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Reaction of enzymes with starch granules: kinetics and products of the reaction with glucoamylase ☆

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Abstract

The reaction of glucoamylase with starch granules from seven botanical sources (waxy maize, maize, barley, tapioca, amylomaize-7, shoti, and potato) and with four potato starches modified with acid in four types of alcohols (methanol, ethanol, 2-propanol, and 1-butanol) were studied using three concentrations of enzyme (2, 20, and 200 units mL^{-1}). The kinetics of the formation of D-glucose were followed over 32 h for the three enzyme concentrations. The starches showed a wide degree of variance in their susceptibility to enzyme hydrolysis. They divided into three groups: waxy maize starch was the most susceptible being converted into 50, 95, and 98% D-glucose in 32 h for the three concentrations of enzyme, respectively; an intermediate group (barley, maize, and tapioca starches) was converted into 10–15, 60, and 75–80% D-glucose in 32 h for the three concentrations of enzyme, respectively; and the third and least susceptible group (amylomaize-7, shoti, and potato starches) was converted into 2–8, 9–16, and 13–21% D-glucose in 32 h for the three concentrations of enzyme, respectively. The percent conversion for the modified potato starches was proportional to the amount of enzyme and the degree of modification. The 100X (200 U mL^{-1}) amount of enzyme gave 13, 17, 21 and 27% D-glucose in 32 h for potato starch modified in the four alcohols, respectively.

The number and size of the granules were determined over 32 h of reaction using 10X (20 U mL^{-1}) enzyme for waxy maize starch and 100X enzyme for the other starches. The number of granules for waxy maize, barley, maize, and tapioca starches significantly increased in the first part of the reaction and then decreased; this was paralleled by a decrease of 40–50% in the size of the granules. The number and size of the potato and shoti starch granules did not change very significantly and the number and size of amylomaize-7 starch granules decreased slightly but steadily to about 25% of the number and size of the native granules. The morphologies of the

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granules during reaction were studied by scanning electron microscopy. Extensive conversion into 50% D-glucose for the starches in the first two groups showed the classical "Swiss cheese" shell structure with many deep holes into the granules. The least susceptible starches in the third group did not show much change in morphology, except for some minor surface pitting.

Keywords: Enzymes; Starch granules; Glucoamylase

1. Introduction

Starch granules are generally thought to be resistant to amylase digestion, although as early as 1879 [1], they were reported to be digested by amylases. Stamberg and Bailey [2] reported a 10% conversion of raw starch in 24 h with alpha-amylase. Sandstedt and Gates [3] reported a study of the digestion of raw starch by amylases from four sources (malt, fungi, bacterial, and pancreas) and found that pancreatic alpha-amylase was the most effective followed in order by malt, bacterial, and fungal amylases. A later report by Leach and Schoch [4] on the action of bacterial (*Bacillus subtilis*) amylase with starches from various botanical sources showed that their starches had widely different susceptibilities. Waxy maize was the most susceptible and high-amylose maize the least.

Glucoamylase is a fungal (*Aspergillus niger* or *Rhizopus niveus*) exo-acting enzyme that produces D-glucose from the nonreducing-ends of starch chains. It has a broad specificity for hydrolysis of α-glycosidic linkages, hydrolyzing both $(1 \rightarrow 4)$-α-glucosidic linkages and $(1 \rightarrow 6)$ branch α-glucosidic linkages when they occur at the nonreducing-ends of the chains. In 1971, Manners [5] reported that glucoamylase hydrolyzes starch granules to only a limited extent. Rasper et al. [6] showed that the degradation of starch granules by glucoamylase varies with the type of starch. Shetty et al. [7] used highly purified *R. niveus* and crude *A. niger* glucoamylases to determine the extent of wheat starch gelatinization. Smith and Lineback [8] followed the action of *R. niveus* glucoamylase on wheat and corn starch granules visually with the scanning electron microscope and chemically by the amount of D-glucose released. The scanning electron micrographs showed that wheat starch was attacked along the equatorial grove of the pancake-shaped granule and that extensive reaction with maize starch produced a Swiss-cheese appearance with many deep holes into the granules.

It is now generally agreed that ungelatinized granules do undergo some reactions with amylases and that there are differences between the types of starches and between the types of amylases. The actual degree and rate of reaction, however, has not been generally determined in most studies. We, therefore, have undertaken a quantitative study of the action of *R. niveus* glucoamylase on several types of starch granules to determine the relative susceptibilities and extent of reaction. The types of native starches were maize, waxy maize, barley, tapioca, amylomaize-7, shoti, and potato and on four alcohol/acid modified potato starches. The starches were carefully selected to give a broad spectrum of starch types: high amylopectin; high amylose; A-, B-, and C-type X-ray patterns; commercially important starches, such as maize, potato, barley, and tapioca; and one starch, shoti, for its unusual granule morphology. Kinetic studies have been made for the formation of D-glucose at various times, using three concentrations of

enzyme, each differing by an order of magnitude from the other; the number and size of the granules are reported during the course of reaction; the per cent of enzyme adsorbed onto the granules was determined; the amount of D-glucose in the granule and the amount released into the reaction medium were determined; and the scanning electron micrographic appearance of the granules at various stages of reaction were obtained.

2. Material and methods

Enzymes.—Glucoamylase from *Rhizopus niveus* was obtained in pure-grade from Seikagaku Kogyo Co., Ltd (Tokyo, Japan). Its activity was determined by reaction at 37°C with soluble starch (50 mg mL^{-1}) that was buffered with 100 mM pyridine acetate (pH 5.0). Aliquots were taken with time (0–20 min) for determining the amount of D-glucose released. The glucose was determined by a microsample plate glucose oxidase assay [9]. One international unit (IU) was the amount of glucoamylase that released 1 μmol D-glucose per min.

Isoamylase from *Pseudomonas amylodermosa* was obtained from Hayashibara Biochemical Laboratories, Ltd (Okayama, Japan). Its activity was determined by reaction at 37°C with potato amylopectin (50 mg mL^{-1}) buffered with 0.1 M pyridine acetate (pH 5.0). Aliquots were taken with time and the increase in the reducing value was measured by a microsample-plate copper bicinchoninate assay [9]. One international unit (IU) of isoamylase was that amount of enzyme that released 1 μmol of maltose equivalent per min.

Starches.—Soluble starch was prepared by heating 25 g of potato starch in 100 mL of anhydrous EtOH containing 1 mL concentrated HCl for 1 h at 65°C [10].

Waxy maize and maize starches were obtained from American Maize Co. (Hammond, IN). Amylomaize-7 (Hylon VII, high amylose maize starch with 70% amylose), tapioca, and potato starch were obtained from National Starch and Chemical Co. (Bridgewater, NJ). Large granule barley starch was a gift from Dr J. Jane who obtained it from Professor K.S. Poutanen (VTT Food Research Laboratory, Espoo, Finland). Shoti starch is from the white turmeric tuber of the curcuma species [11] and was a gift to the Laboratory of Carbohydrate Chemistry and Enzymology at Iowa State University by the late Dr T.J. Schoch. Modified potato starch was prepared by reacting potato starch (12% moisture) in anhydrous MeOH, EtOH, 2-propanol, and 1-butanol with 0.36% HCl for 72 h at 20°C. The starches were filtered and washed with 95% EtOH until the washings were neutral to litmus [12].

