

# Enzymatic and Acidic Hydrolysis of Cationized Waxy Maize Starch Granules

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## ABSTRACT

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A series of wet-cationized starch granules from waxy maize with different degrees of substitution (DS) were solubilized with either 2.2M HCl (lintnerization) or with the  $\alpha$ -amylase of *Bacillus amyloliquefaciens*. The maximum rate of the enzymatic hydrolysis occurred in starches with intermediate DS. It appeared that the cationic substituents interfered with the binding to the active site of the enzyme at high levels of substitution. The DS remained fairly constant in the granular residues after the enzymatic attack. The rate of the acidic hydrolysis increased with increasing DS but the final level of solubilization slightly decreased. The DS of the residual starch material decreased to 40% of the original level, showing

that a large part of the cationic groups was found within the amorphous parts of the granules. A dry-cationized sample with a high DS was also treated with the acid and lost a major part of its substituents at low levels of lintnerization. Probably most of the substituents were associated with the surface and channels of these granules. The cationized starches possessed branches that were resistant to isoamylase attack and the samples also contained  $\beta$ -amylolysis resistant dextrans. The proportion of resistant dextrans in the granular residues decreased after lintnerization, but remained constant after the enzymatic hydrolysis.

Starch derivatives are used in a range of commercial applications from food to paper manufacturing. The substitution pattern on the D-glucosyl units of the starch molecules has been investigated but less is known about the location of the substituents inside the starch granules or on the starch polymers. Enzymatic treatments of the starch components have shown that introduced substituents and cross-linkages interfere with various enzyme actions (Chan et al 1984, Fischer and Piller 1977, Fischer and Piller 1978, Hood and Mercier 1978, Jane et al 1992). As a result, different hydrolysis patterns are obtained with the modified starches when compared with those of their native counterparts. This strategy has been used for structural investigations of cross-linked hydroxypropylated manioc starch (Hood and Mercier 1978), methylated potato starch (Steeneken and Woortman 1994, Burgt et al 1998), hydroxypropylated potato starch (Kavitha and BeMiller 1998), and of various oxidized potato starches (Torneport et al 1990, Zhu and Bertoft 1997, Zhu et al 1998). A general conclusion from these studies is that the starches preferentially are substituted in the amorphous parts of the semicrystalline starch granules so that amylose and the regions around the branches in amylopectin become modified.

Cationized starch is used in large quantities by the paper industry as wet-end additives and for sizing. Commonly the quaternary ammonium reagent 2,3-epoxypropyltrimethylammonium chloride reacts with the hydroxyl groups of the starch under alkaline conditions (Kweon et al 1996). An alternative dry cationization method (Hellwig et al 1992, Khalil and Farag 1998) has received interest because of reduced environmental pollution. The D-glucosyl residues are preferentially monosubstituted at position C-2, though C-3 and C-6 may also be substituted. Small amounts of disubstituted residues also exist (Wilke and Mischnick 1995, Wilke and Mischnick 1997).

In this work, we have characterized the substitution pattern of the amylopectin of waxy maize starch after traditional wet-cationization and after dry cationization by studying the dextrin mixtures obtained after hydrolysis with isoamylase and  $\beta$ -amylase. Lintnerization, by which the amorphous parts of the granular starch are solubilized in dilute hydrochloric acid (Robin et al 1974), was used to investigate the substitution patterns inside the granules. The acidic solubilization was compared with the solubilization of the granules by  $\alpha$ -amylase.

## MATERIALS AND METHODS

### Starch and Enzymes

Commercial waxy maize starch was a gift from Raisio Chemicals Oy (Finland). The  $\alpha$ -amylase from *Bacillus amyloliquefaciens* [(1 $\rightarrow$ 4)- $\alpha$ -D-glucan glucohydrolase; EC 3.2.1.1] was purchased from Boehringer-Mannheim (Germany) and had an activity of 600 U/mg. Sweet potato  $\beta$ -amylase [(1 $\rightarrow$ 4)- $\alpha$ -D-glucan maltohydrolase; EC 3.2.1.2] with an activity of 880 U/mg (26 mg/mL) was from Sigma (Germany). Isoamylase of *Pseudomonas amyloclavata* (glycogen 6-glucohydrolase; EC 3.2.1.68) with an activity of 71,000 U/mg (1 mg/mL) was obtained from Hayashibara (Japan).

### Wet Cationization

Waxy maize starch granules were suspended in water (40% w/v) at 42°C and was adjusted to pH 11.0 with NaOH (2.5% w/v). A commercial etherifying agent containing 2,3-epoxypropyltrimethylammonium chloride (RAISACAT, Raisio Chemicals Oy) was added to the slurry. The reaction was stopped after 20 hr by adjusting to pH 6.5 with H<sub>2</sub>SO<sub>4</sub> (25% v/v). An aliquot (30 mL) of the slurry was filtered and washed with ethanol and water (1:1, 200 mL) and then with ethanol (50 mL). The sample was dried at 105°C for 1 hr before the nitrogen content was analyzed using the Kjeldahl method. The degree of substitution was calculated as: DS = (162.15 × N%)/(14 × 100) after subtracting the nitrogen content (0.026%) of the control starch. The rest of the slurry was filtered (604 Rundfilter, Schleicher & Shueller), washed with water and ethanol, and finally dried in acetone.

Damaged granules in the native and cationized samples (100 g/L) were removed by repeated centrifugation for 5 min at 14 × g. The collected supernatants were then centrifuged for 10 min at 14 × g and the granules were washed with methanol and dried in acetone. The dry weight of the samples was measured with a Sartorius thermo control balance.

### Dry Cationization

Waxy maize starch (2,000 g) was put into a cylindrical container. During vigorous mixing at increased pressure (1.5 atm) and temperature (60°C), a NaOH solution (17% w/v, 180 g) was sprayed onto the starch in small portions. The mixing continued until the starch was dry, after which the cationizing chemical was added in small portions. After 5 hr, an aliquot of the granules (30 g) was mixed with ethanol (600 mL) in a blender (Polytron) at 2000 rpm for 30 sec and then filtered. After repeating this blending procedure three times, an aliquot (5–7 g) of the sample was dried for 2 hr at 105°C and the nitrogen content was determined.

Aggregated granules in the main part of the dry-cationized starch were removed by fractionation through two sieves with pore sizes

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of 500  $\mu\text{m}$  and 150  $\mu\text{m}$ , respectively, giving three size-classes of the material with diameters of  $>500$   $\mu\text{m}$ , 500–150  $\mu\text{m}$ , and  $<150$   $\mu\text{m}$ . The fractions were washed with ethanol, and solid citric acid was added in small portions ( $\leq 1\%$  w/v) to neutralize remaining NaOH.

### Lintnerization of Starch Granules

Native and cationized starch granules were solubilized in 2.2M HCl (2.5 g of starch/100 mL of acid) at 35°C. The suspensions were stirred carefully every day. Aliquots taken at specific time intervals were centrifuged and the carbohydrate content in the supernatant was measured using the phenol-H<sub>2</sub>SO<sub>4</sub> reagent (Dubois et al 1956). The lintnerization was continued to two stages until  $\approx 30$  and 80% of the granules had been solubilized. The granular residues were then collected by centrifugation for 5 min at 1,400  $\times g$ , neutralized with 0.1M NaOAc, and washed three times with deionized water. Finally, the residues were suspended in a small volume of deionized water and lyophilized.

### Enzymatic Hydrolysis of Starch Granules

Native and cationized starch granules (50 mg) were allowed to equilibrate in 0.002M NaOAc (pH 6.5, 5 mL) at 25°C for 20 hr. The samples were then mixed and centrifuged for 2 min at 700  $\times g$ , after which a portion of the supernatant (4 mL) was removed and replaced by water (3.167 mL) and a solution of  $\alpha$ -amylase (60

U/mL, 0.833 mL in 0.1M NaOAc buffer, pH 6.5), giving a final enzyme concentration of 10 U/mL. The samples were incubated at 25°C, and aliquots were taken at intervals up to 24 hr for measurement of solubilized carbohydrates with the phenol-H<sub>2</sub>SO<sub>4</sub> reagent.

