

α -Amylolysis of Large Barley Starch Granules

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ABSTRACT

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The α -amylolysis of large (volume average 16 μm) barley starch granules was studied by measuring the amount of carbohydrates solubilizing during hydrolysis, and the changes in morphology and molecular structure of the granule residues by scanning electron microscopy, particle-size analysis, size-exclusion chromatography, X-ray diffraction, and differential scanning calorimetry. X-ray diffraction showed that, in the earlier stages of α -amylolysis, both amorphous and crystalline parts of the granules were equally solubilized. More extensive hydrolysis caused a gradual decrease in A-type crystallinity and degradation of the granular

structure. Scanning electron microscopy revealed that hydrolysis proceeded through pinholes, and pitted and partially hollow granule residues were formed. The lipid-complexed amylose was less susceptible to α -amylolysis than free amylose and amylopectin. Lipid-complexed amylose started leaching out of the granule residues only after half of the starch had solubilized due to the α -amylase treatment. Even though scanning electron microscopy indicated that there were intact granules left throughout the hydrolysis, the results obtained suggested that α -amylolysis of large barley starch granules proceeded rather evenly among the granules.

Most of the earlier studies on the hydrolysis of native starch granules concentrated on detecting the changes of the granule surface structure by scanning electron microscopy. Below gelatinization temperatures, large barley starch granules hydrolyze through pinholes from the inside out (MacGregor and Ballance 1980, Bertoft and Kulp 1986). Hydrolysis is rather uneven; while many granules are hydrolyzed extensively, others show only minor damage (MacGregor and Morgan 1986).

Unlike mineral acids, α -amylases can solubilize both amorphous and crystalline regions from starch granules (Leach and Schoch 1961). Solubilization of crystalline regions occurs through disentanglement of crystalline chains when α -amylase attacks the amorphous parts nearby (Colonna et al 1988). No preferential attack on either amylose or amylopectin has been detected (Leach and Schoch 1961, Colonna et al 1988). Amylose in native barley starches, however, occurs in two forms: as lipid-free amylose (FAM) and lipid-complexed amylose (LAM) (Morrison 1988; Morrison et al 1993a,b). Thus, nonwaxy cereal starches consists of three distinct components: 1) highly crystalline regions formed from double-helical amylopectin chains, 2) solid-like regions formed from lipid-complexed amylose, and 3) completely amorphous regions associated with amylopectin branches and possibly the lipid-free amylose (Morgan et al 1995). Lipid-complexed amylose may be enriched near the granule surface (Morrison and Gadan 1987, MacDonald et al 1991).

Lipid-complexed amylose is more resistant to α -amylase than free amylose in solution. Complete digestion of the complex is only obtained when a relatively large excess of enzyme or long conversion times are used (Holm et al 1983, Biliaderis and Galloway 1989, Galloway et al 1989). Hydrolysis conditions as well as structural characteristics of the complexing agent affect the hydrolysis of lipid-complexed amylose (Holm et al 1983, Jane and Robyt 1984, Eliasson and Krog 1985, Biliaderis and Galloway 1989).

Although the hydrolysis of lipid-complexed amylose in solution is widely studied, little is known about the accessibility of lipid-complexed amylose during hydrolysis of starch. We have previously studied the accessibility of starch granules to α -amylase during different phases of gelatinization (Lauro et al 1993). In this study, the α -amylolysis of granular barley starch was studied to determine the accessibility of starch components, especially the lipid-complexed amylose.

MATERIALS AND METHODS

Commercial barley A-starch (Ohrakas) was obtained from Primalco Ltd. (Koskenkorva, Finland). Starch content was 99.5% (dwb), determined enzymatically with Megazyme total starch assay procedure. *Bacillus licheniformis* α -amylase was a product of Megazyme (E-BLAAM). Its activity was 3,000 U/mL as reported by the manufacturer (on Ceralpha Reagent at pH 6.0 and 40°C).

α -Amylolysis of Starch Granules

The starch-buffer (0.1M ammonium acetate, pH 6) suspensions were tempered to the hydrolysis temperature of 30°C. α -Amylase solution was added, leading to an the enzyme dosage of 0-1,500 U/g of starch and the final starch concentration of 5% (w/v). Minimal magnetic stirring was used to prevent sedimentation. After 24 hr, the hydrolysis was stopped by adjusting to pH 2 with 1M HCl for 30 min and then back to pH 6 with 1M NaOH. Insoluble starch was separated by centrifuging (10,844 \times g, 10 min) and freeze-drying. Six replicates were made with each enzyme dosage.

