Hydrolysis of Large and Small Starch Granules from Normal and Waxy Barley Cultivars by Alpha-Amylases from Barley Malt[1]

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ABSTRACT

α-Amylase I, the minor α-amylase component in malted barley, was more efficient than the major component, α-amylase II, in solubilizing the four types of starch granules studied. Large starch granules from normal barley were particularly resistant to hydrolysis by α-amylase II. No difference was detected by scanning electron microscopy between the action patterns of the two α-amylases on the same starch granules. No characteristic pinholes were observed in degraded small starch granules of either normal or waxy barley, both of which were hydrolyzed by surface erosion. Large degraded granules from normal barley contained many pinholes and appeared to be hydrolyzed from the inside out. Large granules from waxy barley appeared to be degraded by a combination of surface erosion and internal digestion via pinholes from the granule surface.

Starch, the major storage polysaccharide of cereal grains, exists in the form of discrete particles or granules. In mature barley these granules can be separated into two distinct size populations, small granules of < 5 μm in diameter and large granules of 15–20 μm in diameter (MacGregor et al 1971, May and Buttrose 1959). Although 90% of the starch granules are small, they account for only 10% of the total weight of the starch (Bathgate and Palmer 1972).

During germination of barley, hydrolytic enzymes such as α-amylase are synthesized by the embryo and aleurone cells and are then secreted into the endosperm (Briggs 1964, MacLeod et al 1964, Paleg 1960). There, during normal malting, the enzymes start to hydrolyze starch granules. Although strong evidence suggests that small granules are preferentially degraded during the malting process (Kiribuchi and Nakamura 1973a, 1973b; Palmer 1972), no quantitative information is available on the relative rates of hydrolysis, by cereal α-amylases, of large and small granules from barley. Furthermore, α-amylase from germinated or malted barley is not a single entity but a complex mixture (MacGregor 1978). The action of individual components of this mixture on starch granules is unknown but may be of considerable significance to the degradation of starch granules during malting.

Studies on nutritional evaluation of cereal grains have shown that starch granules from waxy sorghums (ie, sorghums containing starch composed only of amylopectin) are more readily digested than is granular starch from normal sorghums (Harbers and Davis 1974, Sullins and Rooney 1975, Tovar et al 1977). Additional evidence shows that starch granules from waxy barley may be more susceptible to enzymic attack than is starch from normal barley (Goering and Eslick 1976). These results confirm earlier findings that waxy starches in general may be more susceptible than normal starches to hydrolysis by α-amylases (Leach and Schoch 1961).

The objective of the present study was to determine the relative rates at which large and small starch granules from isogenic lines of waxy and normal barley were hydrolyzed by two α-amylases purified from germinated barley.

MATERIALS AND METHODS

Starch Granules

Starch granules from near-isogenic lines of normal and waxy Manchurian barley were extracted, purified, and fractionated into large-granule and small-granule populations by methods described previously (MacGregor 1979).

α-Amylase

The enzyme source was green malt prepared from Conquest barley using steeping and germination units described previously (Bettner et al 1962). Extraction, heat treatment, glycogen precipitation, separation of α-amylases I and II by ion-exchange chromatography on carboxymethyl cellulose and subsequent purification of α-amylase I were all as described previously (MacGregor 1977). α-Amylase II fractions from the initial fractionation on carboxymethyl cellulose were pooled, concentrated, and dialyzed against TRIS-HCl buffer (0.04 *M*, pH 8.0, 0.001 *M* $CaCl_2$). The enzyme was then loaded on to a column of diethylaminoethyl cellulose and eluted with the following linear gradient: 1,500 ml of TRIS-HCl buffer (0.04 *M*, pH 8.0, 0.001 *M* $CaCl_2$, 0.05 *M* NaCl) and 1,500 ml of above buffer but containing 0.18 *M* NaCl.

Fractions containing α-amylase were pooled, concentrated, and dialyzed into acetate buffer (0.2 *M*, pH 5.5, 0.001 *M* $CaCl_2$).