Large batches of enzymatically treated granules (6 g) were prepared under identical conditions by incubation for 5–6 hr at which point 30–40% of the starch had solubilized. The granular residues were collected by centrifugation (10 min at 700  $\times g$ ), washed repeatedly with water and with ethanol, and finally dried in acetone.

The effect of annealing and drying conditions on enzyme susceptibility of native starch granules was also tested. Starch suspensions (40% w/v) in either water or a NaOH solution (pH 11) were incubated at 42°C for 20 hr (to resemble the conditions for wet cationization). The suspensions in NaOH were then neutralized with sulfuric acid (25% v/v) and all samples were washed with water. The samples were then either washed with ethanol and dried in acetone or dried by lyophilization. The samples were finally treated with  $\alpha$ -amylase for 5 hr on an analytical scale as described above.

### Enzymatic Analyses of Starch Components

Intact starch granules or residues of granules prepared by enzymatic or acidic hydrolysis were boiled for 15–30 min in water (5 mg/mL, 0.65 mL). The samples were diluted with water (0.25 mL), and 0.1M NaOAc buffer (pH 3.5, 0.1 mL), after which a freshly diluted (5 $\times$ ) isoamylase solution (10  $\mu\text{L}$ ) was added. The reaction was stopped after incubation overnight at room temperature by boiling the digests in a water bath. Aliquots were taken for chromatographic analyses as described below. For a portion of the digest (0.3 mL), the pH was increased to 4.8 with 0.005M NaOAc, the volume was adjusted to 0.59 mL with 0.1M NaOAc buffer (pH 4.8), and  $\beta$ -amylase (2  $\mu\text{L}$ ) was added. After incubation overnight at room temperature, the reaction was stopped by boiling. Lintnerized samples were also treated with  $\beta$ -amylase without prior isoamylolysis.

### Gel-Permeation Chromatography

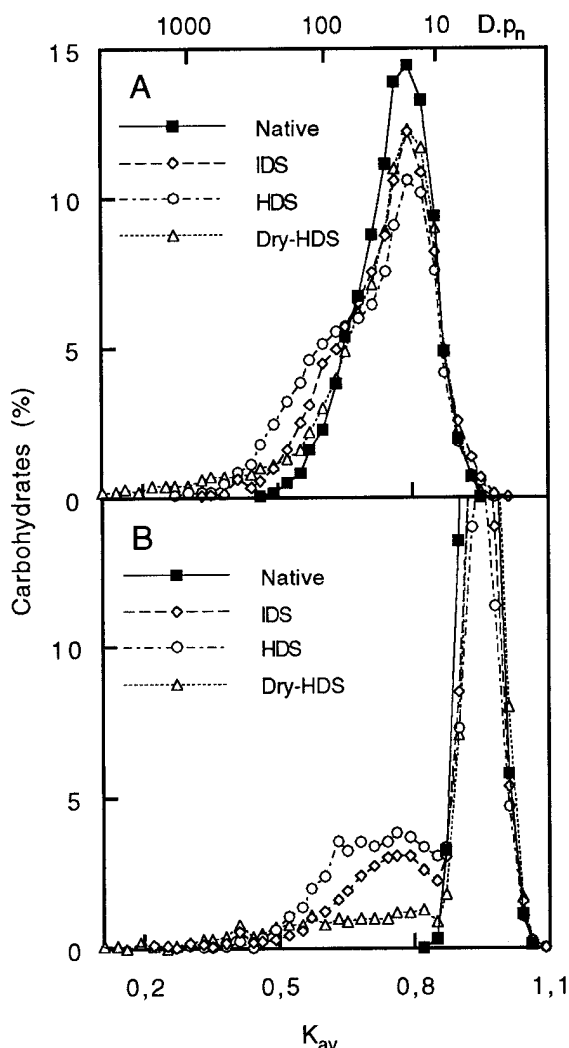
Samples (0.2 mL) with a carbohydrate concentration of  $\approx 1.6$  mg/mL in  $\approx 0.5$ M KOH were eluted through a column (1  $\times$  80 cm) of Sepharose CL-6B (Pharmacia) with 0.5M KOH at a flow rate of 0.5 mL/min. Collected fractions (0.5 mL) were analyzed with the phenol-H<sub>2</sub>SO<sub>4</sub> reagent (Dubois et al 1956). The column was calibrated with dextrans of known average degree of polymerization (dp) as described by Bertoft and Spof (1989). The dp of the samples was calculated as  $\sum c_i / \sum (c_i dp_i)$ , in which  $c_i$  is the carbohydrate concentration and  $dp_i$  is the dp of fraction  $i$ .

### High-Performance Anion-Exchange Chromatography (HPAEC)

Ion-exchange chromatography was performed on a CarboPac PA-100 anion exchange column (250  $\times$  4 mm) in combination with a CarboPac PA-100 guard column using a Dionex HPLC system (series 4500i, Dionex) with pulsed amperometric detection (PAD) as described by Koch et al (1998), except for a small modification of the gradient. The sample (25  $\mu\text{L}$ , 0.36–1.7 mg/mL) was applied in 75% eluent A (150 mM NaOH) and 25% eluent B (150 mM NaOH containing 500 mM NaOAc) and then eluted with a linear gradient: 0–1 min a gradient of eluent B from 25–34%; 1–6 min from 34–45%; 6–55.4 min from 45–67%; and 55.4–80.4 min from 67–90%. The column was qualitatively calibrated for linear dextrans with glucose, maltose, a series of malto-oligosaccharides from maltotriose to maltoheptaose (Boehringer-Mannheim), and with debranched waxy maize starch.

### Scanning Electron Microscopy (SEM)

Intact or residual starch granules were mounted on a specimen holder with carbon cement and coated with gold. The samples were then examined by a Cambridge S360 scanning electron microscope (Cambridge Instruments, UK).



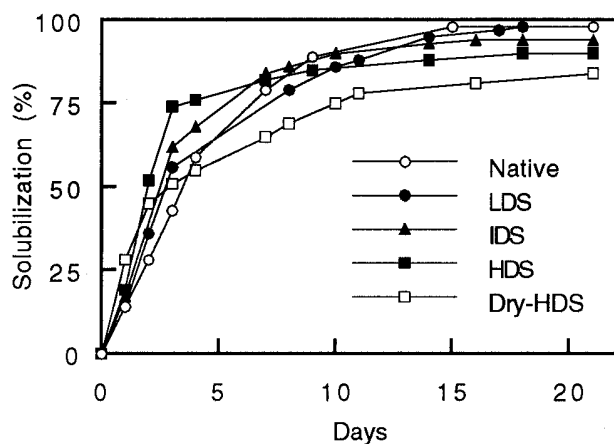
**Fig. 1.** Fractionation on Sepharose CL 6B of native and cationized waxy maize starch after (A) isoamylolysis and (B) successive  $\beta$ -amylolysis. Low (LDS), intermediate (IDS), and high degree of substitution (HDS).

## RESULTS AND DISCUSSION

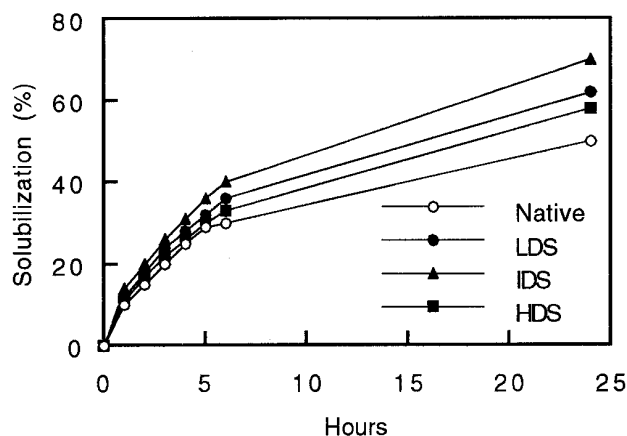
### Characterization of Cationized Samples

The waxy maize starch was wet-cationized into three levels of substitution. The samples had a low (LDS), an intermediate (IDS), and a high degree of substitution (HDS), with DS values of 0.003, 0.022, and 0.046, respectively. The latter sample represented a DS commonly used in commercial applications. The dry-cationized sample that was prepared contained many granules that had partly gelatinized and aggregated. These aggregates were removed from the preparation by fractionation through two sieves with pore sizes of 0.50 and 0.15 mm, respectively, giving three size fractions. When analyzed for nitrogen content, the cationic substituents showed uneven distribution in the sample. Thus, the intermediate size fraction (particles 0.15–0.50 mm) possessed a DS value of 0.104, whereas the large (>0.50 mm) and small (<0.15 mm) size fractions had DS values of 0.045 and 0.046, respectively. The small size fraction represented 68% of the total weight of the sample and was designated Dry-HDS. This sample was used for further studies because it contained individual and apparently intact granules (examined in a light microscope) with a DS comparable to the wet-cationized HDS sample. It should be noted, though, that the composition of the Dry-HDS sample not necessarily was representative of the whole batch of dry-cationized starch.