Chemical Analyses

Soluble carbohydrates were determined using the phenol-sulfuric acid method (Dubois et al 1956) using glucose as standard. Analyses were done in duplicate. Results were given as percent of the original starch, a conversion factor 0.9 was used to convert values from glucose to starch. Standard error was \pm 2%. Total and FAM contents were determined colorimetrically according to the method of Morrison and Laignelet (1983). LAM content was obtained by subtracting FAM from total amylose. Phospholipid content was analyzed by phosphorous analysis (Morrison 1964, Tester and Morrison 1990). These analyses also were done in duplicate. Standard error was \pm 0.2% for amylose and \pm 0.1% for lipids.

Size-Exclusion Chromatography

Molecular weights were analyzed by size-exclusion chromatography using two column combinations: 1) μ Hydrogel 2000, 500, and 250; and 2) μ Hydrogel 250 and 120 (Waters Corp. Millford, MA). The starch (50 mg) was suspended in distilled water (1.25 mL)

TABLE I
Effect of α -Amylolysis on Molecular Weight (MW)
of Barley Starch and Amylose

Enzyme Dosage (U/g of starch)	Residual Starch (% of original)	MW Starch ($\times 10^6$ g/mol)	MW Amylose ($\times 10^3$ g/mol)
0	100	180	880
50	54	31	230
400	30	25	190
1000	11	27	200
1500	6	22	200

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and dissolved by addition of 2M NaOH (1.25 mL) with magnetic stirring for at least 6 hr at room temperature. Before analysis, the dissolved samples were diluted 1:10 with 1M NaOH. Detection was performed with refractive index detector and with the second column combination with a diode array detector monitoring wavelength at 230–300 nm. The molecular weight of the whole sample was determined by laser-light scattering (measuring angles 15° and 90°) with an analysis program (PD 2000, Precision Detectors, Amherst, MA). Amylose molecular weight was determined using postcolumn iodine coloring and spectrophotometric detection as described by Suortti et al (1998).

Differential Scanning Calorimetry

Differential scanning calorimetry was performed in a Mettler TA thermal analyzer system with a DSC 30S cell. The starch was weighed (7–9 mg) in a medium-pressure crucible and mixed with water to a moisture content of 70% (w/w). The sealed pans were scanned at a rate of 10°C/min from 10 to 150°C, then cooled down to 10°C, and rescanned to 150°C. Each sample was run in triplicate. Standard error was ± 0.4 J/g for ΔH and $\pm 0.6^\circ\text{C}$ for T_p .

X-ray Analysis

For X-ray analysis, the freeze-dried samples were conditioned at 60% rh and 20°C for one week. Diffractograms were then recorded on a Philips PC-APD diffractometer PW3710 equipped with an Anton Paar TTK temperature chamber. Diffractograms were recorded in the reflection mode in the angular range 4–40° (2 θ). The Cu K α -radiation (λ 1.542 Å), generated at 40 kV and 50 mA, was made monochromatic using a 15 μm of Ni-foil. The diffractometer

was equipped with a 1° divergence slit, a 15-mm beam mask, a 0.2-mm receiving slit, and a 1° scatter slit. Scattered radiation was detected using a proportional detector.

Scanning Electron Microscopy

For scanning electron microscopy, starch granules and granule residues were sprayed onto double-sided tape on a microscope stub. Samples were analyzed by taking images on an environmental scanning electron microscope (ESEM 2020, Electro Scan 1996) at 20 kV and 5 TORR (666.6 Pa).

Particle-Size Measurement

Particle-size of the granule residues were measured using a Coulter LS 230 particle size analyzer. Before analysis, starch samples were suspended in ion exchange water (4 mg/mL) by ultrasound treatment for 20 min. Analyses were done in duplicate. The results are shown as total volume of particles as a function of diameter.

RESULTS

The α -amylolysis (24 hr, 30°C, 0–1,500 U/g) of barley A-starch granules was studied by measuring the amount of carbohydrates solubilized during hydrolysis, and the changes in morphology and molecular structure of the granule residues by scanning electron microscopy, particle-size analysis, size-exclusion chromatography, X-ray diffraction, and differential scanning calorimetry.