Preparations of both enzymes were divided into small volumes (2–5 ml) and kept frozen until used. Subsequent analysis by isoelectric focusing showed that neither preparation contained β-amylase and that neither α-amylase contained a trace of the other.

α-Amylase Activity

This was determined as described previously, using the β-limit dextrin of waxy maize starch as substrate (Briggs 1961, MacGregor et al 1971).

Scanning Electron Microscopy

Intact and degraded granules were sprinkled onto double sided tape on a microscope stub and coated with gold (100 A). Samples were analyzed on a JEOL 35c scanning electron microscope at an accelerating voltage of 10 kv. Photomicrographs were taken on Plus-X pan Kodak film.

Amylose

Amylose determinations were carried out using an amperometric titration technique (Coton et al 1955, Larson et al 1953).

Gelatinization Temperature

This was determined using a Zeiss microscope with long working distance objectives and a Mettler FP5 temperature control unit equipped with a Mettler FP52 heating block. Temperatures were raised through the gelatinization range at 1°C per min. Temperatures were noted when 50 and 90% of the granules were no longer birefringent.

Protein

The method of Mitcheson and Stowell (1970) was used for digesting samples, and nitrogen was determined with Nessler reagent (Williams 1964). Nitrogen values were multiplied by a factor of 6.25 to give protein values.

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Hydrolysis of Starch Granules

Suspensions of starch granules and α-amylase were prepared by one of the procedures outlined below.

Procedure One. This was used to determine initial rates of hydrolysis (carried out at 20°C) and effect of temperature on hydrolysis.

Starch granules (40 mg) were shaken on a Labquake shaker (Labindustries, Berkeley, CA) with acetate buffer (20 ml, 0.1 *M*, pH 5.5, 0.001 *M* $CaCl_2$) containing bovine serum albumin (BSA) at 0.5 mg/ml. When a uniform suspension had been obtained and equilibrated at the required temperature, α-amylase II (250 μl, 65,000 IDC units) was added.

Procedure Two. This was used to determine the relative rates of hydrolysis of starch granules by α-amylases I and II (carried out at 35°C).

Starch granules (25 mg) were shaken with a mixture of acetate buffer (6.25 ml, 0.2 *M*, pH 5.5, 0.001 *M* $CaCl_2$, 1 mg/ml of BSA) and calcium chloride solution (0.001 *M*, 6.25 − X ml, where X is the volume of enzyme that was added). Equal activities (38,000 IDC units) of α-amylases I and II were added to the starch suspensions.

All digests were prepared in duplicate with appropriate starch blanks. After addition of enzyme, the starch was maintained in suspension by gentle shaking and, at intervals, portions (2 ml) were removed from each digest, brought to pH 3.0 with dilute HCl to stop enzyme activity, and centrifuged (15,000 × *g*, 10 min). An appropriate portion of the supernatant solution was analyzed for total carbohydrate by an automated orcinol-sulphuric acid technique (LaBerge et al 1973). The method was calibrated with standard glucose solutions, but the quantity of carbohydrate material present in the supernatant solutions was expressed in terms of starch.

Starch granules in the centrifuged pellet were washed twice with acidulated water (diluted HCl, pH 3.0) and twice with distilled water and were freeze-dried.

TABLE I
Properties of Starch Granules

	Normal		Waxy	
	Large	Small	Large	Small
Protein, %[a]	0.27	0.53	0.08	0.17
Amylose, %	23.6	20.4	4.1	1.6
Gelatinization temperature, °C	56.7–58.8	63.4–65.1	61.6–64.0	64.6–66.4
Granule size, μm	10–20	1–2	10–20	1–2

[a]Protein = N × 6.25.