The components of the native and cationized starches were characterized by hydrolysis with isoamylase and a successive treatment with  $\beta$ -amylase. The unit chain distribution profile analyzed



**Fig. 2.** Acidic hydrolysis of native and cationized waxy maize starch granules in 2.2M HCl at 35°C. Low (LDS), intermediate (IDS), and high degree of substitution (HDS).



**Fig. 3.** Enzymatic hydrolysis of native and cationized waxy maize starch granules with the  $\alpha$ -amylase of *Bacillus amyloliquefaciens*. Low (LDS), intermediate (IDS), and high degree of substitution (HDS).

on Sepharose CL 6B of the completely debranched native starch is shown in Fig. 1A. After  $\beta$ -amylolysis, only the peak for maltose was obtained (Fig. 1B). The low cationized sample (LDS) had a very similar profile after the isoamylolysis (not shown), whereas the IDS and HDS samples possessed increased amounts of dextrans with dp up to  $\approx$ 1,000 that were resistant to the enzyme. This resulted in increased dp values in the isoamylolysis mixtures of 18.7–22.9 (Table I). As a result of the incomplete debranching, the molar amount of dextrans formed in the mixtures decreased from 5.3 to 4.4 with increasing DS. From the difference between the native and the cationized samples, the relative amount of resistant branches was estimated as [(moles of dextrans formed from the native sample) – (moles of dextrans formed from the cationized sample)]/(moles of dextrans formed from the native sample)  $\times$  100. As shown in Table I,  $\approx$ 6% of the branches were resistant to isoamylolysis in the IDS sample and the HDS sample possessed 17% resistant branches. In accordance with other investigations on modified starches (Hood and Mercier 1978, Kavitha and BeMiller 1998), this suggested that the waxy maize amylopectin was substituted close to the branch points, thereby blocking the action of the isoamylase.

The Dry-HDS sample was clearly different from the corresponding wet-cationized HDS sample, despite the similar substitution level. The Dry-HDS starch possessed only  $\approx$ 8% resistant branches and the dp was 20.4 (Table I). In the gel-permeation chromatogram, a small amount of large dextrans with dp > 1,000 was obtained (Fig. 1A).

**TABLE I**  
Characterization of Components in Debranched Native and Modified Waxy Maize Starches

| Parameter                                  | Native | Wet-Cationized <sup>a</sup> |      |      | Dry HDS |
|--|--------|-----------------------------|------|------|---------|
|  |        | LDS                         | IDS  | HDS  |         |
| After isoamylolysis                        |        |                             |      |      |         |
| dp <sup>b</sup>                            | 18.7   | 18.7                        | 19.9 | 22.9 | 20.4    |
| Dextrans (mole) <sup>c</sup>               | 5.3    | 5.3                         | 5.0  | 4.4  | 4.9     |
| Resistant branches (mole%) <sup>d</sup>    | ...    | 0                           | 6    | 17   | 8       |
| Successive $\beta$ -amylolysis             |        |                             |      |      |         |
| $\beta$ -Amylolysis limit (%) <sup>e</sup> | 100    | 94                          | 74   | 59   | 83      |
| Resistant dextrans <sup>f</sup>            |        |                             |      |      |         |
| Weight %                                   | ...    | 6                           | 26   | 41   | 17      |
| dp   | ...    | 30.0                        | 32.5 | 31.5 | 42.5    |
| Mole <sup>c</sup>                          | ...    | 0.2                         | 0.8  | 1.3  | 0.4     |
| Mole % <sup>g</sup>                        | ...    | 4                           | 16   | 30   | 8       |

<sup>a</sup> Low (LDS), intermediate (IDS), and high degree of substitution (HDS).

<sup>b</sup> Degree of polymerization.

<sup>c</sup> Relative molar amounts calculated as weight %/dp.

<sup>d</sup> Estimated from difference in molar amounts of dextrans after debranching of cationized starch and native starch.

<sup>e</sup> Amount of maltose produced estimated from Fig. 1B.

<sup>f</sup>  $\beta$ -Amylase resistant dextrans with dp > 10.

<sup>g</sup> Proportion of  $\beta$ -amylase resistant dextrans with dp > 10 in isoamylolyzates.

**TABLE II**  
Level of Substitution of Native and Cationized Waxy Maize Starch Granules Before and After Solubilization in Acid or with  $\alpha$ -Amylase

| Sample <sup>a</sup> | Solubilized Carbohydrates (%) | N (wt%)         | Relative Content Substituents (%) |
|---------------------|-------------------------------|-----------------|-----------------------------------|
| Native starch       | 0                             | 0.026           | ...                               |
| LL                  | 29                            | na <sup>b</sup> | ...                               |
| HL                  | 78                            | na              | ...                               |
| Dry-cationized HDS  | 0                             | 0.423           | 100                               |
| LL                  | 31                            | 0.071           | 17                                |
| HL                  | 84                            | 0.096           | 23                                |
| Wet-cationized HDS  | 0                             | 0.420           | 100                               |
| LL                  | 32                            | 0.274           | 65                                |
| HL                  | 82                            | 0.166           | 40                                |
| Enzymatically       | 40                            | 0.356           | 85                                |

<sup>a</sup> Solubilized to low and high lintnerization level (LL and HL, respectively). High degree of substitution (HDS).

<sup>b</sup> Not analyzed.

A conversion of the linear chains into maltose by  $\beta$ -amylase is often used to show that the debranching is complete. When the wet-cationized LDS sample was treated with  $\beta$ -amylase, the hydrolysis limit was 94% despite the apparent absence of resistant branches (Table I). Thus it appeared that the sample contained substituted chains that were resistant to  $\beta$ -amylolysis. The dp range of these chains was 10–100 (not shown) with an average dp of 30, suggesting that the substitution was found far from the reducing end side of the chains. On a molar level, the resistant chains represented  $\approx$ 4% of the debranched waxy maize amylopectin. Because 0.3% of the glucosyl residues in the sample were substituted (DS = 0.003), it could be estimated that each modified chain contained  $\approx$ 1.4 cationic groups on the average  $[(100 \times \text{dp} \times \text{DS})/(\text{mole}\% \text{ of resistant chains})]$ .

The samples with higher DS values possessed more dextrans that were resistant to  $\beta$ -amylolysis (Fig. 1B) and, thus, lower  $\beta$ -limit values (Table I). In the HDS sample, 41% of the sample by weight was resistant to hydrolysis. This represented 30% on a molar level of the dextrans in the isoamylolysis mixture. The average dp of the resistant dextrans was, however, similar in all the wet-cationized samples regardless of the DS-values. This suggested that the locations of the substituents within the dextrans were similar in all three samples. The Dry-HDS sample possessed much lower amounts of dextrans resistant to the attack of  $\beta$ -amylase. The size-distribution of these dextrans was broad, however, and the average dp was higher (42.5) than in the wet-cationized samples (30.0–32.5). This result suggested that the Dry-HDS sample contained fewer cationized starch molecules, but a higher density of substituents in the modified polymers.