During α -amylolysis, solubilization of barley starch $\leq 94\%$ occurred with the enzyme dosages used (Table I). Scanning electron microscopy of the granules revealed that α -amylolysis caused formation

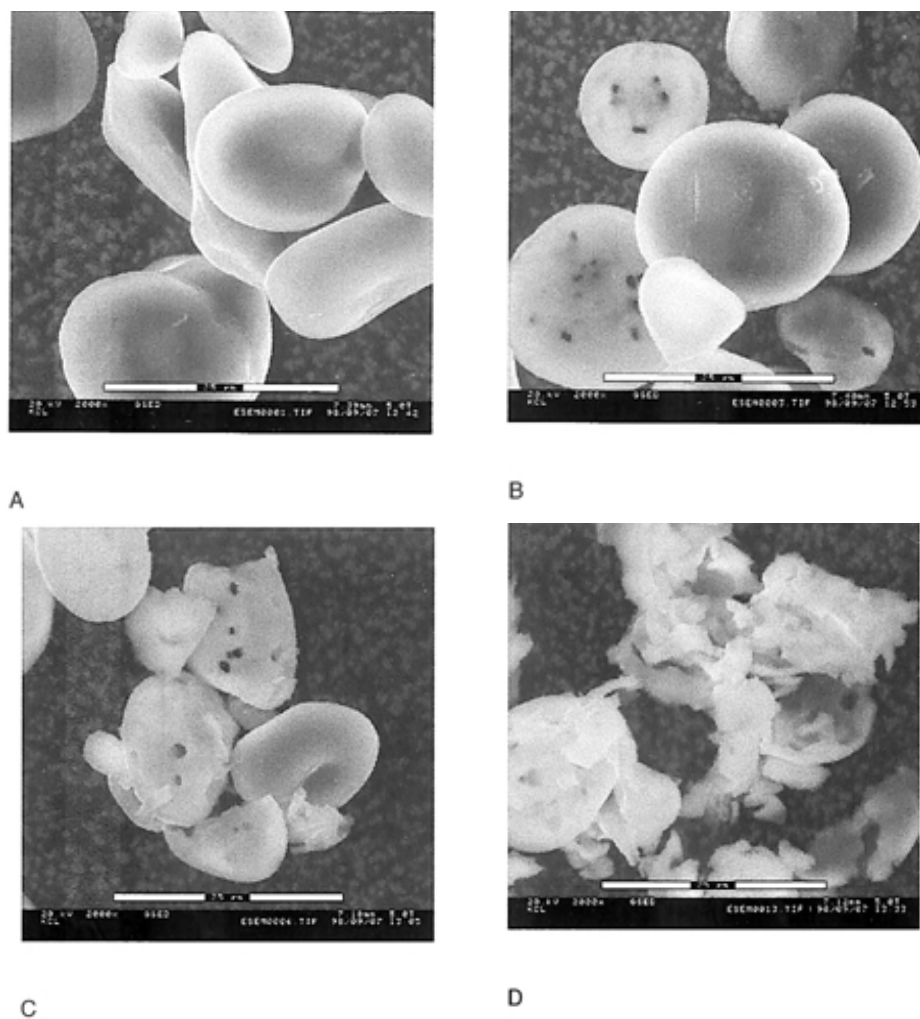


Fig. 1. Scanning electron microscopy of α -amylase-treated barley starch granules. **A**, reference; **B**, 50 U/g; **C**, 400 U/g; **D**, 1,500 U/g.

of pinholes even at low degrees of solubilization (Fig. 1A and B). When hydrolysis was more extensive, partially hollow granule residues and granule fragments were present (Fig. 1C and D). However, even when the residue represented only 6% of the original starch, it contained a few, apparently intact granules. A commercial sample of large barley A-starch granules was used in the hydrolysis experiments, and the volume average particle size of the reference sample was 16 μm , and the size range was 4–33 μm (Fig. 2A). α -Amylolysis caused only a slight decrease in the average particle size; volume average particle size was 14 μm when 6% of the original starch was left in the residue (Fig. 2A–C). When 54% of the starch was left in the residue, the particle-size distribution was similar to the original one. Extensive hydrolysis caused an increase in the amount of small (<10 μm) and large (>30 μm) particles, particle-size range at 2–60 μm (Fig. 2C).

The X-ray analysis of the reference sample (treated without the enzyme) and the granule residues after amylolysis showed an A-type diffraction pattern typical of cereal starches (Fig. 3). No differences in the diffraction pattern of native barley starch and the reference sample were detected, meaning that the changes observed in the crystallinity of granule residues were due to the α -amylolysis and not to an annealing effect caused by incubating starch in excess water at 30°C for 24 hr. Up to \approx 50% of solubilization, no change in the total crystallinity was observed (Fig. 3B and C). After that, the total crystallinity of the granule residues decreased, and eventually the residue was almost amorphous according to the X-ray data (Fig. 3D).