Reaction of glucoamylase with starch granules.—Starch granules (100 mg) were suspended in 1.4 mL of 50 mM acetate buffer (pH 4.6) and 0.2 mL of 0.2% NaN_3. The reaction was initiated by the addition of 0.4 mL of enzyme that had 4-, 40-, or 400-IU of activity; the reaction was conducted at 37°C; aliquots (0.3 mL) were taken at various times (0–32 h) after stirring the mixture to obtain a homogeneous suspension of starch granules and reaction solution. The reaction was stopped by the addition of 55 μL of 0.2 M HCl to the aliquots to give a pH of 2. The suspension was centrifuged for 1 min in a micro centrifuge and divided into supernatant and packed starch granules. The granules were suspended in 3 mL of water and placed into a boiling-water bath for 10 min to

ensure inactivation of the enzyme. The solution was then neutralized by the addition of 10 μL of 0.02 M NaOH containing 0.1 M Tris-HCl and the starch was solubilized by autoclaving for 20 min at 121°C. The original supernatant was placed in a boiling-water bath for 5 min to completely inactivate the enzyme. The cooled supernatant solution (250 μL) was neutralized by the addition of 70 μL of 0.2 M NaOH containing 0.1 M Tris-HCl. The amount of D-glucose in the original supernatant and in the solubilized starch solution was determined by the glucose oxidase assay.

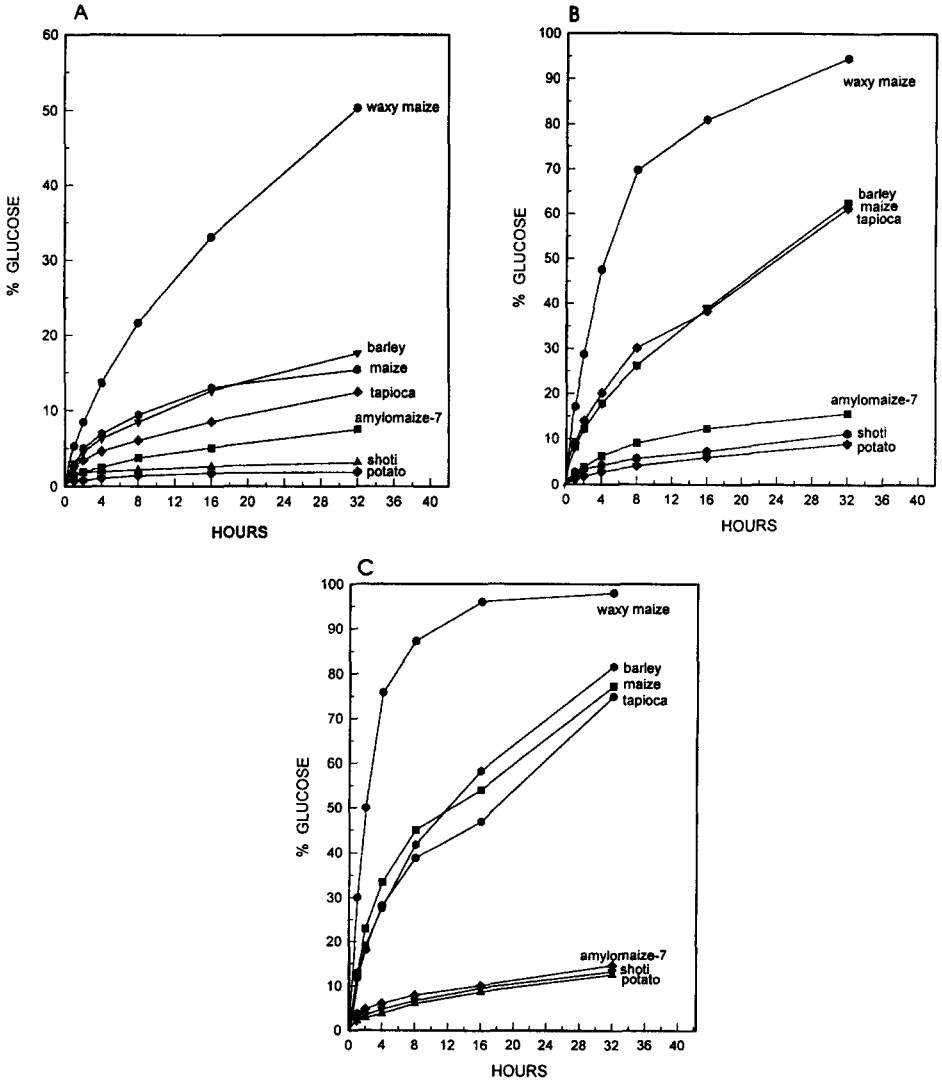

Fig. 1. Kinetics of glucoamylase hydrolysis of native starch granules: (A) hydrolysis with 2 IU mL^{-1} (1X) enzyme; (B) hydrolysis with 20 IU mL^{-1} (10X) enzyme; (C) hydrolysis with 200 IU mL^{-1} (100X) enzyme.

Scanning electron microscopy of starch granules.—Scanning electron micrographs were obtained for native starch granules, modified starch granules, and starch granules that had reacted with glucoamylase for 32 or 288 h. The starch granules were suspended in 95% EtOH and one drop of each suspension was applied to adhesive metal tape attached to a specimen stud. The starch granules were evenly distributed on the surface of the tape, and the ethanol evaporated. The samples were then coated with 3:2 gold–palladium. The micrographs were obtained in the Microscopy Laboratory of the Department of Botany, Iowa State University using a JEOL JSM-35 scanning electron microscope (JEOL, Tokyo, Japan).

Number and size of the granules in the reaction.—The starch granules were reacted with the following amounts of glucoamylase: waxy maize starch (20 U mL^{-1}); and all of the other starches (200 U mL^{-1}). Uniform samples of granules in the glucoamylase reaction mixture (50 μL) were taken at various times (0–32 h, 5–7 aliquots/digest); 180 μL of 0.01 M HCl was added to stop the enzyme reaction. The aliquots were centrifuged and the supernatant separated from the granules. The granules were washed three times with 250 μL of 0.1 M sodium phosphate buffer (pH 6.7) and then suspended in 0.5 mL of water. The granules were counted and sized using an Epics Profile Flow Cytometer (Coulter Corp., Hialeah, FL). Three separate determinations were made for each sample and the mean and standard deviation determined.

Determination of the amount of glucoamylase adsorbed onto the granules.—An amount of enzyme was added to the starch granules (50 mg mL^{-1}) to give 2 IU mL^{-1}; the mixture was incubated at 37°C and aliquots (0.3 mL) were removed at 10, 30, and 60 min by centrifugation. The supernatants were assayed for glucoamylase activity. The per cent of adsorbed glucoamylase was determined by the following equation: $(T\text{-}S)/T \times 100$, where T is the total activity added per aliquot volume and S is the activity assayed per aliquot volume.

Isoamylase analysis of starch granules.—The degree of polymerization (dp) was determined [13] by measuring the total carbohydrate by the phenol–H_2SO_4 method [9] and the reducing value by the copper bicinchoninate method [9] for native starch granules, native starch granules after reaction with isoamylase, glucoamylase-reacted starch granules, and glucoamylase-reacted starch granules after reaction with isoamylase.