#### Solubilization of Starch Granules

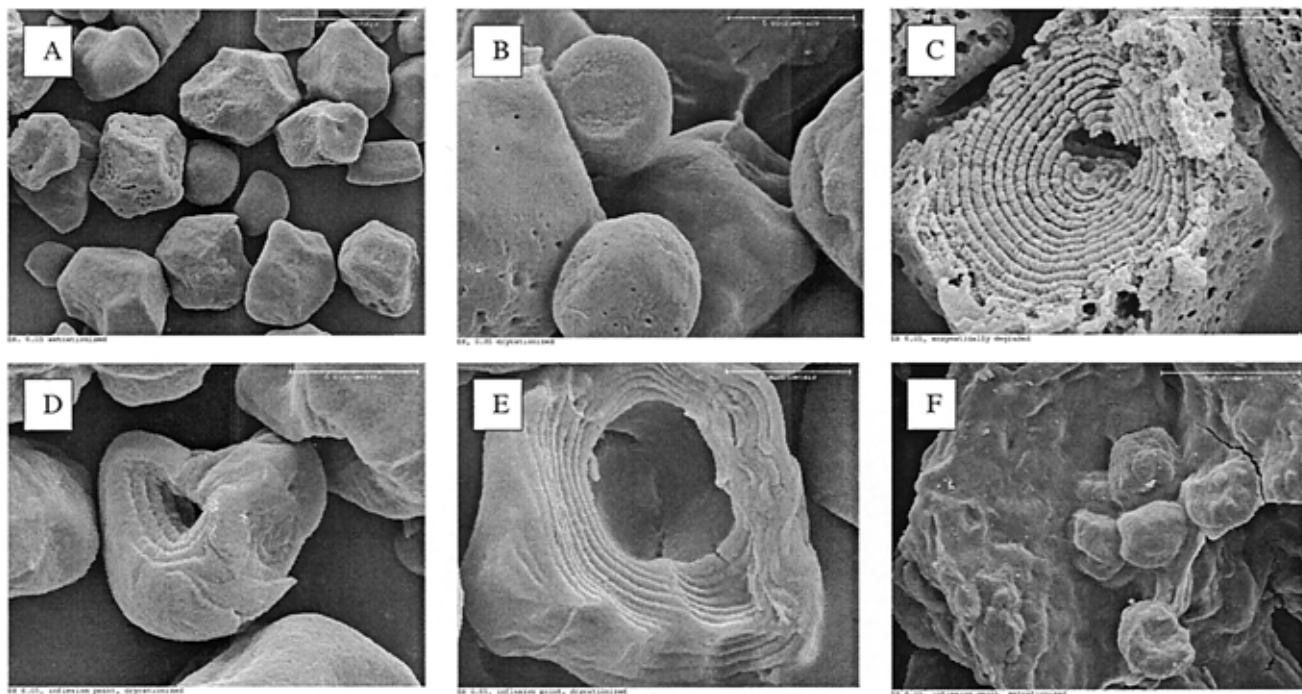
When the native starch granules were treated in 2.2M HCl (lintnerization), the solubilization was fast initially (Fig. 2). During this phase, the amorphous parts of the granules are hydrolyzed (Robin et al 1974, Jenkins and Donald 1997). After four days, when 59% of the starch had been solubilized, the rate decreased, and after 15 days almost all (98%) of the starch granules had dissolved. This

high value was possibly partly due to small crystalline remnants of the granules that remained in the supernatant after centrifugation, thus causing an overestimation of the solubilized carbohydrates. Overall, however, the lintnerization proceeded by a pattern similar to that described earlier in several reports (Biliaderis et al 1981, Colonna et al 1988, Jacobs et al 1998).

Both the rate and the level of the initial stage of the lintnerization increased with increasing DS in the series of the wet-cationized starches (Fig. 2). Thus, it appeared that the cationic substituents had decreased the amount of crystallinity inside the granules, or alternatively, made the amorphous parts more susceptible to the acid. The rate of the second stage, however, decreased with increasing DS and the final amount of acid resistant material increased to 10% in the HDS sample. The changes in the granules that promoted this resistance remained unclear. The Dry-HDS sample was lintnerized at a high rate during the first two days, after which the rate became slow and the starch then possessed the highest resistance to acid. At day 21, only 84% of the granules of the Dry-HDS starch had become solubilized.

The granular residues were collected after a low lintnerization (LL) and a high lintnerization (HL) level at which  $\approx$ 30 and 80% of the granules had been solubilized, respectively. The nitrogen content of the Dry-HDS residues had decreased to only 17% of the original level in the LL sample (Table II). This showed that the major part of the cationic groups were found in the most acid-labile areas. The relative nitrogen content then remained at this low level during the later stages of the lintnerization. In the wet-cationized HDS sample, the substitution level decreased more slowly. At the LL level, the relative nitrogen content was 65% of the original value and when 82% of the starch had solubilized, the nitrogen content had decreased to 40%. Because most (if not all) of the residues that remained at the HL level were crystalline, a substantial part of the cationic substituents was found within the crystalline starch.

The wet-cationized granules were also solubilized enzymatically with the  $\alpha$ -amylase of *B. amyloliquefaciens*. Again, the modification affected the rate of the hydrolysis. This time, however, the

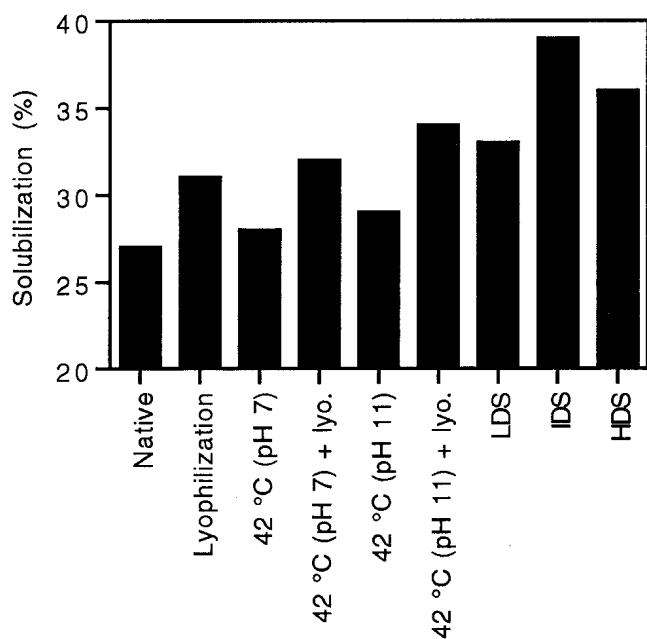


**Fig. 4.** Scanning electron micrographs of cationized waxy maize starch granules. Low (LDS), intermediate (IDS), and high degree of substitution (HDS). **A**, Wet-cationized HDS granules (bar 20  $\mu\text{m}$ ); **B**, dry-cationized HDS granules (bar 5  $\mu\text{m}$ ); **C**, wet-cationized HDS granules after solubilization to 40% with  $\alpha$ -amylase (bar 5  $\mu\text{m}$ ); **D and E**, dry-cationized HDS granules at a low level of lintnerization (bar 5  $\mu\text{m}$ ); **F**, wet-cationized HDS granules at a high level of lintnerization (bar 20  $\mu\text{m}$ ).

solubilization of the HDS sample was slower than that of the LDS sample, whereas the IDS sample was most hydrolyzed (Fig. 3). Cationization increases the swelling power of starch granules (Kweon et al 1997) and therefore, probably, a moderate modification of the starch granules made them more susceptible to the enzymatic attack, whereas higher DS interfered with the enzyme-substrate complex formation or with the adsorption of the enzyme to the granular starch. Thus, in terms of biodegradability, there existed an optimal DS of the starch granules. Kweon et al (1997) reported an increased rate of hydrolysis with the  $\alpha$ -amylase from porcine pancreas of several cationized starches (DS 0.030–0.035), except for waxy maize starch granules. In our investigation, in which we used the  $\alpha$ -amylase of *B. amyloliquefaciens*, all cationized waxy maize starches were better attacked than the native granules.