Analyses by differential scanning calorimetry showed that the gelatinization enthalpy (10.8 J/g) of the reference sample was the same, and the gelatinization peak temperature (67.4°C) was 2° higher than in the original barley starch. The α -amylolysis further increased the gelatinization temperature to 75°C when 6% of the starch was left. The gelatinization enthalpy of the residual starch decreased due to the hydrolysis, first only slightly, and after half of the starch was solubilized, more clearly, to one-third of the original value when \approx 10% of the starch was left in the residue (Fig. 4).

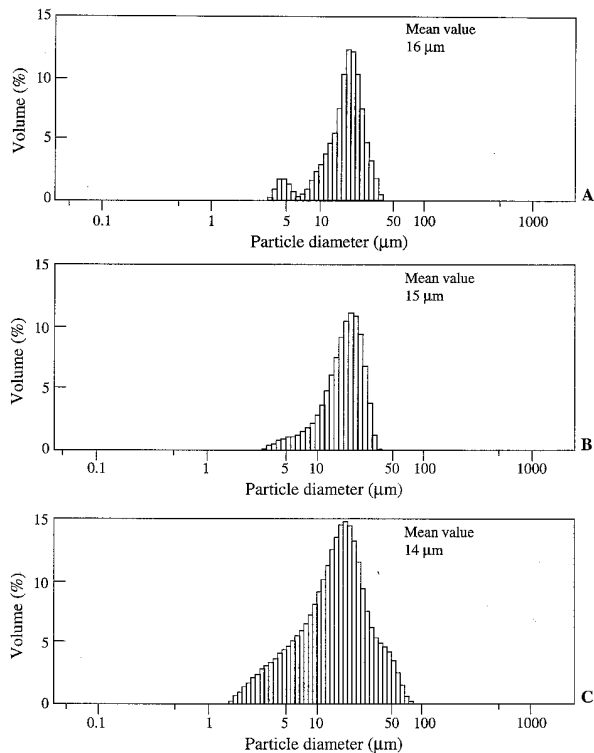


Fig. 2. Particle-size distributions of α -amylase-treated barley starch granules. A, reference; B, 50 U/g; C, 1,500 U/g.

The weight average molecular weights (MW) of starch were analyzed using size-exclusion chromatography. With the column combination μ Hydrogels 2000, 500, and 250, amylopectin eluted at \approx 27 min and amylose at 41 min (Fig. 5A). The MW of the reference sample was 180×10^6 g/mol (Table I). When half of the starch was solubilized, the MW of the residual starch had decreased to 31×10^6 g/mol, and the original high molecular weight amylopectin eluting at 27 min had disappeared (Fig. 5B). More extensive hydrolysis further decreased the MW of the residual starch. Due to the amylolysis, formation of molecules in the molecular weight range 2,000–15,000 g/mol, at peak \approx 54 min (Fig. 5C) was observed. The molecular weight of amylose in the reference sample was 880×10^3 g/mol, and it was reduced to 230×10^3 g/mol when half of the starch was solubilized (Fig. 6, Table I). After that, the molecular weight of amylose decreased only slightly to 180×10^3 g/mol when 6% of the original starch was left in the residue.

A more detailed resolution of the smaller molecules formed during amylolysis was obtained by using the column combination μ Hydrogel 250 and 120. The formation of these molecules was clearly observed as a peak at 24–25 min when 6% of the starch was left in the residue (Fig. 7C). To verify the carbohydrate nature of these molecules, UV-spectrophotometric detection at 235 nm was used. At this wavelength, carbohydrates give much lower response than, for instance, proteins. The response of these molecules was much higher than that of pure glucose polymers (Fig. 8).

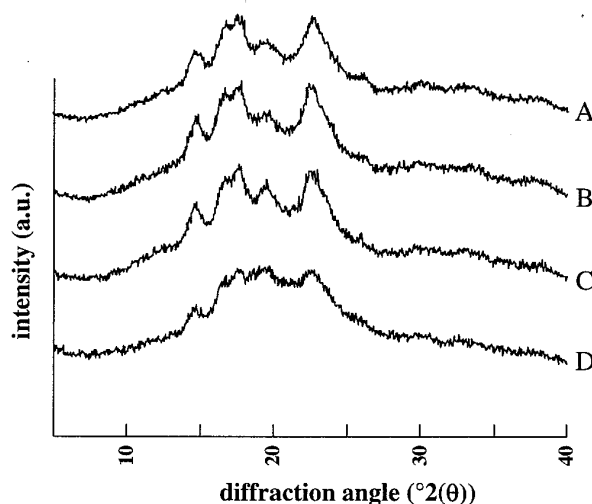


Fig. 3. X-ray chromatograms of α -amylase-treated barley starch granules. A, native barley starch; B, reference; C, 50 U/g; D, 1,500 U/g.