3. Results

The kinetics of the reaction of 1X (2 IU mL^{-1}), 10X (20 IU mL^{-1}), and 100X (200 IU mL^{-1}) of *R. niveus* glucoamylase are shown in Figs 1(A)–(C). Waxy maize starch gave 50% conversion into D-glucose in 32 h of reaction with 1X of enzyme. The other starches gave considerably less conversion [Fig. 1(A) and Table 1]. Reaction of 10X amount of enzyme [Fig. 1(B)] clearly shows three classes of starch: waxy maize in the first class was nearly completely (94.5%) converted into D-glucose; barley, maize, and tapioca starches made up an intermediate group that was converted into 62.5, 61.2, and 59.0% D-glucose, respectively in 32 h [Fig. 1(B) and Table 1]; and the third group consisted of amylomaize-7, shoti, and potato starches, which were converted by 10X

Table 1
Percent conversion into D-glucose in 32 h of reaction of starch granules with three concentrations of glucoamylase [a]

Starches	Amount of glucoamylase		
	2 IU mL^{-1}	20 IU mL^{-1}	200 IU mL^{-1}
Waxy maize	50.3	94.5	98.0
Barley	17.6	62.5	81.7
Maize	15.4	61.2	77.3
Tapioca	12.4	59.0	75.0
Amylomaize-7	7.5	15.6	20.9
Shoti	3.2	11.2	14.7
Potato	1.9	8.9	12.6

[a] Reaction was at pH 4.6 and 37°C.

enzyme into 15.6, 11.2, and 8.9% D-glucose, respectively, in 32 h [Fig. 1(B) and Table 1].

Reaction with 100X amount of enzyme [Fig. 1(C)] also shows reactions of three classes of starch. Waxy maize starch was converted into 95% D-glucose in 16 h and then slowly increased to 98% in 32 h: the second class of barley, maize, and tapioca were converted to 81.7, 77.3, and 75.0%, respectively, in 32 h [Fig. 1(C) and Table 1]; in the third class, amylomaize-7, shoti, and potato starches remained relatively resistant to reaction, even with this relatively high amount of enzyme, only being converted into 20.9, 14.7, and 12.6% D-glucose, respectively, in 32 h of reaction.

The modified potato starches gave similar kinetic curves (data not shown), and similar to native potato starch were converted into D-glucose in relatively low amounts, but in higher amounts than unmodified potato starch (Table 2). As with the native starches, the amount of conversion increased with the increase in the amount of enzyme. For methanol-modified potato starch the amount of conversion went from 3.2% D-glucose for 1X amount of enzyme to 13.3% D-glucose for 100X of enzyme; and for 1-butanol-modified potato starch, the per cent conversion to D-glucose went from 15.3% for 1X of enzyme to 27.1% for 100X amount of enzyme. These conversions into D-glucose represent some increase over that of native potato starch that had a conversion

Table 2
Per cent conversion into D-glucose in 32 h of reaction of acid–alcohol modified potato starch with three concentrations of glucoamylase

Potato starch modified [a] in	Starch dp	Amount of glucoamylase		
		2 U mL^{-1}	20 IU mL^{-1}	200 IU mL^{-1}
Methanol	1717	3.2	10.2	13.3
Ethanol	185	4.7	11.9	16.6
2-Propanol	90	8.0	16.5	20.8
1-Butanol	75	15.3	25.7	27.1

[a] Modified for 72 h in the various alcohols with 0.36% HCl at 20°C [12].

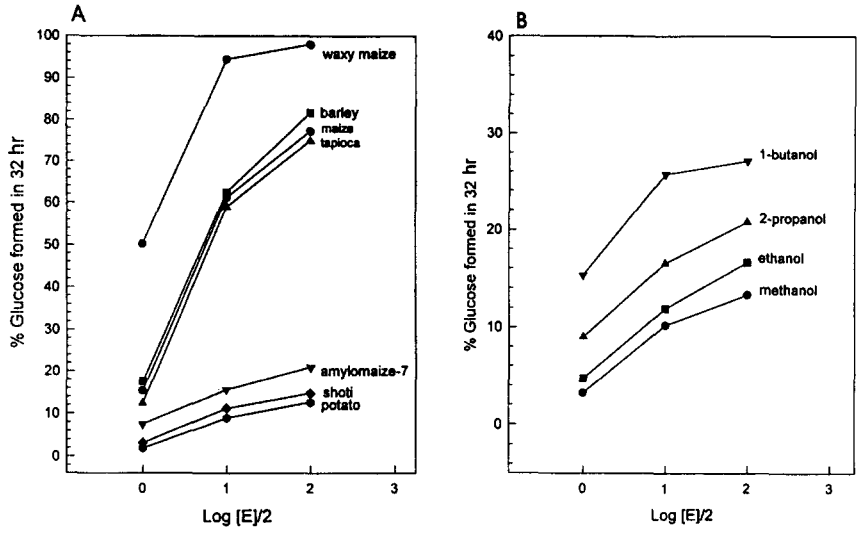

Fig. 2. Effect of the 10-fold increases in the amount of enzyme: (A) hydrolysis of native starch granules; (B) hydrolysis of acid/alcohol modified potato starches.

into D-glucose of 1.9 and 12.6% for 1X and 100X amounts of enzyme. The degree of potato starch granule modification increases in going from methanol to 1-butanol and the per cent conversion into D-glucose by the action of glucoamylase likewise increases for the alcohol-modified potato starch granules (Table 2).

The effect of the 10-fold increases in the concentration of glucoamylase on the per cent conversion of the native starch granules in 32 h of reaction are summarized in Fig. 2(A). This figure shows the relative effects of the increase in the concentration of enzyme and also illustrates the three classes of granule susceptibility for glucoamylase hydrolysis. Fig. 2(B) shows the effect of the 10-fold increase in the amount of glucoamylase on the reaction of the four modified potato starches. This figure illustrates the increase in susceptibility of the potato starches modified in the four alcohols, as well as the effect of the amount of enzyme.

It should be pointed out that if the native starch granules had been solubilized, 1X amount of enzyme (2 IU mL^{-1}) would have converted all of the starch into D-glucose in about 2.5 h for each of the starches; 10X amount of enzyme would have converted all of the starch into D-glucose in 0.25 h or 15 min; and 100X amount of enzyme would have converted all of the starches into D-glucose in 0.025 h or 1.5 min. In the reactions with the native starch granules, 1X amount of enzyme had only converted 10% of the waxy maize starch granules into D-glucose in 2.5 h and less than 1% of the potato starch granules into D-glucose in 5 h. Clearly, the native starch granules are converted into D-glucose much more slowly and to a much lesser amount than the conversion of solubilized starches. Further, there are significant differences in the different types of starches for glucoamylase hydrolysis. If the waxy maize starch granules are allowed to react long enough, it will almost be completely converted into D-glucose. The time can be shortened by increasing the amount of enzyme 10-fold. The reaction, however, does

Table 3
Per cent D-glucose released into the supernatant and percent remaining in the granule after 2 and 32 h of reaction with glucoamylase

Starches		Amount of enzyme					
		1X		10X		100X	
		2 h	32 h	2 h	32 h	2 h	32 h
Waxy maize	S [a]	87.1	89.5	89.0	99.8	91.6	99.8
	G	12.9	10.5	11.0	0.2	8.4	0.2
Barley	S	92.0	93.7	89.6	95.8	89.4	96.8
	G	8.0	6.3	10.4	4.2	10.6	3.2
Maize	S	81.3	92.7	91.8	96.7	93.6	96.6
	G	18.7	7.3	8.2	3.3	6.4	3.4
Tapioca	S	88.6	93.3	93.2	97.2	92.4	97.7
	G	11.4	6.7	6.8	2.8	7.6	2.3
Shoti	S	77.8	81.2	91.5	92.3	84.4	93.9
	G	22.2	18.8	8.5	7.7	15.6	6.1
Amylomaize-7	S	81.2	92.1	84.7	90.8	80.1	91.1
	G	18.8	7.9	15.3	9.2	19.9	8.9
Potato	S	65.1	75.8	77.0	91.4	72.7	90.0
	G	34.9	24.2	23.0	8.6	27.3	10.0
Methanol	S	68.4	85.9	78.7	92.1	76.5	90.4
Potato	G	31.6	14.1	21.3	7.9	23.5	9.6
Ethanol	S	84.2	90.3	81.0	92.3	83.9	93.1
Potato	G	15.8	9.7	19.0	7.7	16.1	6.9
2-Propanol	S	92.2	94.7	88.1	92.2	84.3	90.7
Potato	G	7.8	5.3	11.9	7.8	15.7	9.3
1-Butanol	S	95.3	94.9	90.9	92.1	88.7	92.6
Potato	G	4.7	5.1	9.1	7.9	11.3	7.4

[a] S is % in the supernatant; G is % in the granule.

not give a 10-fold increase in the rate of the amount of D-glucose produced in an equal amount of reaction time. Likewise a significant increase also is observed in the rate and amount of D-glucose formed from barley, maize, and tapioca starches when the amount of enzyme is increased 10-fold. Although there also is an increase in the conversion of amylomaize-7, shoti, and potato starch with 10-fold increases in the amount of enzyme, the overall conversions remained relatively low [Fig. 2(A)].