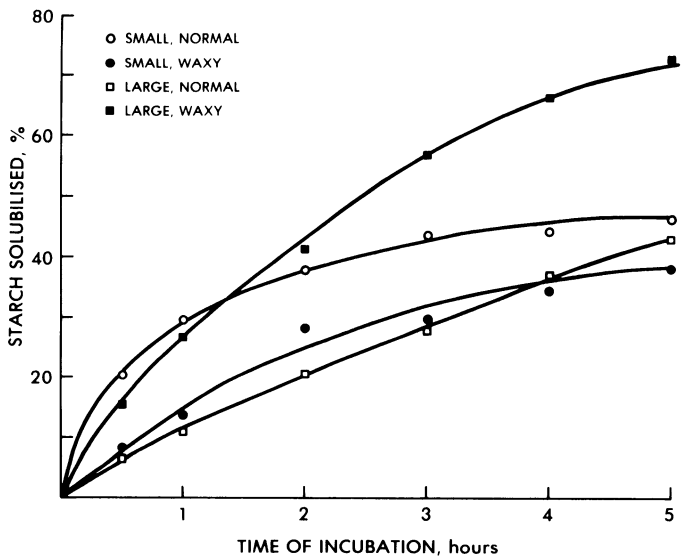

Fig. 1. Solubilization of starch granules at 35°C by α-amylase I.

RESULTS AND DISCUSSION

Properties of the starch granules used in this study are shown in Table I. The protein content of isolated starch granules is often used as a measure of granule purity. All samples analyzed contained low protein levels, but the small granules contained twice as much as the large granules did. This is a common finding (Slack et al 1979), but the cause is not clear. It may be because small granules contain more protein as an integral part of the granule structure or because more protein is adsorbed to the much larger surface area of small granules and is more difficult to remove during starch purification. Small granules do appear to be associated with a thick protein matrix in barley endosperm cells (Palmer 1972).

The amylose content of small granules from normal barley starch was slightly lower than that of large granules from the same starch. This is in agreement with other reports (Goering and DeHaas 1974, Kano 1977). No evidence was found to support the idea that small granules have a very high amylose content (Bathgate et al 1973). Granules from waxy barley had very low amylose contents, and the amount present in small granules represents only a trace amount. Values for large granules were higher and agree with earlier findings (Banks et al 1970). Preliminary observations by light microscopy indicated that a small number of the large waxy granules gave a blue stain with iodine and so perhaps contained normal amounts of amylose. The detectable amylose content of this sample could be from these granules. This tentative observation is in agreement with previous findings (Banks et al 1970), but a final answer to the nature of amylose in waxy barley starches must await further research.

In normal starch, small granules had higher gelatinization temperatures than did large granules, but the difference was small and similar to that reported by others (Goering and DeHaas 1974). High values for small granules reported by Bathgate and Palmer (1972) may have been caused by the Congo Red method used to determine the gelatinization temperatures. Waxy starch granules had slightly higher gelatinization temperatures than did normal granules, suggesting that they have a stronger, more cohesive physical structure.

Instability of the α-amylases caused problems during preliminary experiments on the hydrolysis of starch granules. Results were not reproducible even over short periods of hydrolysis (1–5 hr), and the extent of hydrolysis was always low. Addition of BSA at a concentration of 0.5 mg/ml of digest solved this problem. No further improvement was noted at higher levels of BSA. The precise mechanism of this stabilization is not known, but the added protein probably just protects the active enzyme configuration (Hao et al 1977).

The method used for maintaining starch in suspension during hydrolysis by α-amylase was very important. Vigorous shaking inactivated the enzyme, whereas even gently stirring with a magnetic stirrer damaged the granules and gave erroneous results. A gentle, tumbling motion just sufficient to keep the starch

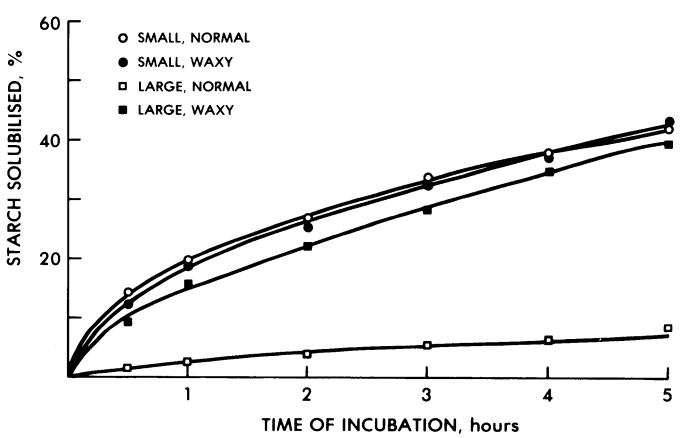

Fig. 2. Solubilization of starch granules at 35°C by α-amylase II.

suspended gave reproducible results.