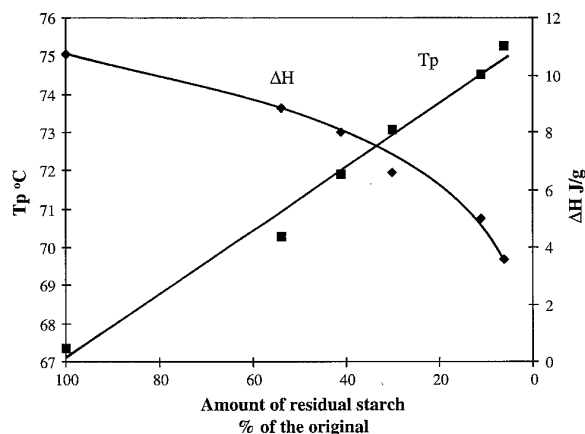


Fig. 4. Gelatinization enthalpies (ΔH , J/g) and peak temperatures (T_p , °C) of barley starch granule residues after α -amylolysis (0–1,500 U/g, 24 hr, 30°C, pH 6).

Accessibility of the lipid-complexed amylose during α -amylolysis was studied by analyzing the amounts of FAM, LAM, and phospholipids in the granule residues. In the reference starch, there was 22% FAM, 6.4% LAM, and 0.9% phospholipids. The FAM content of the granule residues had already decreased in the early stages of the hydrolysis, being 14% when half of the starch was solubilized and virtually zero when 6% of the starch was left (Fig. 9). LAM and phospholipids concentrated in the granule residues until 40–50% of the total starch was solubilized due to the amylolysis (Fig. 10). LAM content increased up to 16% when 30% of the starch was left in the residue and then started to decrease (Fig. 9).

Differential scanning calorimetry was used to study the presence of lipid-complexed amylose in the granule residues. In the first scan from 10 to 150°C, a gelatinization endotherm (ΔH ; T_p 67–76°C) and a second endotherm with a T_p at 105°C ($\Delta H_{ami}(I)$) was seen. In the rescan (10–150°C), only the endotherm >100°C ($\Delta H_{ami}(II)$) was present, and it was smaller than the one in the first run. The endotherm observed at >100°C in the rescan from 10 to 150°C was probably the dissociation of lipid-complexed amylose. The dissociation enthalpy and also the concentration of the lipid-complexed amylose increased as the hydrolysis

proceeded until 30% of the starch was left (Figs. 9 and 11). After that, the concentration and dissociation enthalpy of the lipid-complexed amylose started to decrease. The theoretical values for the dissociation enthalpies ($\Delta H_{ami}(teor)$) at different LAM contents were calculated assuming linear correlation between LAM content and dissociation enthalpy.

DISCUSSION

During α -amylolysis of native barley starch A-granules at 30°C, solubilization of starch $\leq 94\%$ occurred with the enzyme dosages used (0–1,500 U/g of starch). At low degrees of solubilization, the pinholes and partially hollow granule residues seen with ESEM and the almost unchanged particle-size distribution support the earlier findings of MacGregor and Ballance (1980) that the α -amylolysis of large barley starch granules proceeds through pinholes from the inside out. ESEM showed a few intact

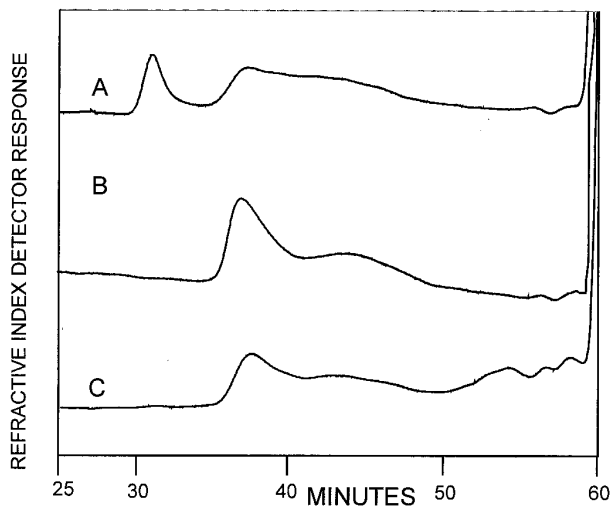


Fig. 5. Effect of α -amylolysis (0–1,500 U/g, 24 hr, 30°C, pH 6) on size-exclusion chromatography of barley starch granules. A, reference; B, 50 U/g; C, 1,500 U/g.

