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Carbohydrate Research 309 (1998) 213–218

CARBOHYDRATE RESEARCH

Note

Structural characterization of oxidized potato starch

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Received 16 December 1997; accepted in revised form 22 May 1998

Abstract

Sodium hypochlorite oxidized (HO) and hydrogen peroxide oxidized (PO) potato starches were fractionated on an ion-exchange chromatography column of DEAE-Sepharose. Bound dextrins represented the major fraction and possessed larger d.p.-values than the minor fraction of unbound dextrins. The HO- and PO-starches were also debranched prior to separation on the ion-exchanger. The proportion of bound chains was much larger in the HO-starch. In both starches only 63% of the bound chains were resistant to beta-amylolysis, suggesting that the non-resistant chains possessed substitutions at the reducing end. The relative molar concentration of modified chains were 34 and 20% in the HO- and PO-starch, respectively. ^{13}C NMR spectra of fractions obtained from the HO-starch showed that carboxylic groups were concentrated on the bound chains. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Oxidation; Potato starch; Dextrins; Ion-exchange

Enzymic methods have been successfully used in structural studies of native starch polysaccharides for several decades [1]. However, the methods have been only little used in the structural investigations of chemically modified starches [2–9]. Introduced substituents have in several cases been shown to interfere with the enzymic reaction in a similar way to the branching points in amylopectin [4–9]. Recently, we investigated the possibility of using enzymes as tools in structural studies of oxidized potato starches [10]. In the present study, the enzymic methods were used in combination with ion-exchange chromatography and ^{13}C NMR spectroscopy to identify modified chains and to locate the positions of substituents along them. During the oxidation process, the starch was extensively depolymerized [11] and became substituted with carboxyl and carbonyl groups [12–15].

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PII S0008-6215(98)00138-4

1. Results and discussion

Background data on the sodium hypochlorite oxidized (HO) and the hydrogen peroxide oxidized (PO) potato starches used in this study are shown in Table 1. Both samples possessed similar chain lengths (c.l.) but slightly different d.p.-values. The HO-sample contained both longer and higher amounts of R-chains (defined as chains resistant to β-amylase attack [10]) than the PO-sample.

The starches were applied to an ion-exchange chromatography column of DEAE-Sepharose after being dissolved in sodium acetate buffer, pH 6.5. The major part of the carbohydrate material in the oxidized samples was bound to the resin (Table 2) showing that charged groups were commonly found among the starch components. On a molar basis ~64% of the dextrins were bound in both samples.

The molecular-weight-distributions of the bound and unbound fractions were very different (Fig. 1). The distribution of the bound dextrins was broad and resembled the original samples [10], whereas unbound dextrins possessed narrow distributions with low d.p. of 17 and 40 for the HO- and PO-samples, respectively (Table 2). The characteristics of the bound dextrins were similar to those of the whole samples (Table 1).

Dextrins without charged groups should not be bound to the ion-exchanger. The existence of the small unbound dextrins indicated, therefore, that they had been produced during the oxidation by simple hydrolysis without further modification of the D-glucosyl residues. However, the beta-amylolysis of the debranched sample was not complete, which suggested the existence of a small number of R-chains in the unbound dextrins (Table 2). The nature of these apparent R-chains was uncertain. The average number of chains in the unbound dextrins was 1.3 for the HO-sample and 1.7 for the PO-sample. Because linear dextrins have a chain number of 1 and a pure solution of branched dextrins have a n.c. of ≥ 2, a part of the dextrins were linear.

Table 1
Some data for sodium hypochlorite oxidized (HO) and hydrogen peroxide oxidized (PO) starches

Parameter	HO-starch	PO-starch
CHO (weight%)[a]	0.22	0.41
COOH (weight%)[b]	1.04	0.50
D.p.[c]	177	130
C.l.[c]	22.9	23.4
C.l. of R-chains[c]	32.4	28.7
R-chains (mole %)[c]	26	20

[a] Substitution of carbonyl groups on dry weight basis.
[b] Substitution of carboxyl groups on dry weight basis.
[c] From reference [10].