The distribution of D-glucose in the supernatant and in the granule was determined. Table 3 summarizes the distributions after reaction for 2 and 32 h for the three concentrations of enzyme. In general, the 1X concentration of enzyme gave a higher percentage of D-glucose inside the granule. There also was a higher percentage in the granule in the first few hours (2 h) of reaction than in the later stage (32 h) of reaction. This indicates that the reactions were taking place inside the granule and that as time of reaction increased and the rate of reaction slowed down, the D-glucose produced in the early stages of the reaction inside the granule diffused from the granule out into the aqueous medium. Some variation was observed for the starches from the different botanical sources. Potato starch gave the highest percentage of D-glucose retained in the granule with 34.9% inside the granule in 2 h of reaction and 24.2% in 32 h of reaction.

Table 4
Per cent of glucoamylase adsorbed onto the different starch granules

Starches	Percent adsorbed [a]
Shoti	62.0 ± 0.1
Waxy maize	54.0 ± 3.6
Tapioca	53.3 ± 1.2
Amylomaize-7	40.7 ± 0.9
Maize	40.3 ± 0.9
Barley	34.1 ± 0.4
Potato	33.1 ± 4.7
Methanol/potato [b]	34.5 ± 2.3
Ethanol/potato	38.4 ± 2.0
2-Propanol/potato	34.5 ± 2.7
1-Butanol/potato	41.7 ± 3.3

[a] Determinations were made in triplicate and the standard deviation calculated.
[b] Potato starch was modified in the four alcohols with 0.36% HCl at 20°C for 72 h.

The next highest was shoti with 22.4% in 2 h of reaction and 18.8% in 32 h of reaction. This was followed by amylomaize-7 and maize with 18.8 and 18.7% in 2 h and 7.9 and 7.3% in 32 h respectively. The species that retained D-glucose least was barley starch. The methanol-modified potato starch retained 31.6% in 2 h of reaction and 14.1% in 32 h of reaction. Thereafter, the percentage of D-glucose retained in the granule decreased with increase in the hydrophobic character of the alcohol. These results suggest that the third class of starches that were not converted into high amounts of D-glucose are not very permeable to diffusion of enzyme into the granule and diffusion of D-glucose out of the granule. As the degree of modification of the potato starch increased, the amount of conversion into D-glucose increased, suggesting that the amount of enzyme diffusion into the granule increase as well as the amount of diffusion of D-glucose out of the granule increased.

The percentage of glucoamylase adsorbed onto the various starch granules is given in Table 4. Shoti starch adsorbed the highest amount (62.0 ± 0.1%) and potato starch the least (33.1 ± 4.7%). The percentage of adsorption increased for potato starch as the degree of acid–alcohol modification, with 1-butanol-modified potato starch adsorbing 41.7 ± 3.3%. The data are not consistent with any apparent structural feature of the granules. Both shoti and potato starches belonged to the third class of starches that were not readily converted into D-glucose by action of glucoamylase and represented the starches that adsorbed the most and the least. It might have been expected that the amount of conversion into D-glucose would have been proportional to the amount of adsorption, but this was not observed. Besides the difference observed for shoti and potato starches, waxy maize starch adsorbed 54.0 ± 3.6% and tapioca starch adsorbed an almost identical amount, 53.3 ± 1.2%, and yet had a significant difference in the amount of D-glucose formed in 32 h for 1X amount of enzyme, 50 and 12.4%, respectively.

The number of granules in the reaction digests over a 32 h period is given in Figs 3(A)–(G). As the reactions progressed, the number of granules increased for five of the starches. The numbers dramatically increased for waxy maize starch, doubling over 4 h;

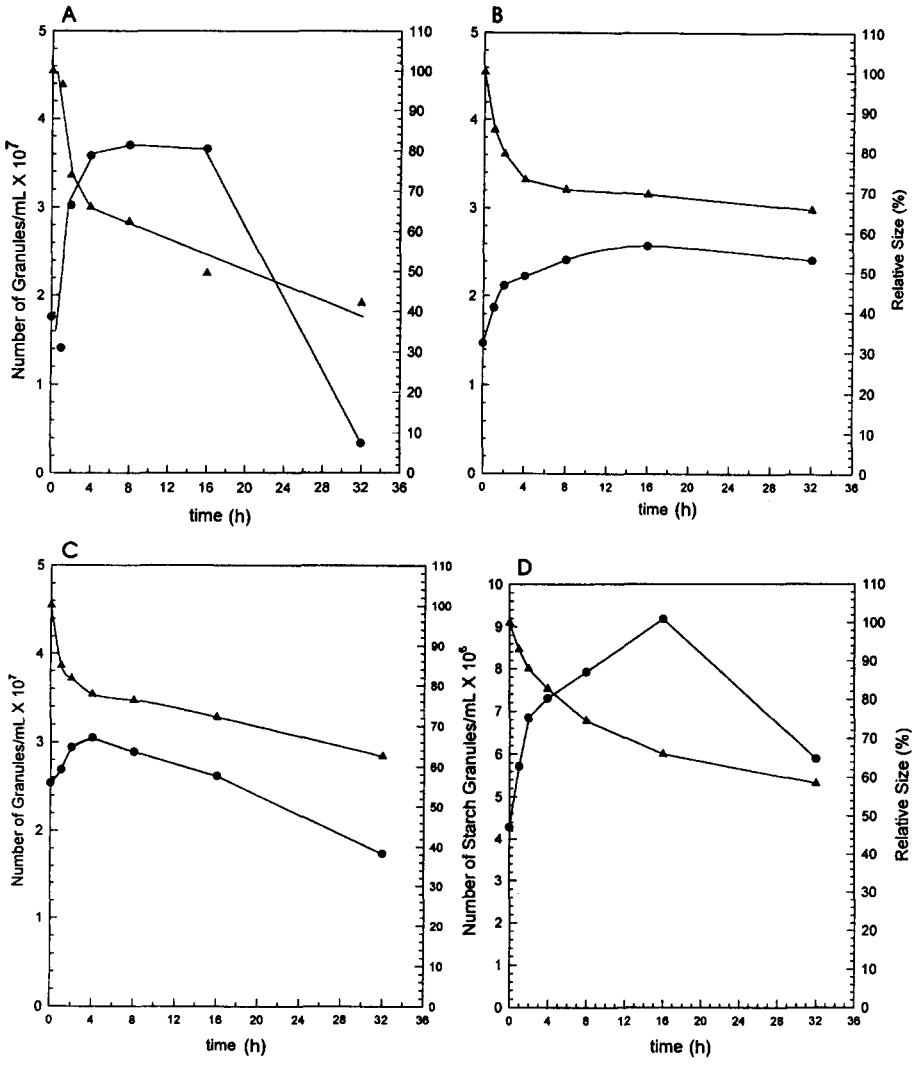

Fig. 3. Number and size of the starch granules during glucoamylase hydrolysis: (A) waxy maize (20 U mL^{-1}); (B) maize; (C) tapioca; (D) barley; (E) potato; (F) shoti; (G) amylomaize-7 were each reacted with 200 U mL^{-1}. -●- number, -▲- relative size.