A batch of  $\alpha$ -amylase treated granular residues of the wet-cationized HDS starch was collected at 40% solubilization. In this sample, the nitrogen content was still 85% of the original level. The enzymatic attack thus proceeded through the granules regardless of the presence of the cationic groups. It is known that the dextrans from waxy maize starch granules dissolved by the  $\alpha$ -amylase have a high molecular weight (Bertoft and Manelius 1992). The substituted parts of the granules could therefore be dissolved without it being necessary for the enzyme to attack close to the cationic groups, which therefore would not interfere with the hydrolysis. It was also shown that the relative crystallinity of wheat starch granules remains at almost the same level after  $\alpha$ -amylase attack (Colonna et al 1988), and thus both crystalline and amorphous parts are solubilized with this method.

When the wet-cationized starch granules were examined by scanning electron microscopy (Fig. 4) their visual appearance was identical to the native granules (not shown). In the dry-cationized sample, the granules were partly aggregated through glue-like bridges on their surface as a result of the addition of hydroxide. Probably, these parts represented highly cationized areas. The enzymatic degradation resulted in the typical erosion through preexisting channels or holes on the surface of the granules (Huber and BeMiller 1997) resulting in porous starch with blotted "growth rings" and channels as previously described for native waxy maize starch



**Fig. 5.** Enzymatic hydrolysis of native waxy maize starch granules after different pretreatments in neutral or alkaline conditions and drying in either acetone or by lyophilization. Low (LDS), intermediate (IDS), and high degree of substitution (HDS). Series of wet-cationized starch with increasing DS was dried in acetone.

(Gallant et al 1972, Gallant et al 1973, Manelius and Bertoft 1996, Franco et al 1998). The lintnerization resulted in shrunken granules, several with a single large hole blotting the growth rings and an empty inner space. At later lintnerization stages, the granules seemed melted together into larger aggregates. There was, however, no visual difference in the lintnerization patterns of the native and cationized starches.

It was claimed that the alkaline treatment of potato starch granules in the production of hydroxyethyl derivatives was a major cause for their higher susceptibility to enzymatic attack, whereas the introduction of the hydroxyethyl substituents further enhanced the degradation (Perera and Hoover 1998). Because the wet-cationization also involved an alkaline treatment of the waxy maize starch granules, we tested whether the conditions in the cationization process could increase the susceptibility to  $\alpha$ -amylase. Native starch granules were treated at pH 11 and 42°C for 20 hr before the addition of  $\alpha$ -amylase. Only a slight increase in the solubilization (27–29%) was obtained in a 5-hr incubation period (Fig. 5). The same result was obtained with a pretreatment of the native granules in water (pH 7) and 42°C (thus resembling light annealing conditions). These granules were dried in ethanol and acetone. However, if the granules were dried by lyophilization, as in the work of Perera and Hoover (1998), the granules became clearly more susceptible to the enzymatic attack, with or without the different pretreatments. When examined in a light microscope,

**TABLE III**  
Characterization of Debranched Native and Wet-Cationized Waxy Maize Starches After Solubilization with  $\alpha$ -Amylase

| Parameter                                  | Native | Wet-Cationized <sup>a</sup> |      |      |
|--|--------|-----------------------------|------|------|
|  |        | LDS                         | IDS  | HDS  |
| Degree of solubilization (%)               | 38     | 37                          | 37   | 40   |
| dp   | 12.1   | 13.0                        | 14.6 | 18.5 |
| Dextrins (mole) <sup>b</sup>               | 8.3    | 7.7                         | 6.8  | 5.4  |
| $\beta$ -Amylolysis limit (%) <sup>c</sup> | 100    | 92                          | 74   | 60   |
| Resistant dextrins <sup>d</sup>            |        |                             |      |      |
| Weight %                                   | ...    | 8                           | 26   | 40   |
| dp   | ...    | 11.4                        | 16.3 | 21.1 |
| Mole <sup>b</sup>                          | ...    | 0.7                         | 1.6  | 1.9  |
| Mole % <sup>e</sup>                        | ...    | 9                           | 24   | 35   |

<sup>a</sup> Low (LDS), intermediate (IDS), and high degree of substitution (HDS).

<sup>b</sup> Relative molar amounts calculated as weight %/dp.

<sup>c</sup> Amount of maltose produced.

<sup>d</sup>  $\beta$ -Amylase resistant dextrins with dp > 10.

<sup>e</sup> Proportion of  $\beta$ -amylase resistant dextrins with dp > 10 in isoamylolyzates.

**TABLE IV**  
Characterization of Native and Modified Waxy Maize Starches at Low Lintnerization (LL) and High Lintnerization (HL) Levels

| Parameter                       | Cationized <sup>a</sup> |      |         |      |         |      |
|---------------------------------|-------------------------|------|---------|------|---------|------|
|                                 | Native                  |      | Wet-HDS |      | Dry-HDS |      |
|                                 | LL                      | HL   | LL      | HL   | LL      | HL   |
| Before isoamylolysis            |                         |      |         |      |         |      |
| dp                              | 44.8                    | 21.9 | 53.4    | 23.3 | 96.8    | 19.3 |
| After isoamylolysis             |                         |      |         |      |         |      |
| dp                              | 14.9                    | 13.9 | 17.0    | 15.1 | 16.9    | 13.8 |
| Dextrins (mole) <sup>b</sup>    | 6.7                     | 7.2  | 5.9     | 6.6  | 5.9     | 7.2  |
| Successive $\beta$ -amylolysis  |                         |      |         |      |         |      |
| Limit (%) <sup>c</sup>          | 97                      | 97   | 76      | 87   | 94      | 98   |
| Resistant dextrins <sup>d</sup> |                         |      |         |      |         |      |
| Weight %                        | 3                       | 3    | 24      | 13   | 6       | 2    |
| dp                              | 15.0                    | 15.0 | 24.0    | 18.6 | 20.0    | 20.0 |
| Mole <sup>b</sup>               | 0.2                     | 0.2  | 1.0     | 0.7  | 0.3     | 0.1  |
| Mole % <sup>e</sup>             | 3                       | 3    | 17      | 11   | 5       | 1    |

<sup>a</sup> Wet- and dry-cationized samples with high degree of substitution (HDS).

<sup>b</sup> Relative molar amounts calculated as weight %/dp.

<sup>c</sup> Amount of maltose produced.

<sup>d</sup>  $\beta$ -Amylase resistant dextrins with dp > 10.

<sup>e</sup> Proportion of  $\beta$ -amylase resistant dextrins with dp > 10 in isoamylolyzates.

the lyophilized granules possessed large interior fissures that probably exposed the granules for the enzyme and resulted in a higher hydrolysis rate. We therefore believe that the higher hydrolysis rate of the modified starch granules, that had been dried in ethanol and acetone, was mostly a true effect of the cationization rather than a secondary result of the conditions in the cationization process.

### Characterization of Amylase-Treated Starch

The components of the native and wet-cationized starch granule residues remaining after a solubilization to  $\approx 40\%$  with  $\alpha$ -amylase were characterized by debranching with isoamylase and gel-permeation chromatography. The native starch was completely debranched as shown by the complete hydrolysis into maltose by a successive  $\beta$ -amylolysis (Table III). The dp of the debranched starch had decreased from 18.7 to 12.1 after the  $\alpha$ -amylolysis, showing that a major part of the amylopectin had been attacked by the enzyme at this stage of the solubilization.

The series of cationized starches in Table III possessed the same resistance to  $\beta$ -amylolysis as before the  $\alpha$ -amylase treatment (Table I), though the dp of the dextrans in the isoamylolysis mixtures was lower. This suggested that the mode of attack performed by the  $\alpha$ -amylase was not affected by the cationized substitutions. Interestingly however, the dp of the  $\beta$ -amylase resistant dextrans, which before the solubilization had comparatively high dp of  $\approx 31$  regardless the DS, now possessed much lower dp. In addition, the dp increased with the DS of the starch (11.4–21.1). On a molar level, the proportion of  $\beta$ -amylase resistant dextrans increased in the isoamylolysis mixtures after the solubilization with  $\alpha$ -amylase despite the slight reduction of the cationic substituents. This was probably an effect of the pronounced reduction in molecular size. Thus, the  $\alpha$ -amylase had been able to attack the modified parts inside the granules between cationized substituents, thereby reducing the average size.