Table 2
Characterization of fractions isolated by ion-exchange chromatography of sodium hypochlorite oxidized (HO) and hydrogen peroxide oxidized (PO) starches

	Unbound dextrins		Bound dextrins	
Parameter	HO	PO	HO	PO
Yield				
(weight %)	7	15	85	78
(mole %)	37	36	63	64
D.p.$_n$	17	40	172	125
C.l.	13	24	24	22
C.l. of R-chains	13	14	34	30
R-chains (mole %)[a]	10	7	26	20
β-Limit after debranching (%)	95	96	64	74
N.c.[b]	1.3	1.7	7.2	5.7

[a] Percent of R-chains within each fraction.
[b] Number of chains calculated as d.p./c.l.

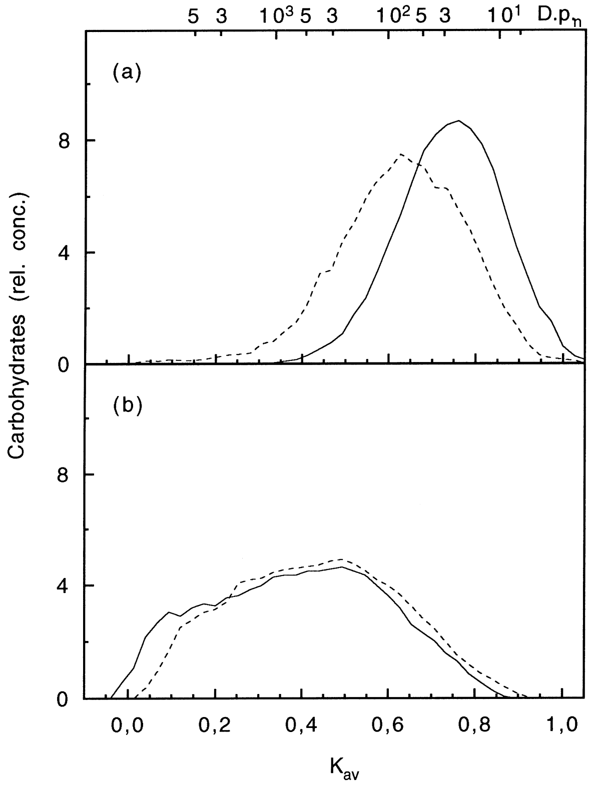

Fig. 1. Molecular-weight-distribution on Sepharose CL 6B of (a) unbound dextrins and (b) bound dextrins from HO-starch (——) and PO-starch (- - - -) after ion-exchange chromatography.

The HO- and PO-starches were also first debranched with isoamylase and the unit chains were then separated on the ion-exchanger. Two types of chains, unbound and bound, were isolated (Fig. 2), of which the former were obtained in higher yield from the PO-starch (Table 3). Their β-limit was $>90\%$ and on a molar basis 4 and 10% of the unbound chains in the HO- and PO-samples, respectively, were resistant to the attack of beta-amylase. The c.l. of these apparent R-chains was similar to the average for all the unbound chains and it was lower than the c.l. of the bound chains.

The yields of bound chains were lower than that of bound dextrins in both samples (Table 3). This showed that a large part of the unit chains in the bound dextrins were not modified, or at least not charged. On a molar basis, R-chains represented ~60% of all bound chains in both samples. Thus, though all chains were charged, ~40% were not resistant to the attack of beta-amylase. The substitutions in these chains should therefore be located at or close to the reducing end. Because the c.l. of the bound R-chains remained practically similar to that for all the bound chains, they were probably either completely resistant or only slightly attacked by the beta-amylase. Thus, the modified D-glucosyl residues were found either close to the non-reducing end or to the reducing end of the chains.

Table 3
Characterization of fractions isolated by ion-exchange chromatography of debranched sodium hypochlorite oxidized (HO) and hydrogen peroxide oxidized (PO) starches

Parameter	Unbound chains		Bound chains	
	HO	PO	HO	PO
Yield				
(weight %)	49	70	50	25
(mole %)	66	80	34	20
C.l.	13	19	25	27
C.l. of R-chains	11	18	27	25
R-chains (mole %)[a]	4	10	62	64
β-Limit (%)	97	91	46	46

[a] Percent of R-chains within each fraction.