thereafter the numbers remained constant for 12 h and then progressively decreased to a low level in the following 16 h of reaction. Barley starch granules also doubled, over 16 h of reaction and then decreased in the following 16 h to a value somewhat above the starting value. Maize starch granule numbers were less dramatic, increasing by about 67% in 16 h, followed by a slight decrease in the next 16 h. Tapioca starch granules had a modest 20% increase in numbers in 4 h and then steadily declined to a value of ca. 80% of that of the starting number. Shoti starch granules also had a 20% increase in the

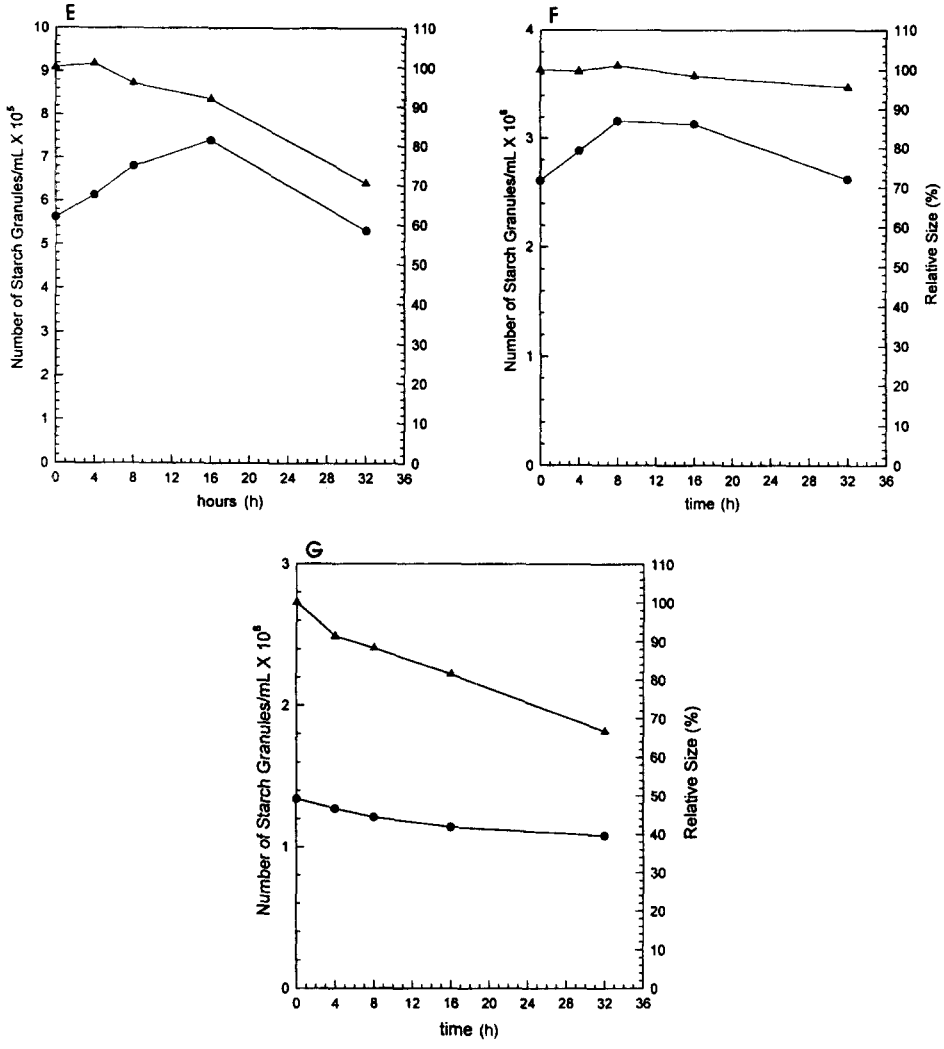

Fig. 3 (continued).

number of granules in 8 h of reaction, which then leveled off and decreased in 32 h to that of the starting number. The number of potato starch granules only slightly increased and then declined in 32 h to that of the starting number. Amylomaize-7 starch was the only starch that did not increase in number of granules and slowly, but steadily decreased to 23% of the number of granules at the start of the reaction. These data indicate that the reaction of glucoamylase breaks the granules into smaller pieces, especially in the reaction of waxy maize and barley starches. It further supports the hypothesis that reaction is taking place inside the granule as the relatively high per cent conversions into D-glucose for barley, tapioca, and maize starches (81.7, 77.3, and