Progress of the hydrolysis of starch granules was followed by measuring the total amount of starch solubilized. This does not necessarily represent the true rate of starch hydrolysis because not all starch scissions will lead to soluble products and not all initial soluble products of hydrolysis need be of the same size. Therefore, even if an α-amylase were to hydrolyze equal numbers of α, 1-4 bonds within two different samples of starch granules, the ensuing rates of solubilization would depend on the size of the initial soluble products formed. Because the major objective of the project was to determine the relative rates of solubilization of the starch granules, no attempt was made to determine the final limits of starch solubilization.

The results for α-amylase I are shown in Fig. 1. Different curves were obtained for each starch, but the large granules from waxy starch were hydrolyzed more extensively than the other granules. After 5 hr, 72.5% of this starch had been solubilized compared to 38.0% for the small waxy granules and 45–46% for the large and small normal granules. Solubilization of large normal granules was almost linear with time, but the corresponding curve for small granules was quite different. Initially, the starch was solubilized very rapidly, but the rate decreased after 1–2 hr. This suggests that either some granules were readily hydrolyzed and others were relatively resistant or that differences in enzyme susceptibility exist within each granule. Distinguishing between these two possibilities is not yet possible.

Results for α-amylase II are shown in Fig. 2. Solubilization of both samples of small granules was almost identical, reaching values of 42–45% after 5 hr. The curve for large, waxy granules was similar but slightly lower at each stage. However, large, normal granules were hydrolyzed extremely slowly, and only 8% of the starch was solubilized after 5 hr. This very slow hydrolysis of the major fraction of barley starch by the major α-amylase component of malted barley was very surprising.

Large starch granules from waxy barley were hydrolyzed much faster than those from normal barley by both α-amylases. These results may partially explain the phenomenon of "self-liquefaction" reported by Goering and Eslick (1976). These workers found that some cultivars of waxy barley contained sufficient α-amylase to hydrolyze starch reserves without the need for germination. The results in Figs. 1 and 2 show that much less α-amylase would be required to effect significant hydrolysis of large granules of waxy starch than of the corresponding granules of normal starch. Results for the small granules were different, but since these granules represent a minor fraction of barley starches (Bathgate and Palmer 1972) they are unlikely to cause a significant change in the relative rates of hydrolysis of total starches from normal and waxy barleys.

The experiments shown in Figs. 1 and 2 were carried out at 35° C, a temperature too low to cause the starch granules to gelatinize (Table I). Therefore, the enzymes were digesting intact starch granules in much the same way that α-amylase hydrolyzes starch granules in barley kernels during the germination stage of the malting process. Only during kilning is the temperature of malt raised above 60° C, but by then the moisture content of the malt is so low that insignificant gelatinization of the starch occurs.

The results in Fig. 2 show that α-amylase II solubilized small granules from normal starch much faster than large granules. This explains the preferential loss of small granules during malting noted by other workers (Bathgate et al 1973, Kiribuchi and Nakamura 1973a, 1973b). Other investigations have shown that at 65° C, large granules are more susceptible than are small granules to attack by malt α-amylase (Bathgate and Palmer 1973, Slack et al 1979). However, at this temperature, large granules are rapidly gelatinized (Table I), and this gelatinized starch is much more