The ^{13}C NMR spectra of the native potato starch and the HO-starch, and of the unbound and bound chains of the latter sample, are shown in Fig. 3. Small differences in the chemical shifts between the spectra were seen, especially an upfield shift of C-1 and C-4 in the spectrum of the

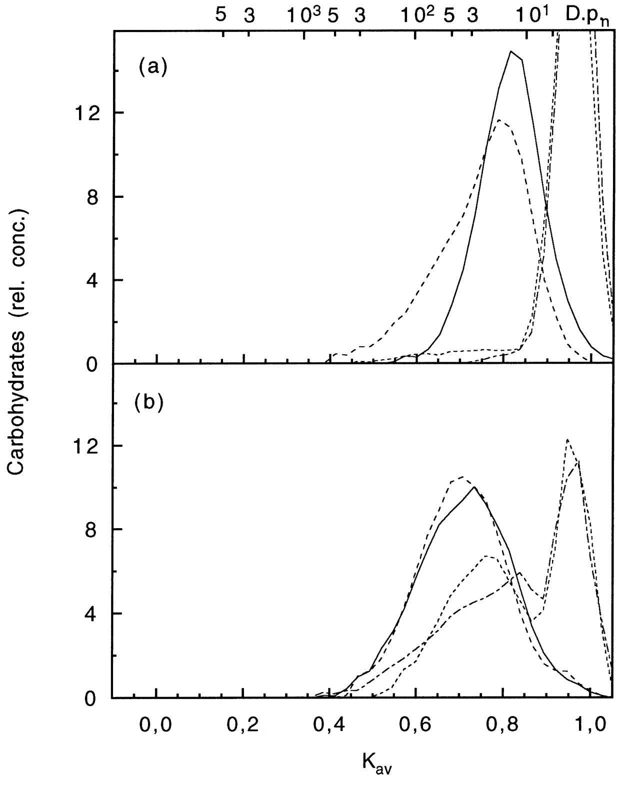

Fig. 2. Distribution on Sepharose CL 6B of (a) unbound and (b) bound chains from HO-starch (——) and PO-starch (- - - -), and after beta-amylolysis of the chains from the HO-starch (-.-) and PO-starch (····).

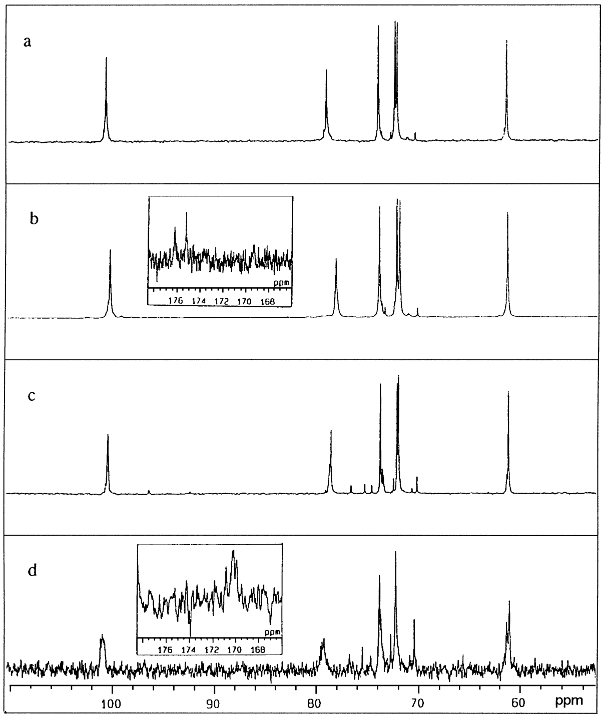

Fig. 3. ^{13}C NMR spectra of (a) native potato starch, (b) HO-starch, (c) unbound chains of HO-starch, and (d) bound chains of HO-starch.