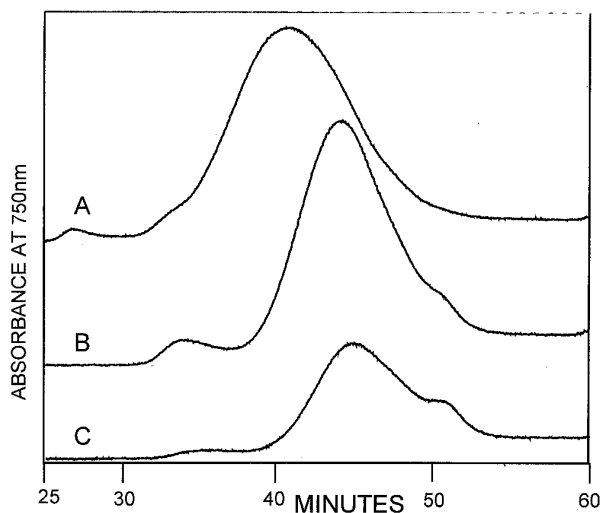


Fig. 6. Effect of α -amylolysis (0–1,500 U/g, 24 hr, 30°C, pH 6) on size-exclusion chromatography of barley starch amylose detected by postcolumn iodine coloring and spectrophotometric detection. A, reference; B, 50 U/g; C, 1,500 U/g.

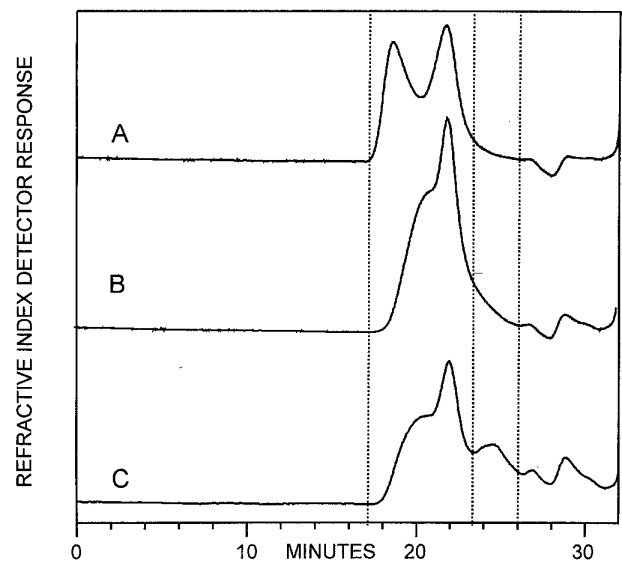


Fig. 7. Effect of α -amylolysis (0–1,500 U/g, 24 hr, 30°C, pH 6) size-exclusion chromatography of barley starch granules. A, reference; B, 50 U/g; C, 1,500 U/g.

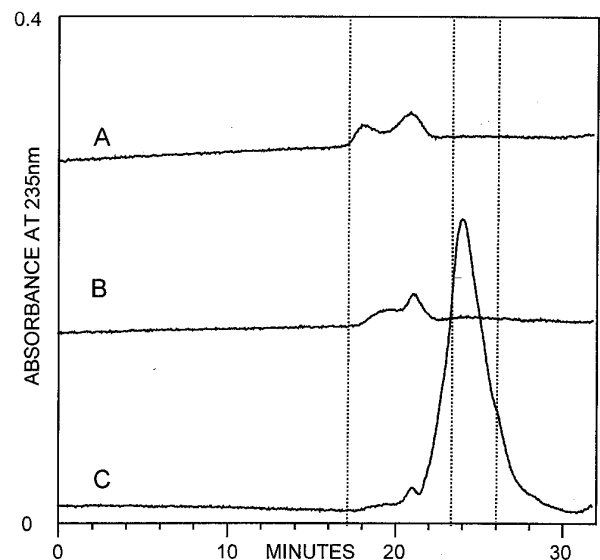


Fig. 8. Application of UV-spectrophotometric detection at 235 nm on barley starch granule residues after α -amylolysis (0–1,500 U/g, 24 hr, 30°C, pH 6). A, reference; B, 50 U/g; C, 1,500 U/g.