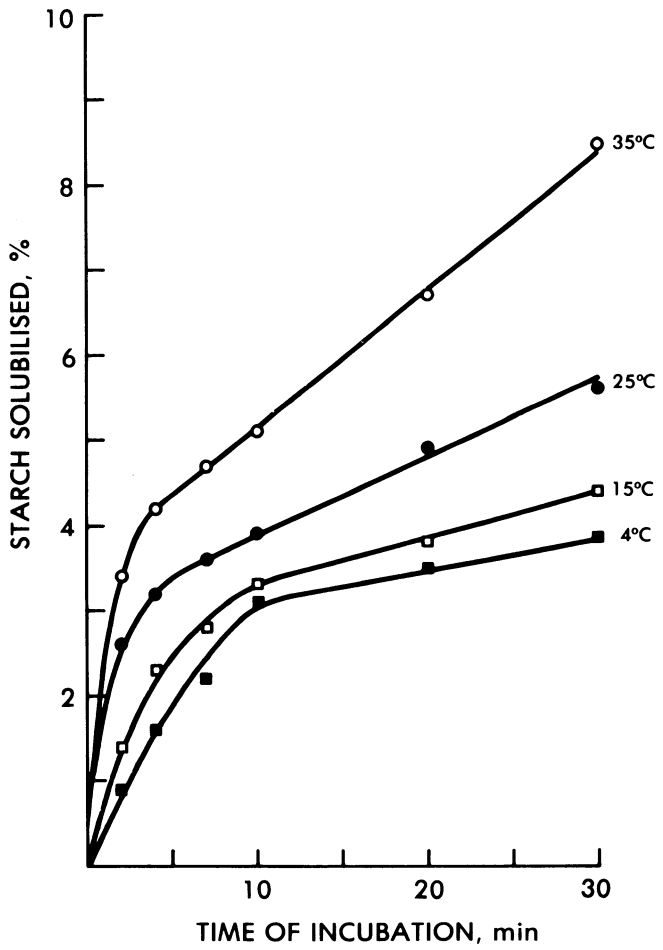

Fig. 3. Effect of temperature on the solubilization of large starch granules from waxy barley by α-amylase II.

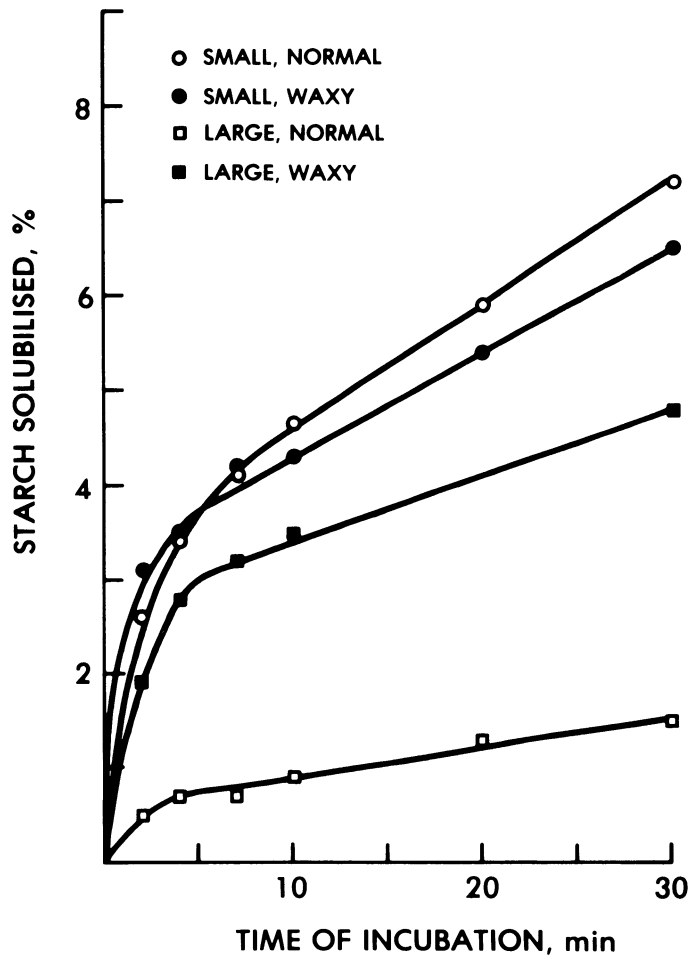

Fig. 4. Initial solubilization of starch granules by α-amylase II at 20° C.

rapidly hydrolyzed by α-amylase than are intact granules. Small granules have a higher gelatinization temperature (Table I), will require a longer period of time to gelatinize at 65°C, and so will be more slowly degraded. Therefore, the fact that at 65°C small granules are hydrolyzed more slowly than large granules is not surprising.

