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Structure of dicarboxyl malto-oligomers isolated from hypochlorite-oxidised potato starch studied by ^{1}H and ^{13}C NMR spectroscopy

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Abstract

The main oxidised component in hypochlorite-oxidised potato starch was isolated by anion-exchange chromatography after enzymatic hydrolysis. The primary structure of the isolated oligosaccharides was determined by ^{1}H and ^{13}C NMR spectroscopy, using homonuclear and heteronuclear two-dimensional techniques. The isolated pentamer and hexamer contained one glucose unit oxidised to a dicarboxyl residue. As the hypochlorite oxidation has occurred at positions C-2 and C-3 of a glucose unit, the introduced carboxyl groups caused ring cleavage between the carbons C-2 and C-3. The ring-cleaved dicarboxyl residue had glycosidic linkages on both sides, implying that this oxidation pathway does not result in depolymerisation. The vicinal coupling constant between H-4 and H-5 in the ring-cleaved dicarboxyl residue was 3.2 Hz, showing that the gauche orientations are preferred. As a result, a different bending of the starch chain is observed and is probably, therefore, one of the reasons why hypochlorite oxidation reduces the tendency to retrogradation. The pK_a values (3.0) were determined from the pH-dependent chemical shifts of H-1, H-4 and H-5 of the dicarboxylic residue.

Keywords: Alpha amylase; Glucoamylase; HPAED-PAD; Oxidised starch, structure; Ring-cleavage

1. Introduction

Starch is a polymeric carbohydrate composed of linear amylose and highly branched amylopectin. Amylose is mainly a linear polysaccharide formed by α-(1→4)-linked D-glucose residues. Amylopectin has a backbone of α-(1→4)-linked D-glucose residues but also contains α-(1→6)-linked branching points. Modified starches are used in the paper industry for coating and surface sizing of paper. Oxidised starches are added as stabilisers in internal sizing emulsions and are used in surface sizing as well as cobinders for coating colours. The oxidation results in a low-viscosity product with reduced tendency to retrogradation and gelling in solution.

Sodium hypochlorite and hydrogen peroxide are commonly used oxidants, which will depolymerise starch during the oxidation process. Depending on reaction conditions, carboxyl and carbonyl groups are introduced to

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starch to different extents and in different proportions [1,2]. The oxidised glucosyl residues within the starch polymer have been claimed to be located either close to the non-reducing or the reducing end [3]. Oxidation of potato starch with bromine at neutral pH mainly results in oxidation at C-2. Some oxidation at C-3 also occurs [4].

In this work we present a procedure for the isolation of the main oxidised component of hypochlorite-oxidised potato starch. The positions of the carboxyl groups in the glucose ring and the primary structure of the isolated oligomers were determined by two-dimensional (2D) ^{1}H and ^{13}C NMR spectroscopy.

2. Results and discussion

Oxidation with alkaline hypochlorite involves treating an aqueous starch suspension with a sodium hypochlorite solution [1]. The amount of carboxyl and carbonyl groups for Raisamyl 302 ESP, a commercial hypochlorite-oxidised starch preparation, was 3.6 and 1.3 per 100 glucose units based on dry weights, respectively. A degree of polymerisation of 177 has earlier been reported for the sodium hypochlorite-oxidised potato starch used [5]. The reducing end groups thus correspond to about 0.6 carbonyl groups per 100 glucose units. Both carboxyl and carbonyl groups appear to have been introduced to the potato starch by the oxidation procedure used.

After alpha amylase and glucoamylase hydrolysis, the hydrolysate of Raisamyl 302 ESP contained a large amount of glucose and minor amounts of malto-oligosaccharides as observed in the HPAEC-PAD chromatogram (Fig. 1A). The ^{1}H NMR spectrum of the enzymatic hydrolysate was dominated by the glucose signals as expected, but also unknown peaks originating from oxidised glucose units were observed (Fig. 2A). The unknown components were isolated from the enzymatic hydrolysate. The acidic and neutral compounds were separated by anion-exchange chromatography. The bound (acidic) compounds were eluted from the column by a linear NaAc gradient. The fractions eluted within 80–140, 170–225 and 255–315 mM salt concentration were combined and named **F1**, **F2** and **F3**, repectively. Fraction **F2** was studied further after removing salts and freeze-drying the sample. The two other fractions were analysed by ^{1}H NMR spectroscopy. Both Fractions **F1** and **F3** contain the same oligomers as **F2** and minor amounts of other, probably oxidised, oligosaccharides.

