified positions 2 and 3 are found at $\delta = 71.3$ and 71.8 as shoulders and C-5 appears at $\delta = 72.9$. The carboxymethylation of positions 2 and 3 leads to a downfield shift and gives a signal at 74.6. The signal of C-4 is found at $\delta = 80.5$. C-1′ appears at δ = 95.6—96.9 and the complete substitution at position C-2 can be assumed because no peak for C-1 (usually at about $\delta = 101$) occurs. The splitting of the C-1′ peak was unexpected. Obviously, functionalized O-2 atoms exist in the CMA samples which influence the C-1′ signal to a different extent. As it is very well known, starch consists of two components, *i.e.* amylose and amylopectin. Thus, the C-1 peaks of these two polymers might be differently influenced by an O-2 functionalization. The signals for C-atoms of the carboxylate groups appear in the range δ = 177.3—178.1. It is interesting to note that both the intensity

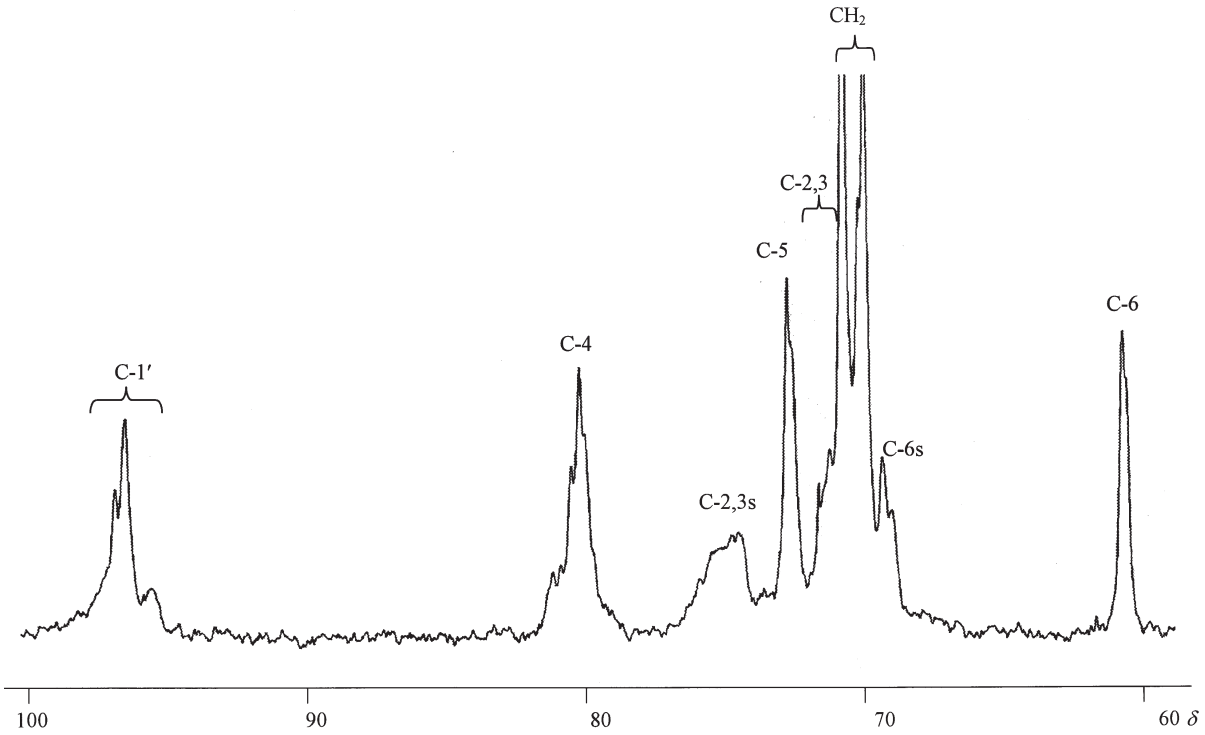

Fig. 2. ^{13}C NMR spectrum of the intact carboxymethylamylose sample *V* measured in D_2O.