The mashing stage of brewing is usually carried out at about 65°C, and results indicate that small granules may be incompletely degraded during this process (Bathgate et al 1973). The above discussion explains this finding.

Starch granules are easily damaged, and such granules are more susceptible to enzyme attack than are intact granules (Sandstedt and Mattern 1960). To ensure that the different rates of starch hydrolysis shown in Figs. 1 and 2 were not caused by varying levels of starch damage, a closer examination was made of the initial stages of hydrolysis.

The effect of temperature on the initial hydrolysis of large granules from waxy barley is shown in Fig. 3. The zero-time

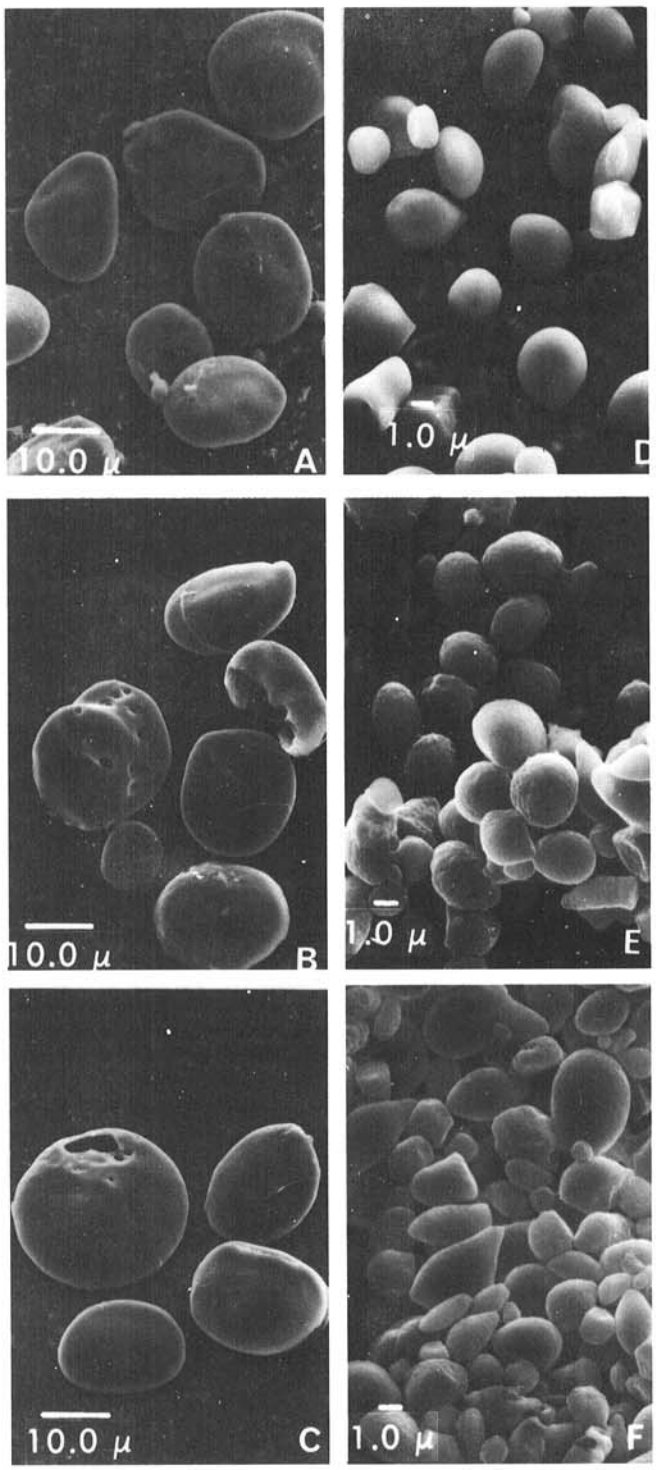