Structure of **F2**.—The structure of the isolated carboxyl group containing Fraction **F2** of enzymatically hydrolysed hypochlorite-oxidised potato starch was determined based on ^{1}H and ^{13}C NMR data. In the 1D ^{1}H NMR spectrum, five groups of resonances are observed at 5.36, 5.26, 5.22, 4.95 and 4.65 ppm in the anomeric region (Fig. 2B). The relative intensities indicated a mixture of a penta- and hexasaccharide. Two major and several minor peaks are also observed in the HPAEC-PAD chromatogram of Fraction **F2** (Fig. 1B). The

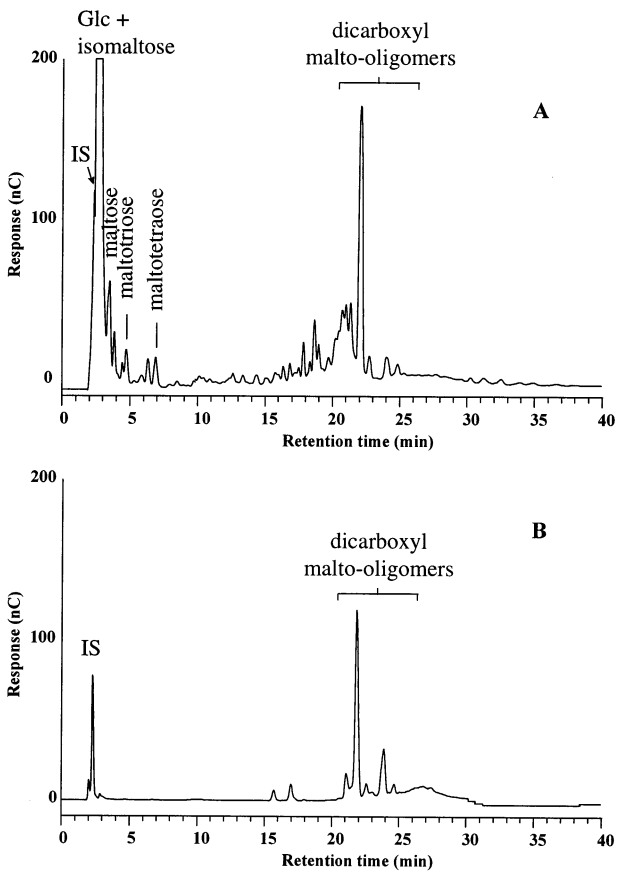

Fig. 1. HPAEC-PAD chromatograms of hypochlorite-oxidised potato starch Raisamyl 302 ESP. Glc, glucose; IS, internal standard. (A) Hydrolysate after enzymatic hydrolyse by alpha amylase and glucoamylase, and (B) isolated dicarboxyl malto-oligomers (**F2**).