granules were left even with higher degrees of starch solubilization. According to Colonna et al (1988), wheat starch granules were not equally susceptible to α -amylolysis, and even after 91% solubilization, most of the residual granules were intact with only a few surfaces pitted. In their study, large granules were hydrolyzed preferentially. In our study, only large barley starch granules were used, thus the hydrolysis could be expected to be more uniform. Also, to prevent sedimentation we used minimal magnetic stirring instead of shaking, and this could have lead into some mechanical damage of the granules, possibly making the hydrolysis more uniform. On the other hand, Bertoft et al (1993) showed that in the α -amylolysis of granular pea starch, almost all granules were already fragmented after 7% solubilization. Studies with granular corn starch have shown that most of the granules became highly eroded and fragmented even at the early stages of amylolysis (Leach and Schoch 1961, Planchot et al 1995). Thus, the evenness of the hydrolysis also seems to be strongly dependent on the starch origin.

When the hydrolysis was more extensive, the formation of small particles observed in particle-size analysis indicated granule fragmentation due to the α -amylolysis. These fragments were also seen in ESEM. The observed increase in the amount of large particles at the later stages of the hydrolysis was most likely due to aggregation of the granule fragments when suspended in water because no large particles were observed with ESEM. The α -amylolysis of barley starch granules thus differed from that of wheat starch granules during which all large granules (diameter > 20 μ m) disappeared when half of the starch had solubilized (Colonna et al 1988). On the other hand, incubation with glucoamylase has been shown to initially break barley starch granules into two fragments of approximately equal size, and the hydrolysis then takes place within the granules (Kimura and Robyt 1995)

It has been suggested that α -amylases, unlike acid, are able to simultaneously solubilize the amorphous and crystalline areas of starch granules (Leach and Schoch 1961, Colonna et al 1988). This was also the case in our study because no increase in the crystallinity due to the α -amylolysis was observed with X-ray analysis. The gradual decrease in the crystallinity and the decrease in the gelatinization enthalpy detected with DSC at the later stages of the hydrolysis means that extensive hydrolysis effectively destroyed and solubilized the crystalline areas of starch.

When studying the gel-permeation chromatograms, it has to be kept in mind that the chromatograms of the granule residues do not contain all of the material of the reference sample because part of the starch was solubilized during α -amylolysis. The reduction of the molecular weight of starch and amylose due to the α -amylolysis was very clear. Because the smaller molecules (MW 2,000–15,000 g/mol) formed during the hydrolysis also had response at 235 nm much higher than pure glucose polymers (pullulans), it is unclear whether the appearance of these molecules is real or at last partly due either to starch proteins concentrating or amylase being adsorbed in the granule residues. In that case, the molecular weights of the residual starch and amylose remained the same after the reduction, observed when half of the starch was solubilized from 180×10^6 g/mol to 31×10^6 g/mol and from 880×10^3 g/mol to 230×10^3 g/mol, respectively. Also, Bertoft and Manelius (1992) reported that in the α -amylolysis of maize starch granules, the length of amylose chains decreased notably, and in the α -amylolysis of granular smooth pea starch, the granular residues contained increased amounts of dextrans at 50–300 degrees of polymerization (Bertoft et al 1993). Our results are in disagreement with the results obtained for the hydrolysis of wheat starch granules. According to Colonna et al (1988), even after 91% hydrolysis, the molecular size distribution of wheat starch granules was similar to that of the original starch, with the exception of a small peak at a lower molecular weight area, which was also observed in our study. In their study, the hydrolysis proceeded granule by granule, leaving mostly intact granules in the residue, thus explaining the unchanged molecular size distribution.

Only a slight decrease in the total amylose content of the granule residues was observed when half of the starch was solubilized, indicating equal solubilization of amylose and amylopectin. The preferential solubilization of free amylose was clearly observed as the amount of free amylose in the granule residues decreased continuously during the hydrolysis, whereas all phospholipids and lipid-complexed amylose remained in the granule residues even when 40–50% of the starch was solubilized. The increased dissociation enthalpy of lipid-complexed amylose observed using DSC confirmed the survival of the complex during α -amylolysis. The measured values ($\Delta H_{\text{aml(II)}}$) correlated well with the theoretical values ($\Delta H_{\text{aml(teor)}}$). The difference in the size of endotherm