Fig. 5. Scanning electron photomicrograph of intact and degraded large and small starch granules from normal barley. **A,** Intact large granules; **B,** large granules degraded by α-amylase I; **C,** large granules degraded by α-amylase II; **D,** intact small granules; **E,** small granules degraded by α-amylase I; **F,** small granules degraded by α-amylase II.

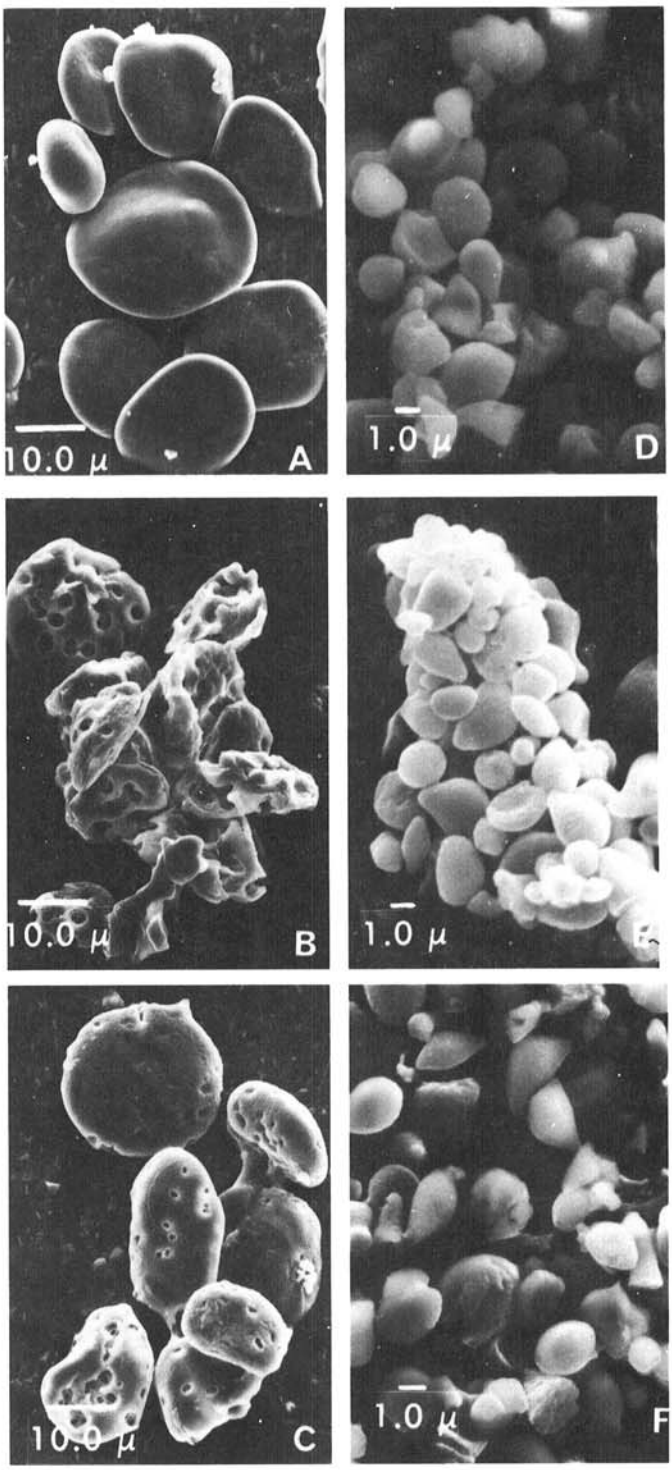

Fig. 6. Scanning electron photomicrograph of intact and degraded large and small starch granules from waxy barley. **A,** Intact large granules; **B,** large granules degraded by α-amylase I; **C,** large granules degraded by α-amylase II; **D,** intact small granules; **E,** small granules degraded by α-amylase I; **F,** small granules degraded by α-amylase II.