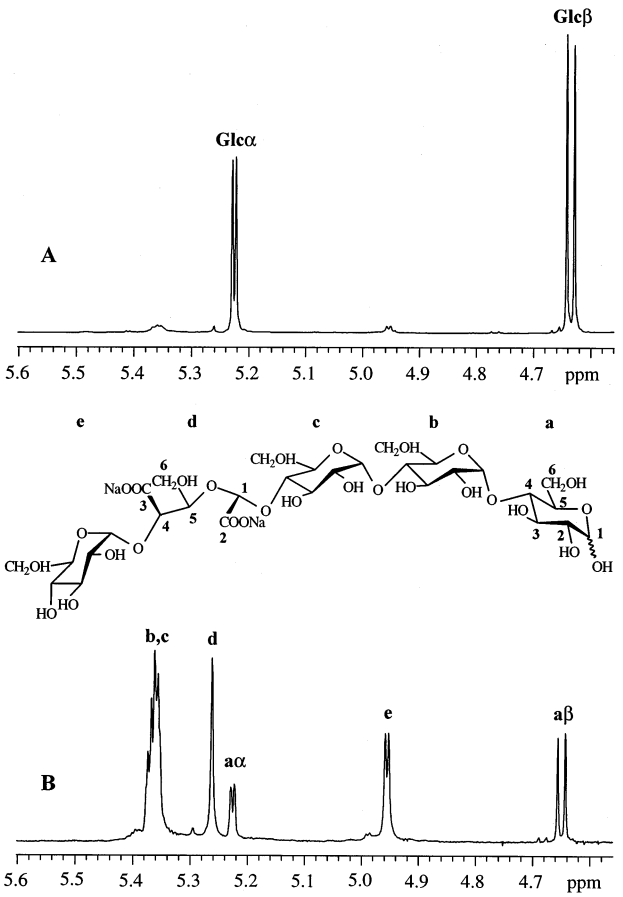

Fig. 2. Anomeric proton region of ^{1}H NMR spectra of hypochlorite-oxidised potato starch Raisamyl 302 ESP. (A) Hydrolysate after treatment with alpha amylase and glucoamylase, and (B) dicarboxyl malto-oligomers (**F2**). The structure of the pentamer part of **F2** is shown in (B); the hexamer has two **b** residues.

monosaccharide units are designated **a**–**e** starting from the reducing end (Fig. 2B). The chemical shifts of the non-hydroxylic protons for residues **a**–**e** are given in Table 1. The assignment was based on phase-sensitive COSY, relay COSY and TOCSY experiments starting from the anomeric protons of residues **a**–**c** and **e**. For H-1 at 5.26 ppm of **d** no homonuclear connection was observed. A spin system of four protons at 4.32, 4.06, 3.74 and 3.80 ppm, with no connection to an anomeric proton, was identified by the homonuclear 2D experiments. An active coupling constant of 12 Hz was measured from the cross-peaks at 3.74 and 3.80 ppm in the phase-sensitive COSY spectrum. In the HMQC spectrum those protons were found to have the same ^{13}C chemical shift, 62.3 ppm, typical for a methylene group. The relative sizes of the signals 5.26, 4.32 and 4.06 ppm indicated that they belong to the same residue.

The ^{13}C chemical shifts are given in Table 2. The assignment was based on heteronuclear correlation spectroscopy (HMQC) from the assigned proton signals. The non-protonated carbon atoms (δ 175.88 and 176.93) were identified from the heteronuclear multiple-bond correlation (HMBC) spectrum (Fig. 3). The chemical shifts of both carbonyl carbons are in good agreement with chemical shifts earlier reported for carboxyl carbons in carbohydrates [3,4,6,7]. In the coupled ^{13}C NMR spectrum C-3 of unit **d** occurred as a triplet ($J_{C,H}$ 2.7 Hz) as expected. In the first acquired ^{13}C spectrum of **F2**, the ^{13}C resonances of C-1, C-2, C-3 and C-4 of residue **d** were broad; the width of the line at half-height was ca. 25 Hz for the carboxyl group signals. The reason for

Table 1
^{1}H NMR data for **F2**

Residue	Chemical shift in ppm[a] ($^3J_{H,H}$ in Hz)						
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
α-Glc*p* **a**	5.22 (3.7)	3.56	3.96	3.63	3.94	3.83	3.83
β-Glc*p* **a**	4.65 (7.9)	3.27	3.76	3.62 (10)	3.58	3.76	3.90
α-Glc*p* **b**	5.37 (3.5)	3.620	3.95	3.64 (10)	3.84	3.84	3.84
α-Glc*p* **b**′	5.357 (3.4)	3.63	3.95	n.d.[b]	n.d.	n.d.	n.d.
α-Glc*p* **c**	5.356 (3.4)	3.65	3.86	3.69 (10)	3.80	3.89	3.88
Dicarboxyl residue **d**	5.26 (-[c])	–	–	4.32 (3.2)	4.06	3.74 (12)	3.80
α-Glc*p* **e**	4.95 (3.6)	3.51 (9.9)	3.78	3.41 (9.6)	3.83	3.75	3.75

[a] Relative to internal acetone at 2.225 ppm (D_2O, 50 °C, pD 7.0), acquired at 600 MHz.
[b] n.d., Not determined.
[c] -, Not relevant.