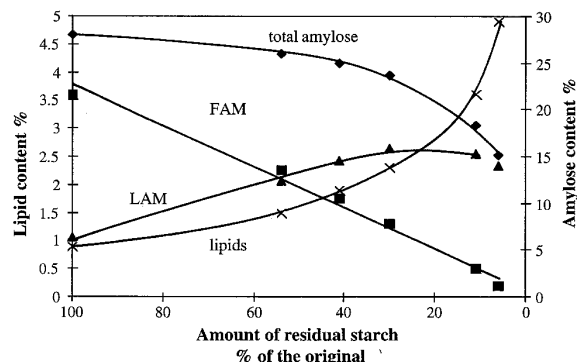


Fig. 9. Amylose and lipid contents of granule residues after α -amylolysis (0–1,500 U/g, 24 hr, 30°C, pH 6). LAM = lipid-complexed amylose; FAM = lipid-free amylose.

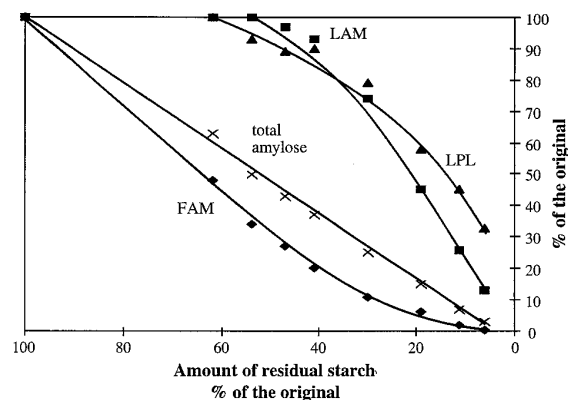


Fig. 10. Yield of lipid-free amylose (FAM), lipid-complexed amylose (LAM), and phospholipids (LPL) in granule residues after α -amylolysis (0–1,500 U/g, 24 hr, 30°C, pH 6).

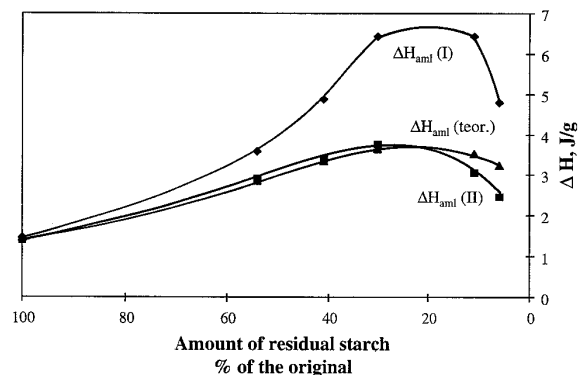


Fig. 11. Dissociation of amylose-lipid complex of barley starch granule residues after α -amylolysis (0–1,500 U/g, 24 hr, 30°C, pH 6) as a function of amount of residual starch. ΔH_{aml} = dissociation enthalpies: (I) scan 10–150°C; (II) rescan 10–150°C; and (teor) theoretical values.

>100°C in the first ($\Delta H_{\text{ami(I)}}$) and second scan ($\Delta H_{\text{ami(II)}}$) could be due to melting of short chain dextrans formed and precipitated during hydrolysis. Also, in solution, lipid-complexed amylose is more resistant to α -amylase than free amylose (Holm et al 1983, Biliaderis and Galloway 1989, Galloway et al 1989). However, total hydrolysis of the complex is obtained with adequate enzyme dosage and time, which was also the case in our study because hydrolysis of LAM was seen at the later stages of the α -amylolysis. Also, Anger et al (1994) found that in the α -amylolysis of granular wheat starch, the lipid-complexed amylose was concentrated in the granule residues.

In conclusion, in the earlier stages of α -amylolysis of large barley starch granules, both amorphous and crystalline parts of the granules were equally solubilized. More extensive hydrolysis caused a gradual decrease in crystallinity and degradation of the granular structure. The lipid-complexed amylose was less susceptible than free amylose and amylopectin. With higher enzyme dosages, LAM and lipids leached out of the granule residues. Scanning electron microscopy showing pinholes and partially hollow granule residues, and the fact that lipid-complexed amylose remained in the granule residues, gives indirect support to the suggestion that there is more lipid-complexed amylose in the outer layers of the barley starch granules (McDonald et al 1991). Even though according to scanning electron microscopy, there were some intact granules left throughout the hydrolysis, these results suggest that α -amylolysis of large barley starch granules proceeds rather evenly among the granules.

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