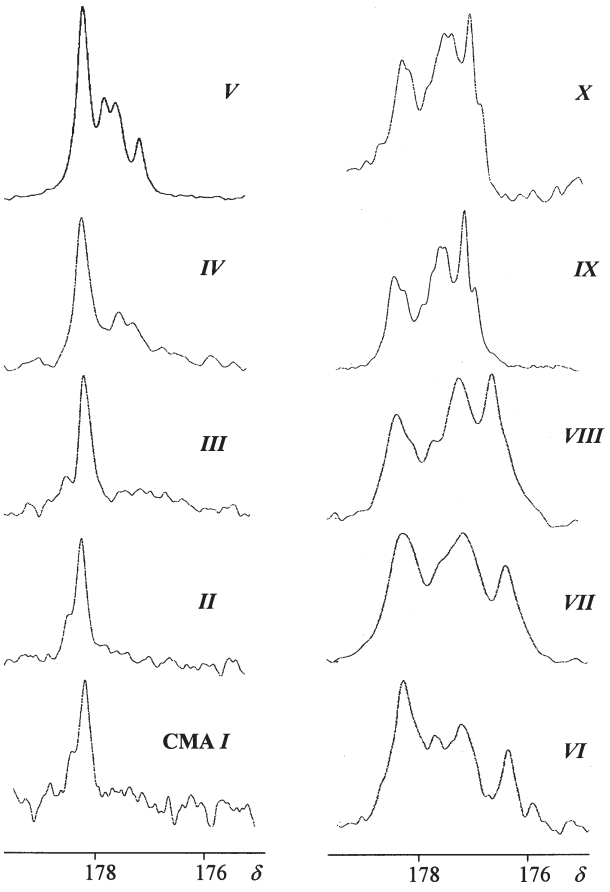

Fig. 3. Carbonyl range of the ^{13}C NMR spectra of the carboxymethylamylose samples *I*—*X*.

and number of signals vary significantly with increasing DS_{CM}. Sections of the ^{13}C NMR spectra of CMA *I*—*X* in the range of the chemical shift characteristic of C-atoms of carbonyl moieties are shown in Fig. 3. While the spectra of samples *I* to *III* show just one signal, the spectrum of sample *IV* possesses an additional shoulder at lower δ values. At higher DS_{CM} a splitting appears to get 3 signals (samples *V*—*X*). Since these signals are separated from the signals of the GU, they might be used to gain information about the DS_{CM} as well.

CONCLUSION

The results clearly show that by a multistep carboxymethylation of amylose, products of very high degree of substitution of carboxymethyl groups (DS_{CM}) up to 2.3 can be obtained which were not accessible up to now. The detailed investigation of the distribution of the carboxymethyl groups by means of ^{1}H NMR spectroscopy reveals that a reactivity *r* of the amylose hydroxyl groups in the order r(O-2) > r(O-6) $\gg$ r(O-3) occurs. Mole fractions of the differently functionalized repeating units, determined by means of HPLC after complete chain degradation, follow the Spurlin statistics. At high DS_{CM} even some 2,3,4,6-tetra-*O*-functionalized units are formed. From the results it unambiguously appears that statistic considerations to evaluate the functionalization pattern of carboxymethyl starch need a more comprehensive structure analysis of the starch material with regard to the

content of amylose/amylopectin, number and length of branches as well as end groups. Moreover, the influence of the slurry medium, applying ethanol and propan-2-ol, on the DS_{CM} will be investigated in further studies.

An unexpected result was that a splitting of the C-1′ signal of CMA samples appeared in the ^{13}C NMR spectra. In order to gain a deeper insight into the molecular structure of carboxymethyl starch, samples starting from different starch materials, *i.e.* with different amylose/amylopectin contents including pure amylose and amylopectin, will be synthesized and the subject of further analytic work in this ongoing research project.

Moreover, the determination of the relative molecular mass and relative molecular mass distribution of the CMS samples using GPC and light scattering is under investigation. The evaluation of the properties with regard to DS_{CM} is studied by means of rheology and the results will be published elsewhere.

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