Table 2
^{13}C NMR data for **F2**

Residue	Chemical shift in ppm[a] ($^{1}J_{C,H}$ in Hz)					
	C-1	C-2	C-3	C-4	C-5	C-6
α-Glc*p* **a**	92.81 (169)	72.24	74.06	78.3	70.93	61.54
β-Glc*p* **a**	96.70 (162)	74.95	77.04	78.2	75.50	61.66
α-Glc*p* **b**	100.46 (172)	72.4	74.18	78.2	72.16	61.40
α-Glc*p* **b′**	100.61 (172)	n.d.[b]	n.d.	n.d.	n.d.	n.d.
α-Glc*p* **c**	100.61 (172)	72.4	73.96	76.84	72.42	61.34
Dicarboxyl residue **d**	102.93 (169)	175.88	176.93	79.50	80.89	62.30
α-Glc*p* **e**	98.57 (171)	72.57	74.45	70.50	73.22	61.47

[a] Relative to external 1,4-dioxane at 67.4 ppm (D_2O, 50 °C, pD 7.0), acquired at 150 MHz.
[b] n.d., Not determined.

this was that metal complexes were formed in solution. The problem could be avoided by adding CDTA, which prevented di- and trivalent metal ions from binding to the carboxyl groups and resulted in narrow ^{13}C resonances.

The cross-peaks observed at the H-1 frequencies in the HMBC and NOESY spectra (Table 3) and at the C-1 frequencies in the HMBC spectrum (Table 4) established the sequence. The ^{1}H and ^{13}C chemical shifts for the **a** and **b** units, i.e., reducing end and internal glucose, are very similar to those observed for maltotriose [8,9]. Furthermore, the chemical shifts of the H-4 and C-4 resonances of unit **e** at 3.41 and 70.50 ppm, respectively, correspond well with the H-4 and C-4 resonances of terminal glucose in maltotriose [8,9]. The α:β ratio for unit **a** is 2:3 as expected. The chemical shifts of **d** C-1 (102.93 ppm) and H-1 (5.26 ppm) differ from earlier reported values (δ ^{13}C 100.7 and δ ^{1}H 4.95) for a corresponding dicarboxyl residue in bromine-oxidised potato starch [4]. Most probably the discrepancy is caused by different cations.

The vicinal coupling constants between H-4 and H-5 ($^{3}J_{4,5}$) in **a**–**c** and **e** (Table 1) indicate a trans orientation of H-4 to H-5. This is expected in the $^{4}C_{1}$ chair conformation of glucose, where all ring non-anomeric protons are in the axial orientation. In the cleaved ring **d** a $^{3}J_{4,5}$ of 3.2 Hz is obtained. As the $^{3}J_{4,5}$ is close to one of the extrema expected from the Karplus relationship, there appears to be no significant motional averaging around the **d** C-4–C-5 bond. A calculation of $^{3}J_{4,5}$ using an empirical generalised Karplus equation [10], where the electronegativity and the position of the α- and β-substituents are taken into account, shows that the gauche orientations are preferred.

IR spectrum.—A comparison of the IR spectra of **F2** and native starch shows that a strong absorption band at 1750 cm^{-1} is only observed for **F2**. This band is the characteristic vibrational fingerprint of C=O stretch of carboxylic acid.

pK_a *value.*—No line-broadening was observed in the ^{1}H NMR spectra during the pH-titration. The largest chemical shift changes were obtained for the well-separated resonances for residue **d** (Fig. 4); this was expected because of the proximity to the carboxyl groups. The pK_a value for both the carboxyl groups was estimated to ca. 3.0. We have earlier reported similar pK_a values for hexenuronic acid (3.03) [6], 4-*O*-methylglucuronic acid (3.14) [6] and 4-*O*-methyliduronic acid (3.17) [7] attached to xylo-oligomers.