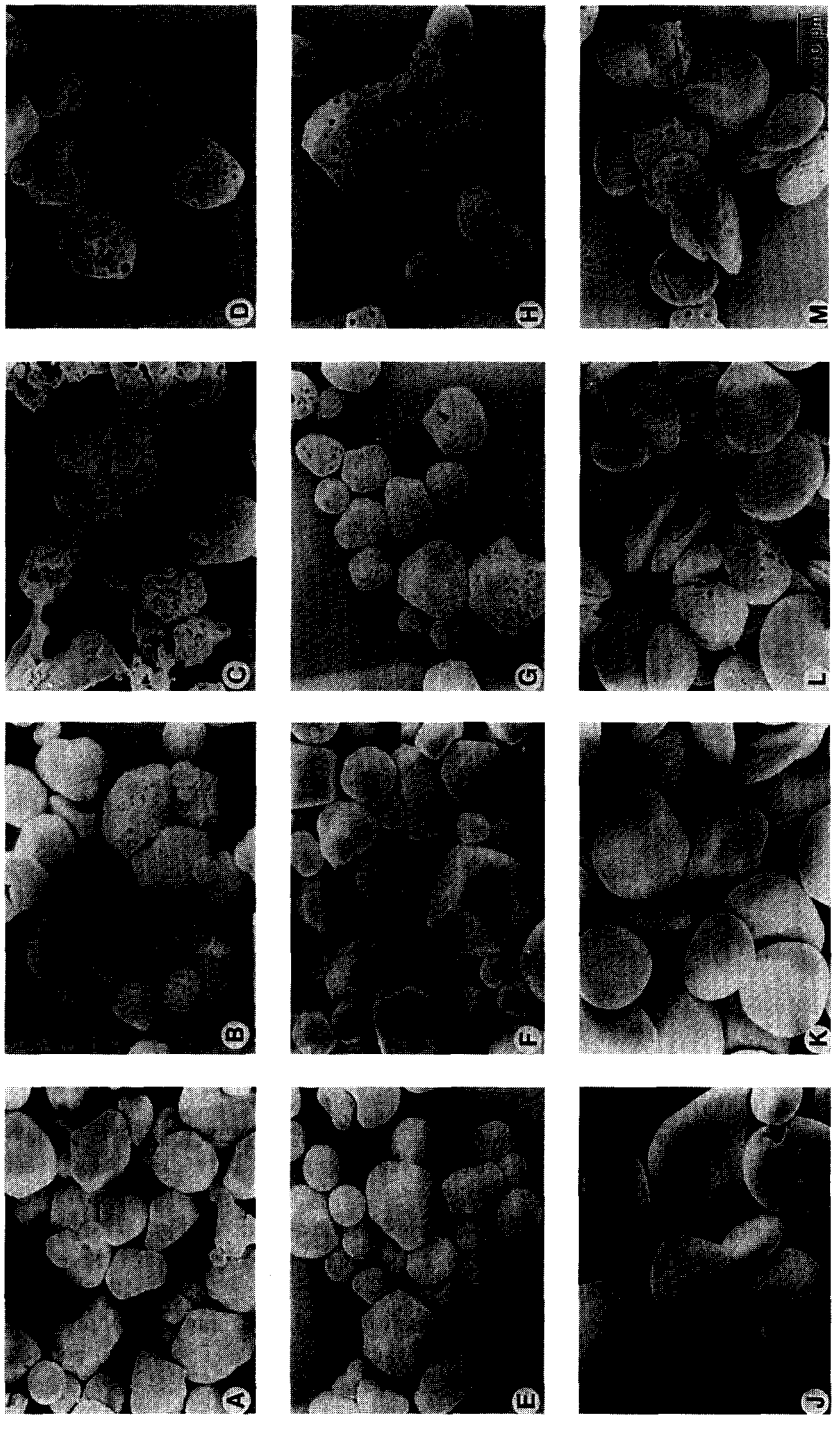

Fig. 4. Scanning electron microscopy of native starch granules at various stages of reaction with glucoamylase. In the first three rows, the micrographs show untreated starch, 1X enzyme for 32 h, 10X enzyme for 32 h, and 100X enzyme for 32 h, respectively, for: (A)–(D), waxy maize starch; (E)–(H), maize starch; (J)–(M), barley starch. In the next four rows, the micrographs show unreacted starch, 1X enzyme for 32 h, 10X enzyme for 32 h, 100X enzyme for 32 h, and 10X enzyme for 288 h, respectively, for: (N)–(S), tapioca starch; (T)–(X), amylomaize-7 starch; (Y)–(c), shoti starch; and (d)–(h), potato starch.

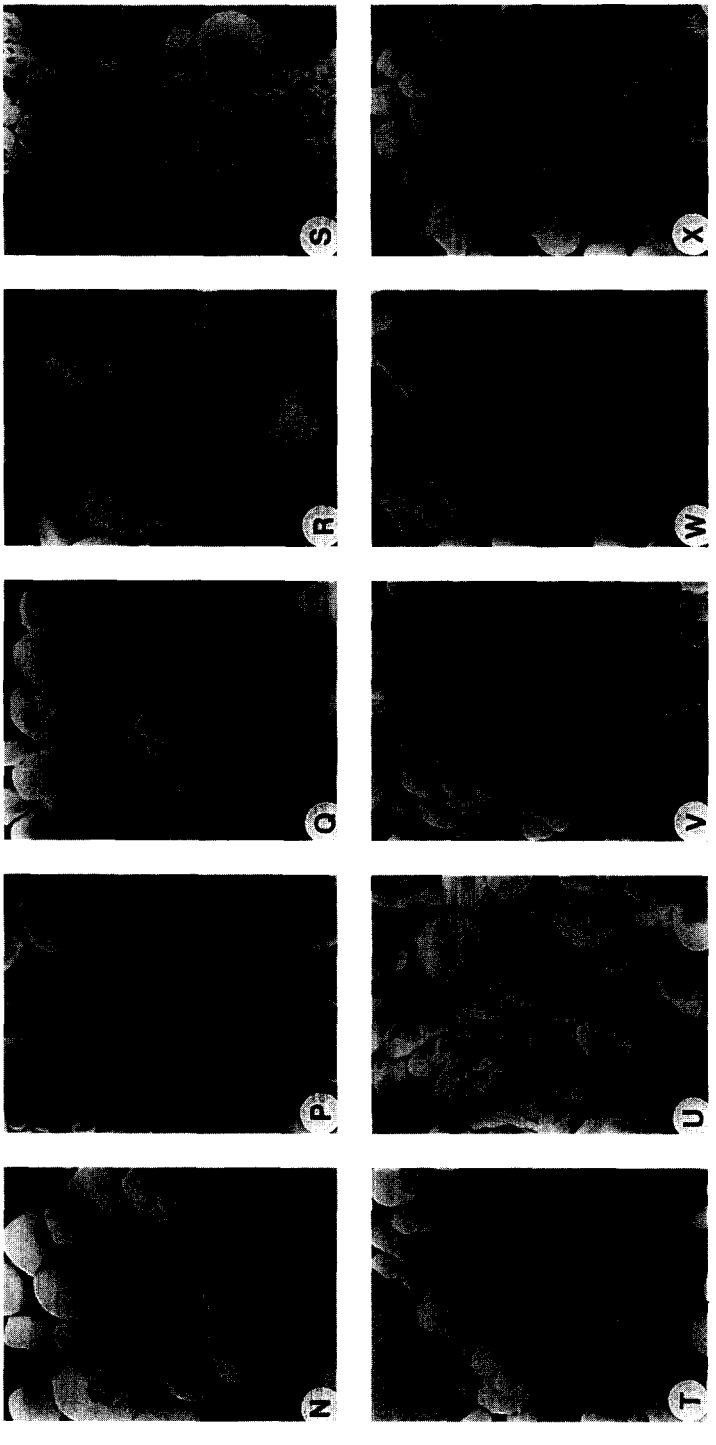

Fig. 4 (continued).

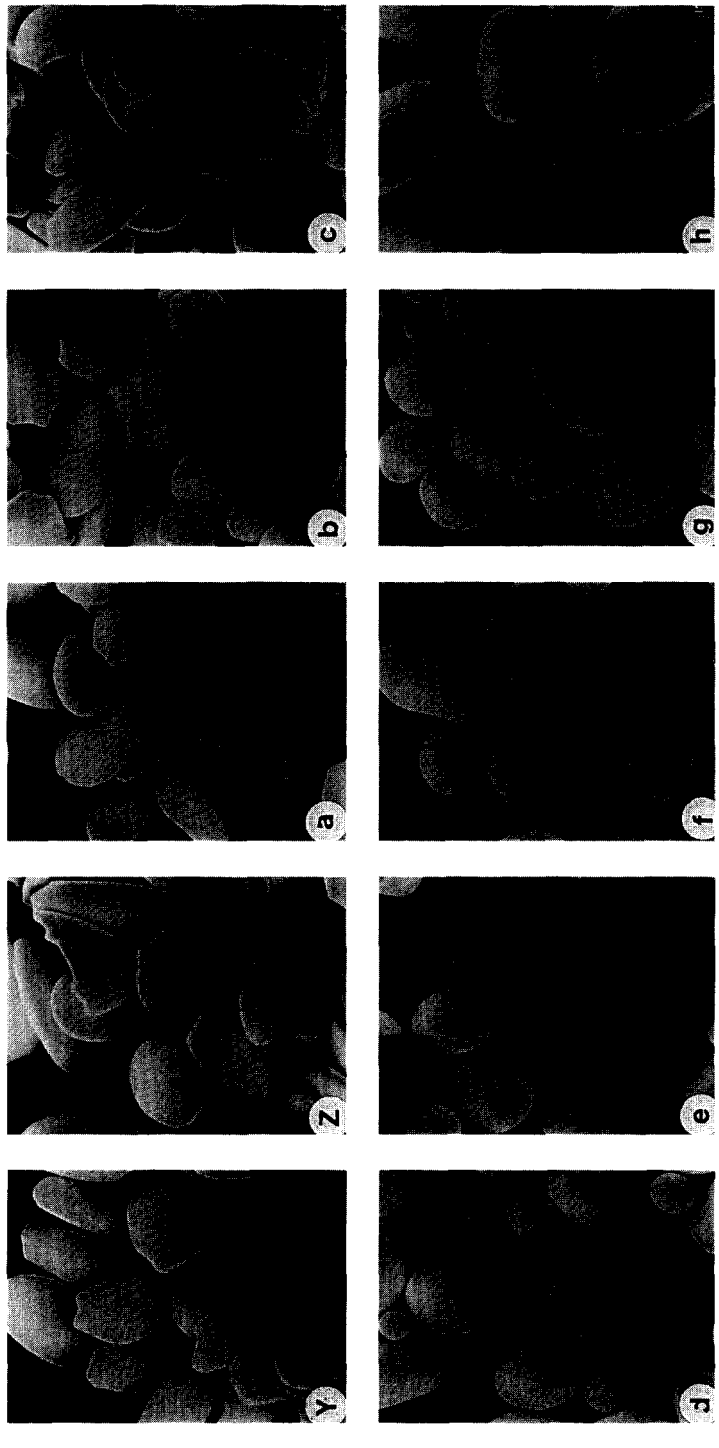

Fig. 4 (continued).