HO-starch (Fig. 3b). The spectrum of the HO-starch was very similar to that of the native starch. However, weak signals assigned to carboxylic carbons were observed at $\delta = 176.0$ and 177.0 ppm (Fig. 3b). The spectrum of the unbound chains showed several signals that could be assigned to carbons in the reducing and the non-reducing end groups, but no carboxyl signals were detected (Fig. 3c). In the spectrum of the bound chains signal broadening was observed which made the interpretation of this spectrum more difficult (Fig. 3d). Weak carboxyl signals were, however, detected. The line broadening may be due to a broad distribution of similar carbons in slightly different surroundings.

The chemical shift ranges and the assignments of the signals in the ^{13}C NMR spectra are given in Table 4. The assignments of the signals were based on data found in the literature [16–20]. However, the main signal in the range $\delta = 75.5 - 73.5$ ppm was assigned to C-2 by Falk et al. [16]. The exact chemical shifts of the signals differed from those given in the references, which could be ascribed to the presence of D_2O in the present samples. This has been reported to induce downfield shifts of the signals without chemical shift crossings [16,21], which facilitates comparison of spectra obtained in different mixtures of D_2O and DMSO-d_6. The signal in the chemical shift range $\delta = 70.7 - 70.0$ ppm was assigned to C-4 in non-reducing end groups and the signals of C-2 and C-6 were found at $\delta = 72.5$ and 61.0 ppm, respectively [17–20]. The spectra in Fig. 3 showed signals on the slopes of the signals for C-2 and C-6 that followed the same intensity variations as that in the chemical shift range $\delta = 70.7 - 70.0$ ppm. Thus, we concluded that the assignment was correct, although the signal at $\delta = 70.7 - 70.0$ ppm was assigned to C-6 in a 1→6 branch by Falk et al. [16].

Table 4
Chemical shift ranges of carbon atoms

Shift range (δ, ppm)	Types of carbons
178–168	COOH
104–98	C-1
98–92	C-1 at reducing end
82–78	C-4
78–75	C-2β, C-3β, and C-5β at reducing end
75–73	C-3, C-5 at non-reducing end
73–70	C-2, C-5
70–70	C-4 at non-reducing end
64–60	C-6

The NMR spectra of the bound chains showed a signal that corresponded to the carboxyl groups (Fig. 3d inset). The spectra of the unbound chains did not possess this signal and suggested that they were not oxidized. Possibly, therefore, the resistance to β-amylase attack of a minor part of the unbound chains in the HO-starch was due to retrogradation.

Table 5 summarizes the types of chains found in the oxidized potato starches. The total amount of R-chains (bound and unbound) was estimated from the figures in Table 3 and agreed with the experimental values of the original HO- and PO-samples (Table 1). This suggested that the unbound R-chains already existed in the original samples, and were not obtained as a consequence of their isolation during ion-exchange chromatography. All detectable modified chains, defined as the bound chains (both R- and non R-chains), represented 34% and 20% in the HO- and PO-starches, respectively. A part of the modified chains were susceptible to the attack of beta-amylase. The amount of these bound non R-chains were 13% in the HO-sample and 7% in the PO-sample. The rest of the chains were then either not substituted, not detectable as modified chains or not R-chains, that is not bound to the ion-exchanger and not resistant to beta-amylolysis. This group of chains constituted approximately ~63% of all chains in the HO-starch and ~73% in the PO-starch and suggested that the PO-starch possessed a higher amount of unmodified chains than the HO-starch.

In conclusion, the methods used in this study offered a possibility to classify and partly characterize the chains that had been modified during the oxidation of starch. It was shown that there was no essential difference between the structures of the HO- and PO-starches in terms of the positions of the substitutions along the chains. Most of the chains were apparently substituted either close to the non-reducing end or to the reducing end.