Amount of dicarboxyl residues in Raisamyl 302 ESP.—The carbohydrate composition was determined by quantitative ^{1}H NMR spectroscopy (Fig. 2A). An estimation of the amount of dicarboxyl residues in Raisamyl 302 ESP was obtained by integration of the anomeric protons. The integral of H-1 for the dicarboxyl residue (δ 5.26 ppm) was ca. 1.4 mol% of the total integral for the anomeric protons. Consequently, the content of carboxyl groups within the dicarboxyl residues was almost 3 per 100 glucose units (twice the amount of dicarboxyl residues). The main oxidation product is thus the dicarboxyl residue.

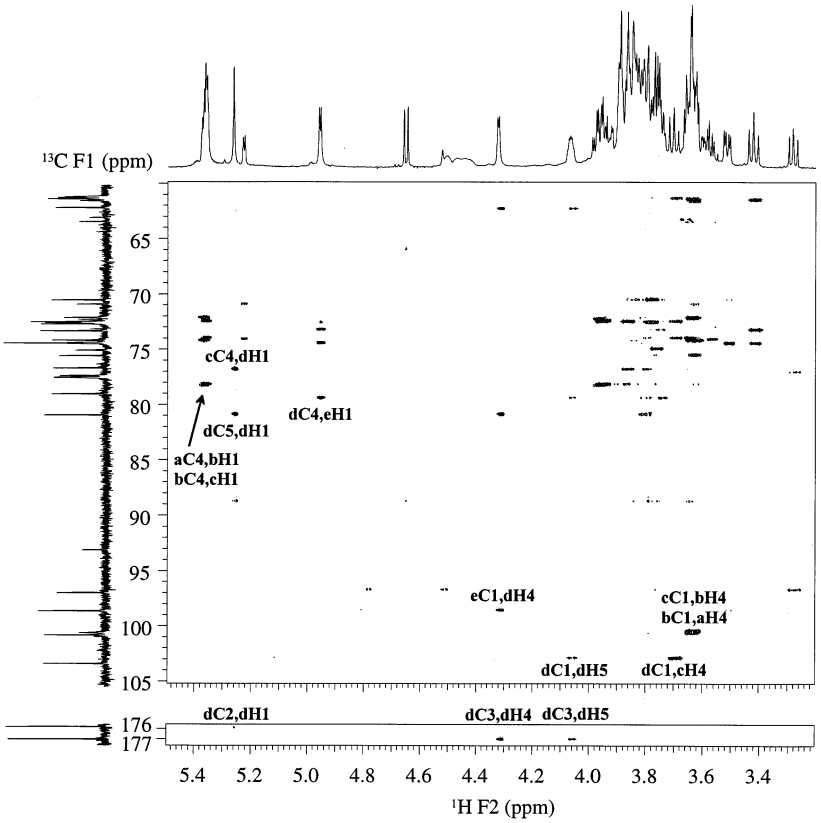

Fig. 3. HMBC spectrum of **F2**. The cross-peaks verifying glycosidic linkages and some of the internal linkages for **d** are indicated. The spectra lined on the sides are 1D ^{13}C and ^{1}H NMR spectra of **F2**.

Ring cleavage and the formation of carboxyl groups at C-2 and C-3 have earlier been found only after oxidation with bromine at neutral pH of potato starch [4]. Unknown minor resonances observed in the ^{1}H NMR spectra of other acidic fractions indicate that minor amounts of other carboxylic groups are expected to be found. The structure of the other oxidised products remains to be elucidated.

3. Conclusions

The hypochlorite oxidation has mainly occurred at positions C-2 and C-3 of a glucose unit. The introduced carboxyl groups cause ring cleavage between the carbons C-2 and C-3. The glycosidic linkages on both sides of the oxidised glucose are intact, indicating that this oxidation pathway does not cause depolymerisation. As could be expected, alpha amylase and glucoamylase can only cleave α-(1→4)-linkages between glucose units or have only a low activity to glycosidic linkages next to the dicarboxyl residue.