intercepts of 2.7–2.8 for the 4, 15, and 25° C hydrolyses agreed very well with the corresponding value of 2.7 obtained at 20° C (Fig. 4). The value of 3.5 obtained at 35° C was higher, but the rapid enzyme reaction at this temperature made determination of the initial rate of starch solubilization difficult. For this reason, the experiments shown in Fig. 4 were carried out at 20° C. The four different types of granules were hydrolyzed with α-amylase II at 20° C. All curves in Fig. 4 show two distinct reaction stages: a rapid solubilization of starch during the first 4–5 min, followed by a much slower, linear solubilization during the following 25 min. The rapid stage is thought to represent solubilization of damaged granules, whereas the slow stage indicates hydrolysis of intact granules (Sandstedt and Mattern 1960). Extrapolation of this latter part of the curves (Fig. 4) to zero time should give values for the amount of starch released from damaged granules. These values were 0.5% for large, normal granules, 2.7% for large, waxy granules, and 3.2% for both samples of small granules. These values are very low and make no significant difference to the results shown in Figs. 1 and 2.

The results in Figs. 1 and 2 show that, in general, α-amylase I is more efficient than α-amylase II in solubilizing intact starch granules, suggesting that the two enzymes may not have the same mode of attack. Scanning electron microscopy was used to analyze intact and degraded granules for differences in their physical structures. Results for intact granules and granules hydrolyzed 5 hr are shown in Figs. 5 and 6.

Large, normal granules showed the now well-known pitting and pinhole effects after degradation by α-amylase I (Fig. 5). These have been thoroughly discussed elsewhere (Fuwa et al 1977, 1978; Kiribuchi and Nakamura 1973a; Maeda et al 1978; Palmer 1972). The enzyme appeared to attack at discrete points on the granule surface, form tunnels into the granule interior, and then hydrolyze the granule from the inside out. Similar but much less extensive effects were produced by α-amylase II. Apart from rate of hydrolysis, no apparent differences in the mechanism of granule disintegration were detected between the enzymes. Similarly, no difference was detectable between the enzymes in their attack on small, normal granules. However, a fundamental difference was found in the way in which large and small granules were hydrolyzed. No pinholes were detected in the small granules, in agreement with earlier observations (Bathgate et al 1973, Kiribuchi and Nakamura 1973b). The surfaces of small granules became rough, and these granules appeared to be hydrolyzed from the outside by surface erosion. The rough texture of these small granules is quite different from the still-smooth surfaces of extensively degraded large granules. These results suggest a fundamental difference in the physical structure of the two types of starch granule.

Both enzymes hydrolyzed small granules from waxy barley in the same way (Fig. 6). Extensive surface erosion but no pinholes were evident after 5 hr. Similarly, large granules from waxy barley degraded by both enzymes had the same features of surface erosion, pinholes, and internal hydrolysis. Surfaces of these granules were rough, deeply pitted, and often had a spongelike texture. They were similar to starch granules in germinated waxy maize (Fuwa et al 1977) and starch granules isolated from various maize mutants hydrolyzed by bacterial α-amylase (Fuwa et al 1978).

Because no difference was detected in the way in which the two α-amylases degraded starch granules, no simple explanation can be offered for the observed differences in rates of hydrolysis (Figs. 1 and 2). Detailed chemical and physical analyses of the initial products of hydrolysis and of the corresponding degraded starch granules are required to solve this problem.

CONCLUSIONS

This report has shown that at 35° C, small starch granules were hydrolyzed faster than large granules by two α-amylases isolated from malted barley. Starch granules from waxy barley were hydrolyzed faster than granules from normal barley. Small and large granules were degraded in different ways, as were large granules from normal and waxy barleys. Although the two α-amylases solubilized the same starch granules at different rates, no difference was apparent in the physical characteristics of the degraded granules.

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