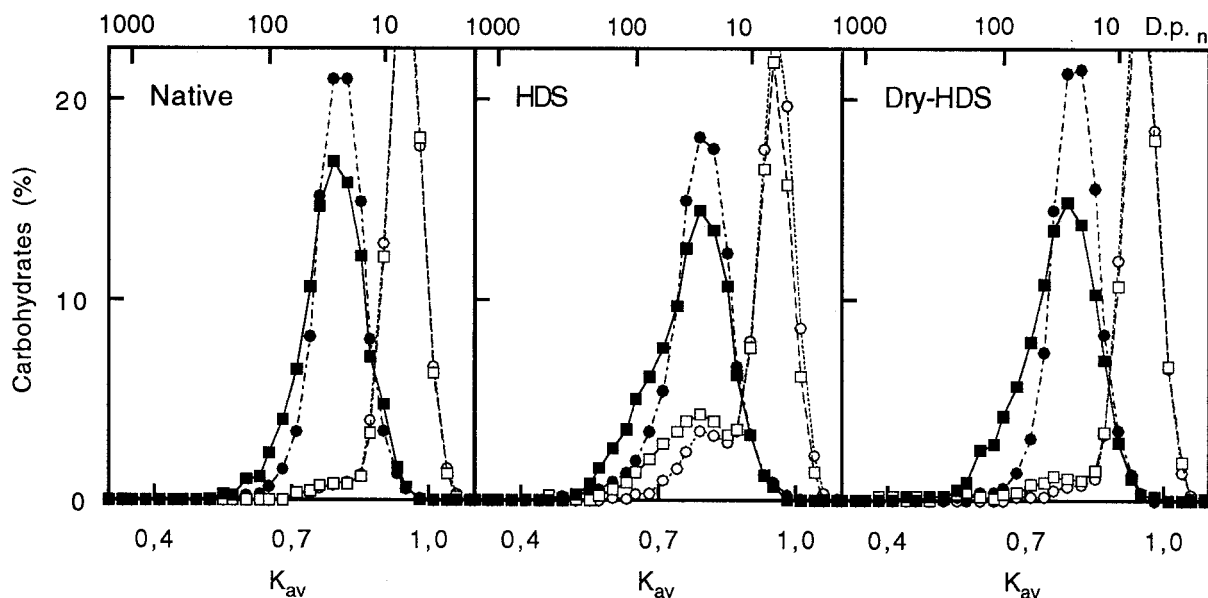
### Characterization of Lintnerized Starches

The starch residues collected at the low lintnerization (LL) level were already extensively depolymerized. The residues in the native starch had a dp of 44.8 and in the wet-cationized HDS sample, the dp was slightly higher (Table IV). The Dry-HDS possessed the highest dp (96.8), but at the high lintnerization (HL) level, at which the size-reduction had continued, the dp was even slightly lower than in the other samples.

The debranching of the lintnerized native starch (Fig. 6) was only close to complete as shown by the  $\beta$ -amylolysis limit of 97% (Table IV). This showed that some very short glucosyl branches (Umeki and Kainuma 1981), which are resistant to attack by isoamylase (Kainuma et al 1978), remained after the acidic hydrolysis. On a molar level, these short-branched dextrans represented  $\approx 3\%$  of the mixtures. The dp of the debranched material was  $\approx 14$ , which corresponded to the results obtained by Kitahara et al (1997) and others and reflected the length of the crystalline lamellae inside the granules. If assuming complete debranching, the average number of chains in the remaining dextrans could be expressed as (dp before isoamylolysis)/(dp after isoamylolysis). At the LL level, the number of chains was  $\approx 3$  and at the HL level it was reduced to 1.5, which showed that, on average, every second dextrin was linear.

After isoamylolysis, the HDS sample possessed dextrans of decreasing dp with increasing levels of lintnerization (Fig. 6, Table IV). The  $\beta$ -amylolysis limit increased from 59% before lintnerization (Table I) to 87% at the HL level (Table IV), which corresponded with the decreased content of cationic groups. As after the  $\alpha$ -amylolysis, the dp of the  $\beta$ -amylase resistant dextrans decreased to 18.6 at the HL level. However, the amount of the resistant dextrans also decreased. At the HL level, they represented only 13% by weight, or 11% by mole in the mixture. The results thus suggested that when the acid had hydrolyzed the amorphous parts (growth rings and lamellae within semicrystalline growth rings), a major part of the cationic substituents were lost and the remaining extensively depolymerized material, representing the crystalline lamellae, contained the residual cationic groups in the granule. The relative density of the substituents in the remaining material was  $\approx 40\%$  of the original (Table II), which suggested a DS of 0.018 (originally 0.046) in the crystalline starch. Obviously, the relative density in the amorphous parts of the granules was higher than the average DS of 0.046.

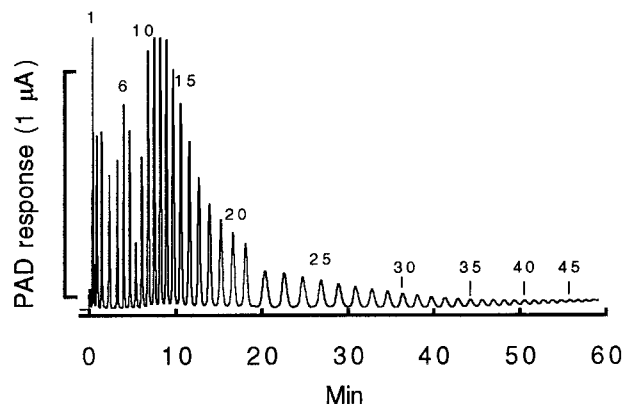
The Dry-HDS sample was very different from the wet-cationized starch. Though the composition of the dextrans in the isoamylolysis mixture at the LL level was similar to the HDS sample, the content of  $\beta$ -amylase resistant dextrans was low (Fig. 6, Table IV). At the HL level the Dry-HDS was similar to the native sample with only traces of resistant material. This was in agreement with the low nitrogen content and suggested again that the starch was preferentially modified at easily accessible sites. In addition to the outer granule surfaces, such sites could also include the channels



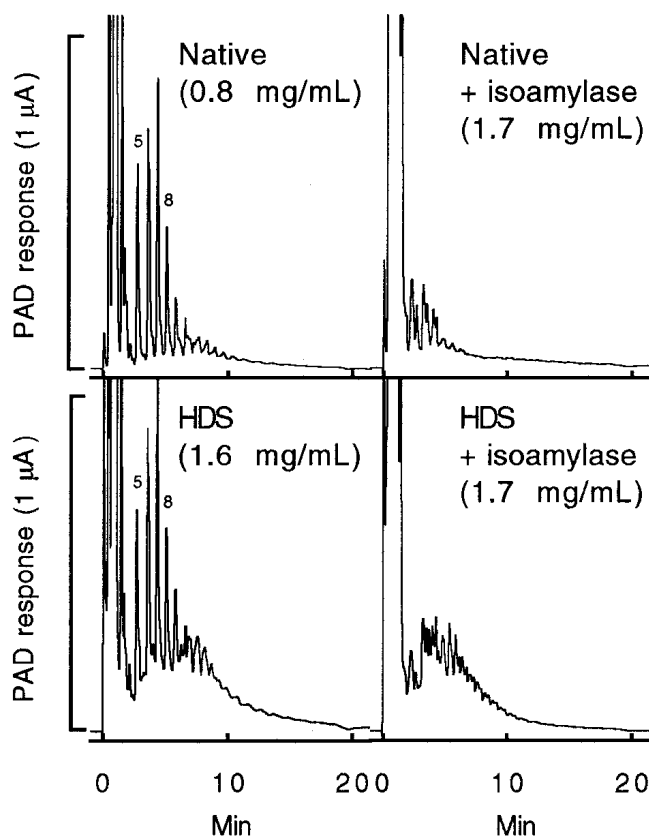
**Fig. 6.** Fractionation on Sepharose CL 6B of lintnerized native, wet-, and dry-cationized starch after isoamylolysis (black symbols) and successive  $\beta$ -amylolysis (white symbols). Samples were taken at a low (squares) and high (circles) levels of lintnerization. High degree of substitution (HDS).

that connect a central cavity in maize granules to the external environment (Huber and BeMiller 1997).