The change of the coupling constant between H-4 and H-5 upon ring cleavage indicates a change in bending of the linear chain. The bending probably prevents side-by-side alignment of linear chains and might thereby be the reason why hypochlorite oxidation reduces the tendency to retrogradation. A major

Table 3
Cross-peaks observed at the H-1 frequencies in the HMBC and NOESY spectra

Residue	HMBC cross-peak	NOESY cross-peak
α-Glc*p* **a** H-1	α-Glc*p* **a** C-3, C-5	α-Glc*p* **a** H-2
β-Glc*p* **a** H-1	n.o.[a]	β-Glc*p* **a** H-2, H-5
α-Glc*p* **b** H-1	α-Glc*p* **b** C-3, C-5; Glc*p* **a** C-4	α-Glc*p* **b** H-2
α-Glc*p* **c** H-1	α-Glc*p* **c** C-3, C-5; α-Glc*p* **b** C-4	α-Glc*p* **c** H-2, H-6′; α-Glc*p* **b** H-4
Dicarboxyl residue **d** H-1	oxGlc **d** C-2, C-5; α-Glc*p* **c** C-4	oxGlc **d** H-5, H-6
α-Glc*p* **e** H-1	α-Glc*p* **e** C-3, C-5; oxGlc **d** C-4	α-Glc*p* **e** H-2, oxGlc **d** H-4

[a] n.o., Not observed.

Table 4
Cross-peaks observed at the C-1 frequencies in the HMBC spectrum

Residue	HMBC cross-peak
α-Glc*p* **a** C-1	n.o.[a]
β-Glc*p* **a** C-1	β-Glc*p* **a** H-2
α-Glc*p* **b** C-1	Glc*p* **a** H-4
α-Glc*p* **c** C-1	α-Glc*p* **b** H-4
Dicarboxyl residue **d** C-1	α-Glc*p* **c** H-4
α-Glc*p* **e** C-1	oxGlc **d** H-4

[a] n.o., Not observed.

cause of the minimum retrogradation is probably the polyelectrolytic properties caused by the anionic character of the carboxyl groups. Probably, a third reason is the extensive depolymerisation of the starch components obtained during the oxidation process.

4. Experimental

Substrate and enzymes.—The commercial oxidised potato starch (Raisamyl 302 ESP) was obtained from Raisio Chemicals (Raisio, Finland). The hydrolysing enzymes used in this work were generous gifts from the suppliers: alpha amylase (EC 3.2.1.1), Termamyl (Novo Nordisk, Bagsvaerd, Denmark) and glucoamylase (EC 3.2.1.3) (ABM, Stockport, UK).

Carbonyl and carboxyl amounts.—The amounts of carboxyl and carbonyl groups were determined by the methods of Smith [11], as described in detail by Parovuori and coworkers [2].

Isolation of **F2**.—The oxidised starch Raisamyl 302 was first hydrolysed to mono- and oligosaccharides by an extensive alpha amylase and glucoamylase treatment. Raisamyl 302 (4.4 g) was suspended in 40 mL of 20 mM NaOAc buffer (pH 5.0) containing 10 mM $CaCl_2$. The suspension was boiled for 10 min. After cooling the reaction mixture to 50 °C, alpha amylase (1000 nkat/g starch) and glucoamylase (5000 nkat/g starch) were added to the reaction mixture. After 20 h of reaction time at 50 °C, the enzymes were inactivated by boiling the reaction mixture for 2 min. The solid materials were removed by centrifugation. The clear solution was loaded onto a column (1 × 20 cm) of Dowex 1 × 2 (strong anion-exchange resin, Fluka, Switzerland), which was equilibrated by ion-exchange water. The bound (acidic) fractions were eluted out from the column by a linear NaAc gradient from 0 to 500 mM. One of the fractions, denoted **F2**, was used for the structure analysis reported here, after desalting by gel filtration (Sephadex G-10 packed in a 1.6 × 26 cm column) and freeze-drying (yield: 18 mg).

HPAEC-PAD analysis.—The high-performance anion-exchange chromatography (HPAEC) was performed with a Dionex DX 500 series chromatograph according to Pellerin and co-workers [12]. The glucose, maltose and isomaltose used as standards were purchased from Fluka (Switzerland).