Table 5
Percentage molar concentration of different types of chains found in oxidized potato starches

Type of chains	HO-starch	PO-starch
Bound R-chains	21	13
Unbound R-chains	3	7
Bound non R-chains	13	7
Total of R-chains	24	20
Total of modified chains	34	20
Other chains	63	73

The samples contained similar types of chains though the amount of modified chains was higher in the HO-starch.

2. Experimental

Enzymes.—Isoamylase of *Pseudomonas amyloderamosa* (glycogen 6-glucanohydrolase EC 3.2.1.68) was purchased from Hayashibara. Sweet potato beta-amylase [(1→4)-α-D-glucan maltohydro-lase; EC 3.2.1.2] was from Sigma.

Sodium hypochlorite oxidation.—A starch slurry of 20–30% solids was prepared and the pH was adjusted to 9–10 with NaOH. Active sodium hydrochlorite (20% of dry starch weight) was added into the slurry in small portions and the temperature was kept at 30–40 °C during the oxidation. After 24 h the starch slurry was neutralized to pH 6 with sulphuric acid.

Hydrogen peroxide oxidation.—A starch slurry was prepared as described above. $CuSO_4$ (0.01%) was added as catalyst after which H_2O_2 (3% of dry starch weight) was added in small portions at 30–40 °C. After 24 h neutralization was done as above.

Ion-exchange chromatography.—Oxidized starch (10 mg/mL) was dissolved in NaOAc buffer (0.001 M, pH 6.5) by heating on a boiling water bath for 10 min with stirring. The sample (~3 mL) was added to a DEAE-Sepharose ion-exchange column (1.5×50 cm). Unbound dextrins were eluted at 0.5 mL/min with the NaOAc buffer in a fraction of 500 mL and concentrated with a vacuum rotor evaporator to 5 mg/mL. The bound dextrins were eluted with 0.5 M KOH in a fraction of 350 mL, neutralized with 2.0 M HCl, desalted, and concentrated to 5 mg/mL.

The oxidized starches were also debranched with isoamylase as described below, but in a preparative scale (30 mg). The unit chains were then fractionated by ion-exchange chromatography into unbound and bound chains, respectively.

Analytical methods.—For debranching, 0.1 M NaOAc buffer (0.1 mL, pH 3.5) and undiluted isoamylase (15 μL) was added to a sample dissolved in water (0.65 mL, 5 mg/mL). The sample was then diluted to 1 mL and incubated overnight at room temperature. After boiling for 3 min, an aliquot (0.3 mL) was treated with an equal volume of water and with 5 M KOH (60μL) before it was analyzed by gel-permeation chromatography.

The pH in an other aliquot (0.3 mL) of the debranched sample was adjusted to 4.8 with 0.2 M sodium acetate and the volume was adjusted to 0.6 mL with 0.1 M sodium acetate buffer, pH 4.8, before beta-amylase (3 μL) was added. The mixture was boiled after incubation over night and analysed by gel-permeation chromatography. R-chains were defined as chains resistant to beta-amylolysis.

Gel-permeation chromatography of samples (~1.5 mg/mL, 0.5 mL) in ~0.5 M KOH was performed on a column (1×80 cm) of Sepharose CL 6B. The samples were eluted with 0.5 M KOH at 1 mL/min and fractions (0.5 mL) were analyzed for total carbohydrates with the phenol-sulphuric acid reagent [22]. The column was calibrated with dextrin standards with known number average degree of polymerization as previously described [23,24].

NMR-spectroscopy.—Samples of native starch (100 mg), HO-starch (100 mg), and the unbound (55 mg) and bound (45 mg) fractions of unit chains of the HO-starch obtained by ion-exchange chromatography, were dissolved in 0.5 mL Me_2SO-d_6 containing D_2O. The center peak of the solvent multiplet was used as an internal standard ($\delta = 39.50$ ppm). The ^{13}C NMR spectra were recorded at 70 °C in a quantitative mode, i.e. with NOE suppression and a pulse delay of 11 s. The spectra were recorded on a JNM-A500 spectrometer operating at 125 MHz.

Acknowledgements

This work was supported by grants from Åbo Akademi University and Magnus Ehrnrooths Foundation.

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