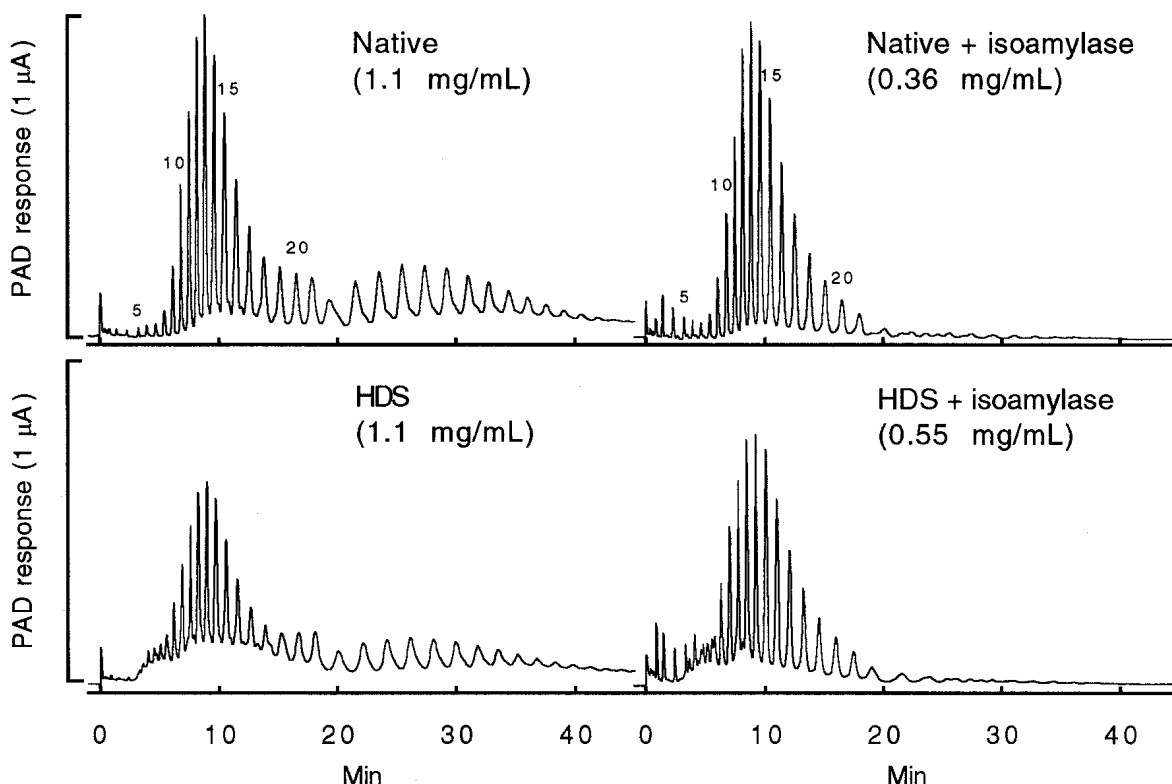
The highly lintnerized native and wet-cationized HDS samples were also analyzed in more detail with high-performance anion-exchange chromatography (HPAEC). With the gradient used, a baseline separation of debranched native waxy maize starch was obtained. A standard chromatogram for the qualitative identification of linear chains is shown in Fig. 7, in which the debranched starch was analyzed together with an added series of glucose to maltoheptaose (dp 1–7). The lintnerized native starch possessed two groups of dextrans (Fig. 8). The major group had a peak corresponding to linear chains of dp 13, whereas the positions of the peaks in the other group were intermediate to linear chains of dp 22–35. After debranching with isoamylase this group of dextrans



**Fig. 7.** Chain length distribution profile with high-performance anion-exchange chromatography of debranched waxy maize starch to which a series of glucose-maltoheptaose was added. Numbers indicate degree of polymerization. Pulsed amperometric detection (PAD).



**Fig. 9.** Distribution profiles with high-performance anion-exchange chromatography of  $\beta$ -amylolysates of lintnerized starches and of debranched lintnerized starches. Concentration of sample applied to ion-exchange column in parentheses. Numbers indicate degree of polymerization. Pulsed amperometric detection (PAD); high degree of substitution (HDS).



**Fig. 8.** Distribution profiles with high-performance anion-exchange chromatography of native and wet-cationized starch at a high lintnerization level before and after isoamylolysis. Concentration of sample applied to ion-exchange column in parentheses; numbers indicate degree of polymerization. Pulsed amperometric detection (PAD); high degree of substitution (HDS).

disappeared, confirming that they were branched in nature (Umeki and Kainuma 1981, Jacobs et al 1998). The debranched sample possessed only the group of chains with  $\approx$ dp 13, together with very small amounts of chains with dp 2–7. Overall, the lintnerized sample was very similar to the corresponding Naegeli amyloextrins of waxy maize starch obtained by hydrolysis in sulfuric acid as described by Kitahara et al (1997) and by Jane et al (1997), who suggested that the remaining singly branched dextrans represent branches that are scattered into the crystalline lamellae in the granules.

The lintnerized native starch was also subjected to a  $\beta$ -amylolysis before and after the debranching (Fig. 9). A main peak for maltose was produced together with larger peaks for glucose and maltotriose. The latter was produced from linear chains with an odd number of D-glucosyl residues, whereas the former probably was produced by fission of maltose by  $\alpha$ -glucosidase, which was present in the  $\beta$ -amylase preparation as a contaminant. Ammeraal et al (1991) showed that branched dextrans of a certain dp are eluted in front of the corresponding linear dextrans. The dp-range of the major  $\beta$ -limit dextrans in the lintnerized starch was accordingly estimated to 5–8. This showed that the position of the branch was found at the reducing end of the dextrans, which agreed with earlier reports (Jane et al 1997, Kitahara et al 1997, Umeki and Kainuma 1981). The dp of the traces of the  $\beta$ -limit dextrans that remained in the debranched sample was  $\approx$ 4–7 (Fig. 9), suggesting that also the glucosyl-branch was at the reducing end.

The major features of the lintnerized wet-cationized HDS sample were similar to the corresponding native starch, but a baseline separation of the peaks in the major group with dp  $\approx$ 13 was not obtained (Fig. 8). This showed that a range of dextrans with cationic substitutions was found within this group. The branched dextrans with dp 22–35 disappeared after isoamylolysis. Thus substitutions close to the branches in this group that would interfere with the enzyme seemed to be absent. The  $\beta$ -amylolysis mixture of the lintnerized HDS starch was very complex, though the size-distribution range of the dextrans was similar to the corresponding native sample (Fig. 9). The debranched sample possessed also a very complex composition of  $\beta$ -amylase resistant dextrans. Compared with the native sample, there was much more substituted material with dp  $\leq$  14. Since the crystalline lamellae in waxy maize starch is 6 nm (Jenkins and Donald 1995), which corresponds to a length of  $\approx$ 17 D-glucosyl residues (Imberty et al 1991), it suggested that the cationic substituents were widely spread into the crystalline lamellae.

## CONCLUSIONS

Cationization of waxy maize starch granules increased the rate of both lintnerization and solubilization of the granules with  $\alpha$ -amylase, though the pattern of the solubilization was not visibly altered. The composition of the starch residues obtained after  $\alpha$ -amylolysis was similar to that of the original starches, showing that the attack could proceed throughout the granules in the presence of cationic groups. However, at a high degree of substitution, the rate of the enzymatic attack decreased.

A major part of the substitutions introduced by the wet-cationization method were found in the amorphous areas inside the granules and close to the branches of the amylopectin, which rendered the (1 $\rightarrow$ 6)-linkages resistant to the attack by isoamylase. The  $\beta$ -amylolysis limit decreased after cationization, which showed that substitutions also were found at external chains. A substantial part of the substituents remained inside the granules after lintnerization and therefore the cationic groups were also spread into the crystalline lamellae. In the dry cationization process, the starch was, to a major part, substituted at the surface and along the channels of the granules, though some of the substitutions also remained after lintnerization.