NMR spectroscopy.—The freeze-dried alpha amylase and glucoamylase hydrolysate were used for determination of the amount of the dicarboxyl residues in Raisamyl 302 ESP. About 14 mg of **F2** were used. Each of the

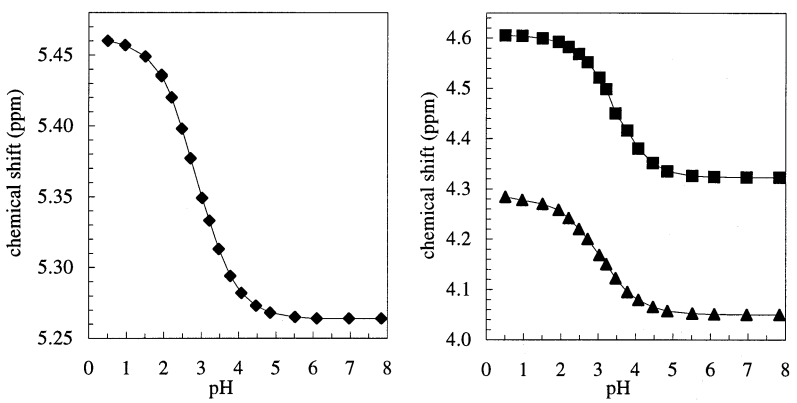

Fig. 4. pH-Dependence of the ^{1}H chemical shift of **d** H-1 (◆), H-4 (■) and H-5 (▲).

samples was dissolved in 0.3 mL D_2O and the pH was adjusted to 7 by the addition of NaOD. The solutions were clear and colourless. In order to remove paramagnetic ions in the **F2** sample, CDTA was added to a final concentration of 5 mM. Shigemi NMR tubes (BMS-005V) were used. The ^1H and ^{13}C NMR spectra were obtained at 599.84 and 150.85 MHz, respectively, on a Varian UNITY 600 MHz spectrometer. 1D ^1H NMR (1D ^{13}C NMR) spectra were recorded using a 75° pulse of 6 μs (60° pulse of 9 μs), a spectral width of 8000 Hz (40,000 Hz), and a repetition time of 20 s for the enzymatic hydrolysate and 7 s for **F2** (1.4 s). Spectra were acquired at 50 °C, except the NOESY spectrum at 12 °C and pH-titration at 27 °C. The chemical shifts are reported relative to internal acetone at 2.225 ppm and external 1,4-dioxane at 67.4 ppm for ^1H and ^{13}C NMR spectra, respectively. Standard pulse sequences and phase cyclings were used to obtain phase-sensitive $^1H,^1H$-correlated spectroscopy (COSY) [13], relayed COSY ($\tau = 90$ ms) [14], total correlation spectroscopy (TOCSY) ($\tau_{mix} = 0.14$ s) [15,16] and nuclear Overhauser effect spectroscopy (NOESY) ($\tau_m = 0.3$ s) [17] 2D spectra. A spectral width of 1700 Hz was employed in both dimensions and the relaxation delay was 2.4 s. For each FID, eight (four for COSY) transients were acquired; the data size was 410 (850 for COSY) in $t_1 \times 2048$ ($\times$ 1024 for TOCSY and NOESY) in t_2. The final data size after Fourier transformation was 2048×4096. The phase-sensitive ^1H-detected HMQC [18] spectrum was acquired over a t_1 spectral width of 9000 Hz and a t_2 width of 1700 Hz with a 800×1024 matrix (zero-filled to 2048 in t_1 and 4096 in t_2) and eight transients per increment. The delay between transients was 3.3 s and the delay for polarisation transfer was set for an estimated average $^1H-^{13}C$ coupling constant of 155 Hz. The multiple-bond $^1H-^{13}C$ shift correlation (HMBC) [19] spectrum resulted from 1000×2048 data matrix size, with 24 scans per t_1 value and a delay time of 2.7 s between scans. A 100 ms delay was used for the evolution of long-range connectivities. The spectral width was 19,000 and 3000 Hz for t_1 and t_2, respectively. Data processing was performed using standard VNMR software.

General methods.—IR spectra were recorded on a Perkin–Elmer 1760 X FTIR spectrophotometer. About 2.5 mg of **F2** in the acidic form (pH 1) or native starch was pressed with KBr to a tablet. For the determination of the pK_a values the desired pH values (not corrected for isotopic effects) were obtained by additions of known amounts of NaOD or DCl.

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