75.0%, respectively) with 100X amount of enzyme do not decrease the number of granules proportionally. An exception, is waxy maize, where the per cent conversion is high (95%) and the numbers of granules are low. The numbers of potato or shoti starch granules do not significantly decrease and are converted by 100X amount of enzyme to 14.7 and 12.6%, respectively.

The relative size of the granules is also shown in Fig. 3. Waxy maize and barley starches decreased by ca. 50% in 32 h of reaction; maize, tapioca, and amylomaize-7 starches decreased in size by ca. 40% each in 32 h of reaction. Potato starch ca. 15% decrease and shoti starch had only a 5% decrease in size in 32 h of reaction.

The morphology of the starch granules was studied by scanning electron microscopy for the different amounts of enzyme and times of reaction. These are shown in Fig. 4. For each starch, the unreacted granules, granules with 1X amount of enzyme for 32 h, 10X for 32 h, 100X for 32 h, and 10X for 288 h are given. Waxy maize starch shows the classical "Swiss cheese" shell appearance with numerous deep holes into the granule with 1X amount of enzyme; this becomes much more pronounced with 10X and 100X amount of enzyme in 32 h and 10X in 288 h of reaction. Maize starch shows only slight changes with 1X enzyme, more observable with 10X enzyme, and significant shell structure with deep holes with 100X amount of enzyme. Barley showed a similar pattern to that of maize. Tapioca starch showed only a very slight number of holes with 10X enzyme, but significant numbers with 100X enzyme. Amylomaize-7, shoti, and potato starches showed only slight surface pitting at 100X amount of enzyme and no deep holes. Although the scanning electron micrographs of the acid/alcohol-modified potato starches (Fig. 5) did not show the "Swiss cheese" deep-holed structures, they had

Table 5
Degree of polymerization of the starches, the starches remaining after glucoamylase hydrolysis, and after isoamylase hydrolysis

Starches		dp_{NAT} [a]	$dp_{GA\ HYD}$ [b]	$dp_{IA\ HYD/GA}$ [c]	$dp_{IA\ HYD/NAT}$ [d]
1X GA [e]	Waxy maize	1720	830 (50%) [f]	14.0	21.6
10X GA [e]	Maize	1760	516 (60%)	17.6	28.2
	Tapioca	4110	949 (60%)	17.1	27.2
	Barley	2110	532 (60%)	19.4	27.9
100X GA [e]	Potato	1030	777 (13%)	17.0	28.2
	Amylomaize-7	320	231 (15%)	53.9	72.0
	Shoti	288	209 (20%)	31.6	49.6
	Maize	1760	398 (77%)	16.5	28.2
	Tapioca	4110	608 (75%)	16.1	27.2
	Barley	2110	503 (81%)	17.8	27.9

[a] dp of native starches.
[b] dp of the starch that remains after glucoamylase hydrolysis; the number in parentheses is the per cent conversion to D-glucose.
[c] dp after isoamylase hydrolysis of the starch remaining after glucoamylase hydrolysis.
[d] dp after isoamylase hydrolysis of the native starch.
[e] 1X, 10X, and 100X indicates the amount of glucoamylase (GA) in the hydrolysis for each starch analyzed in the table.
[f] Percentages in parentheses indicate the amount of conversion into D-glucose.

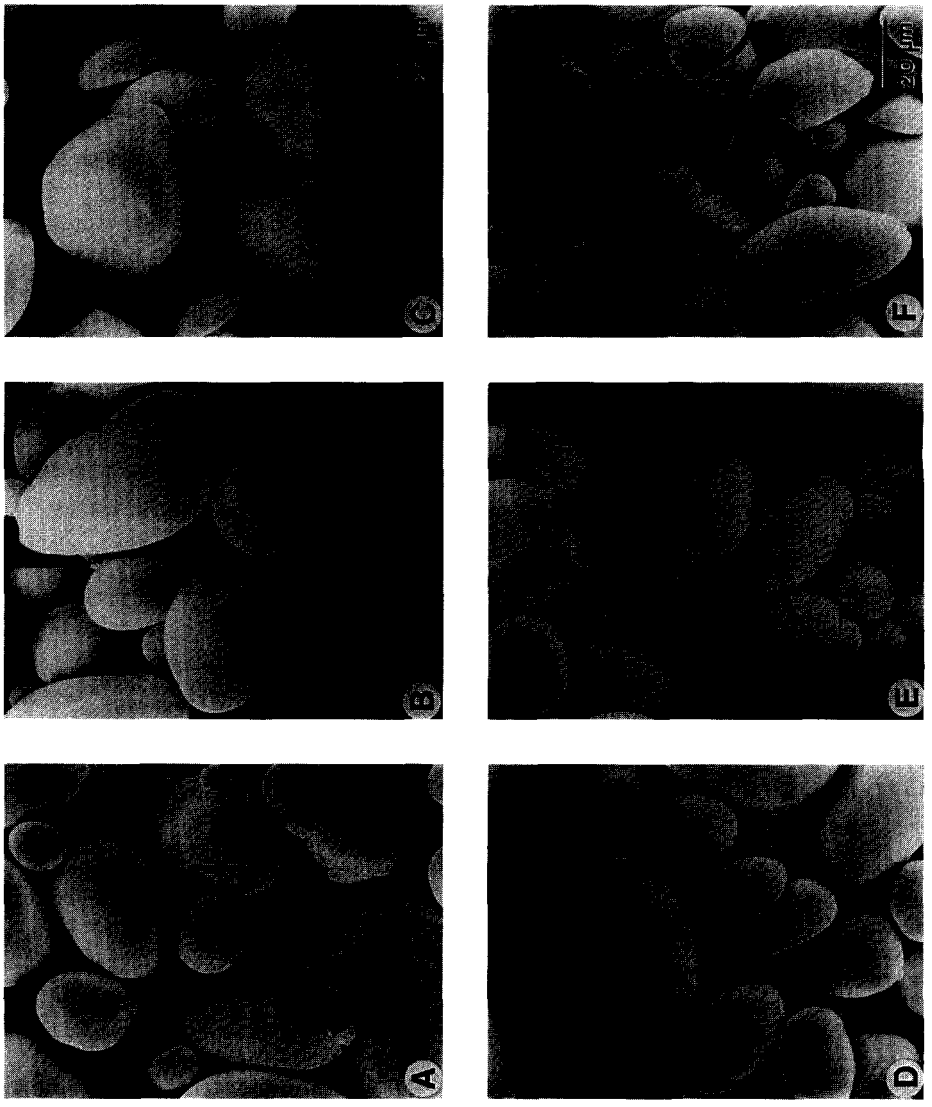

Fig. 5. Scanning electron microscopy of acid/alcohol modified potato starches at various stages of reaction with glucoamylase. Each row is a different modified starch showing granules soaked in buffer for 32 h, 10X enzyme for 32 h, and 10X enzyme for 288 h, respectively, for: (A)–(C), methanol-modified; (D)–(F), ethanol-modified; (G)–(J), 2-propanol-modified; and (K)–(M), 1-butanol-modified.