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## LITERATURE CITED

- Ammeraal, R. N., Delgado, G. A., Tenbarger, F. L., and Friedman, R. B. 1991. High-performance anion-exchange chromatography with pulsed amperometric detection of linear and branched glucose oligosaccharides. *Carbohydr. Res.* 215:179-192.
- Bertoft, E., and Spoof, L. 1989. Fractional precipitation of amylopectin alpha-dextrans using methanol. *Carbohydr. Res.* 189:169-180.
- Bertoft, E., and Manelius, R. 1992. A method for the study of the enzymic hydrolysis of starch granules. *Carbohydr. Res.* 227:269-283.
- Biliaderis, C. G., Grant, D. R., and Vose, J. R. 1981. Structural characterization of legume starches. II. Studies on acid-treated starches. *Cereal Chem.* 58:502-507.
- Burgt, Y. E. M. V. D., Bergsma, J., Bleeker, I. P., Mijland, P. J. H. C., Hoof, A. V. D. K.-V., Kamerling, J. P., and Vliegthart, J. F. G. 1998. Distribution of methyl substituents over branched and linear regions in methylated starches. *Carbohydr. Res.* 312:201-208.
- Chan, Y.-C., Braun, P. J., French, D., and Robyt, J. F. 1984. Porcine pancreatic  $\alpha$ -amylase hydrolysis of hydroxyethylated amylose and specificity of subsite binding. *Biochemistry* 23:5795-5800.
- Colonna, P., Buléon, A., and Lemarié, F. 1988. Action of *Bacillus subtilis*  $\alpha$ -amylase on native wheat starch. *Biotech. Bioeng.* 31:895-904.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350-356.
- Fischer, S. K., and Piller, F. 1977. New knowledge on degradation of starch by hypochlorite. 6. Enzymatic degradation of oxidized starch. *Starch/Staerke* 29:262-265.
- Fischer, S. K., and Piller, F. 1978. New knowledge on degradation of starch by hypochlorite. 7. Study on the effect of hypochlorite oxidation on amylose by means of degradation by  $\beta$ -amylase and determination of the iodine binding capacity. *Starch/Staerke* 30:4-7.
- Franco, C. M. L., Ciacco, C. F., and Tavares, D. Q. 1998. The structure of waxy corn starch: Effect of granule size. *Starch/Staerke* 50:193-198.
- Gallant, D., Mercier, C., and Guilbot, A. 1972. Electron microscopy of starch granules modified by bacterial  $\alpha$ -amylase. *Cereal Chem.* 49:354-365.
- Gallant, D., Derrien, A., Aumaitre, A., and Guilbot, A. 1973. Dégradation in vitro de l' amidon par le suc pancréatique. *Starch/Staerke* 25:56-64.
- Hellwig, G., Bischoff, D., and Rubo, A. 1992. Production of cationic starch ethers using an improved dry process. *Starch/Staerke* 44:69-74.
- Hood, L. F., and Mercier, C. 1978. Molecular structure of unmodified and chemically modified manioc starches. *Carbohydr. Res.* 61:53-66.
- Huber, K. C., and BeMiller, J. N. 1997. Visualization of channels and cavities of corn and sorghum starch granules. *Cereal Chem.* 74:537-541.
- Imberty, A., Buléon, A., Tran, V., and Pérez, S. 1991. Recent advances in knowledge of starch structure. *Starch/Staerke* 43:375-384.
- Jacobs, H., Eerlingen, R. C., Rouseu, N., Colonna, P., and Delcour, J. A. 1998. Acid hydrolysis of native and annealed wheat, potato and pea starches—DSC melting features and chain length distributions of lintnerised starches. *Carbohydr. Res.* 308:359-371.
- Jane, J.-L., Xu, A., Radosavljevic, M., and Seib, P. A. 1992. Location of amylose in normal starch granules. I. Susceptibility of amylose and amylopectin to cross-linking reagents. *Cereal Chem.* 69:405-409.
- Jane, J.-L., Wong, K.-S., and McPherson, A. E. 1997. Branch-structure difference in starches of A- and B-type X-ray patterns revealed by their Naegeli dextrans. *Carbohydr. Res.* 300:219-227.
- Jenkins, P. J., and Donald, A. M. 1995. The influence of amylose on starch granule structure. *Int. J. Biol. Macromol.* 17:315-321.
- Jenkins, P. J., and Donald, A. M. 1997. The effect of acid hydrolysis on native starch granule structure. *Starch/Staerke* 49:262-267.
- Kainuma, K., Kobayashi, S., and Harada, T. 1978. Action of *Pseudomonas* isoamylase on various branched oligo- and polysaccharides. *Carbohydr. Res.* 61:345-357.
- Kavitha, R., and BeMiller, J. N. 1998. Characterization of hydroxypropylated potato starch. *Carbohydr. Polym.* 37:115-121.
- Khalil, M. I., and Farag, S. 1998. Preparation of some cationic starches using the dry process. *Starch/Staerke* 50:267-271.
- Kitahara, K., Eitoku, E., Sukanuma, T., and Nagahama, T. 1997. Some

- properties of branched and linear dextrans from Nägeli amylopectin. *Carbohydr. Polym.* 33:187-194.
- Koch, K., Andersson, R., and Åman, P. 1998. Quantitative analysis of amylopectin unit chains by means of high-performance anion-exchange chromatography with pulsed amperometric detection. *J. Chromatogr. A* 800:199-206.
- Kweon, M. R., Bhirud, P. R., and Sosulski, F. W. 1996. An aqueous alcoholic-alkaline process for cationization of corn and pea starches. *Starch/Staerke* 48:214-220.
- Kweon, M. R., Sosulski, F. W., and Han, H. S. 1997. Effect of aqueous ethanol cationization on functional properties of normal and waxy starches. *Starch/Staerke* 49:202-207.
- Manelius, R., and Bertoft, E. 1996. The effect of  $\text{Ca}^{2+}$ -ions on the  $\alpha$ -amylolysis of granular starches from oats and waxy-maize. *J. Cereal Sci.* 24:139-150.
- Perera, C., and Hoover, H. 1998. The reactivity of porcine pancreatic alpha-amylase towards native, defatted and heat-moisture treated potato starches before and after hydroxypropylation. *Starch/Staerke* 50:206-213.
- Robin, J. P., Mercier, C., Charbonnière, R., and Guilbot, A. 1974. Lintnerized starches. Gel filtration and enzymatic studies of insoluble residues from prolonged acid treatment of potato starch. *Cereal Chem.* 51:389-406.
- Steeneken, P. A. M., and Woortman, A. J. J. 1994. Substitution patterns in methylated starch as studied by enzymic degradation. *Carbohydr. Res.* 258:207-221.
- Torneport, L. J., Salomonsson, B. A.-C., and Theander, O. 1990. Chemical characterization of bromine oxidized potato starch. *Starch/Staerke* 42:413-417.
- Umeki, K., and Kainuma, K. 1981. Fine structure of nägeli amylopectin obtained by acid treatment of defatted waxy-maize starch—Structural evidence to support the double-helix hypothesis. *Carbohydr. Res.* 96:143-159.
- Wilke, O., and Mischnick, P. 1995. Analysis of cationic starches: Determination of the substitution pattern of *O*-(2-hydroxy-3-trimethylammonium)propyl ethers. *Carbohydr. Res.* 275:309-318.
- Wilke, O., and Mischnick, P. 1997. Determination of the substitution pattern of cationic starch ethers. *Starch/Staerke* 49:453-458.
- Zhu, Q., and Bertoft, E. 1997. Enzymic analysis of the structure of oxidized potato starches. *Int. J. Biol. Macromol.* 21:131-135.
- Zhu, Q., Sjöholm, R., Nurmi, K., and Bertoft, E. 1998. Structural characterization of oxidized potato starch. *Carbohydr. Res.* 309:213-218.

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