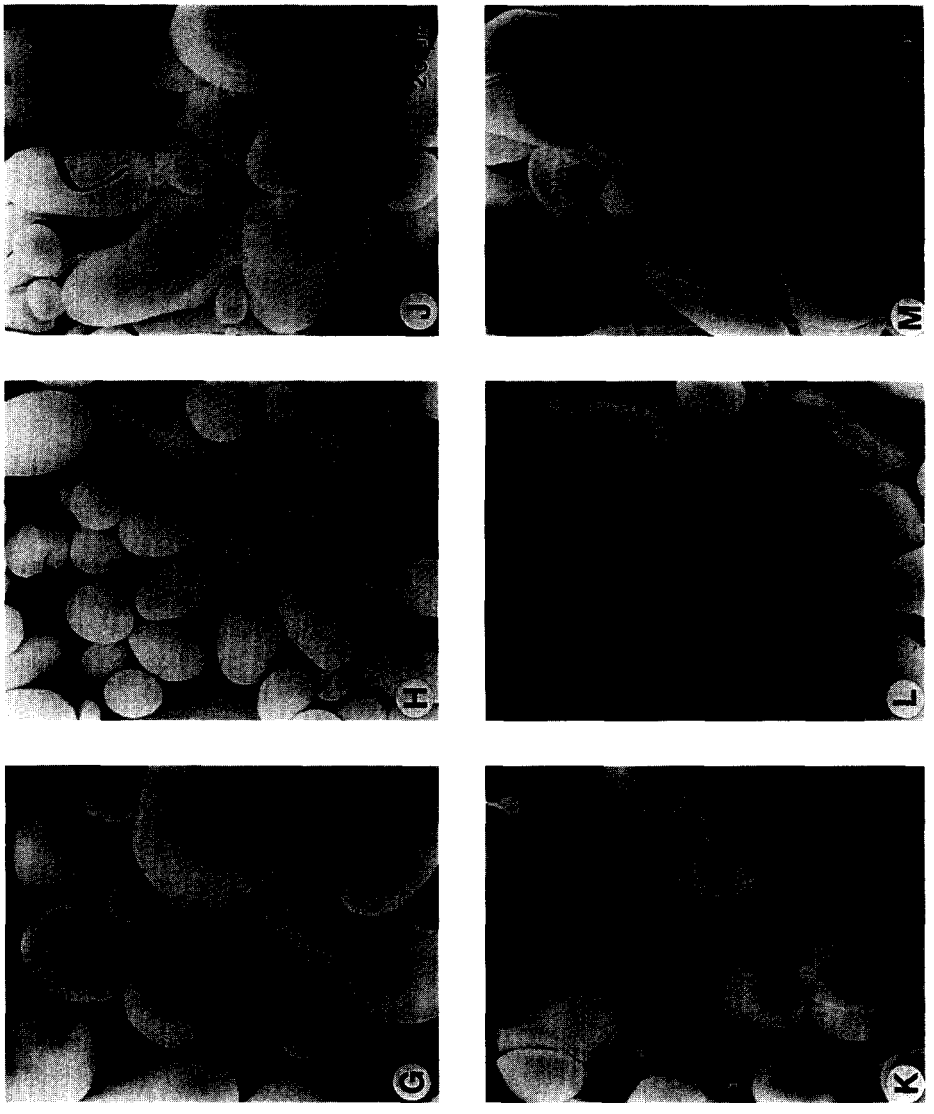

Fig. 5 (continued).

cracks and fissures in the structures that showed a hollow shell structure after reaction with glucoamylase, especially for the 10X enzyme at 288 h of reaction.

The "Swiss cheese" structure with numerous deep holes was first reported by Smith and Lineback [8]. These structures also suggest that the glucoamylase hydrolysis of starch granules occurs inside the granule, at least for the first and second classes of starches and the modified potato starches. The third class that was not very extensively hydrolyzed did not show this type of structure.

Table 5 gives the degree of polymerization (dp) values for the native starches, the dp for the starches remaining after reaction with glucoamylase, the dp of the isoamylase products of the native starches, and the dp of the isoamylase-treated material remaining after reaction with glucoamylase. The dp of all of the native starches as well as the dp of the average chain lengths are significantly decreased after reaction with glucoamylase. These dp values characterize the reactants and products, but nothing specific can be inferred about the structures of either the native starches or of the material that is resistant to glucoamylase hydrolysis.

4. Discussion

It is quite apparent from this study that starch granules from different kinds of botanical sources have widely different susceptibilities to glucoamylase hydrolysis and that all of the starch granules studied are much more resistant to hydrolysis than is solubilized starch. Waxy maize starch, which is devoid of the linear amylose component, was the most susceptible, being converted into 95% D-glucose by 10X enzyme in 32 h. In contrast, the high amylose maize starch, amylomaize-7, was relatively resistant, only being converted into 20.7% D-glucose by 100X enzyme in 32 h. The least susceptible, potato starch, which has a "normal" distribution of 25% amylose and 75% amylopectin, was only converted into 1.9% D-glucose by 1X enzyme in 32 h and 12.6% D-glucose by 100X in 32 h. Other starches, for example barley, maize, and tapioca, which also have a "normal" distribution of amylose and amylopectin of $\sim 25\%$ and $\sim 75\%$, were much more susceptible, being converted into 75–80% D-glucose by 100X glucoamylase in 32 h.

It might have been expected that waxy maize starch would have been the least susceptible, as glucoamylase hydrolyzes the α-(1 $\rightarrow$ 6) linkage about 1/20th as fast as the α-(1 $\rightarrow$ 4) linkage; waxy maize starch has, on a weight basis, a much higher content of α-(1 $\rightarrow$ 6) linkages than the starches with a "normal" distribution of amylose and amylopectin and than the high amylose starch, which has the lowest content of α-(1 $\rightarrow$ 6) linkages. It might, therefore, also have been expected that the high-amylose starch would have been the most susceptible as it has the highest content of α-(1 $\rightarrow$ 4) linkages. Leach and Schoch [4] concluded that the presence of the linear amylose component retards the alpha-amylase hydrolysis of the granule. While this might be supported from the present study for glucoamylase, it does not really appear to be the significant factor in determining the relative susceptibilities of the granules toward hydrolysis. Potato starch, which contains a "normal" distribution of amylose and amylopectin, was even less susceptible than the high amylose maize starch. Other

starches with "normal" amounts of amylose and amylopectin were much more susceptible. The amount of amylose in the granule does not seem to be a contributing factor in glucoamylase susceptibility.

Leach and Schoch [4] also concluded that enzymes do not penetrate into the granule and that their action was limited to certain regions on the surface of the granule. This is in contrast to the present findings and to those of Smith and Lineback [8] that when ca. 50% of the granule has been converted into D-glucose, scanning electron micrographs show numerous deep holes into the granule. At higher degrees of conversion, the granules appear to be shells with the interior hydrolyzed. The penetration of the starch granule by the enzyme is also supported by our study of the per cent of D-glucose inside the granule and outside in the reaction supernatant. In the earlier stages of the reaction, namely 2 h, there was uniformly a higher percentage of D-glucose inside the granule than there was at a much later stage of reaction, namely 32 h, when the diffusion of the D-glucose into the supernatant had had time to take place.

The data on the size and number of starch granules also supports the hypothesis that the granules are primarily hydrolyzed within the granule. The reactions of waxy maize, maize, tapioca, and barley starches all initially gave a doubling of the number of granules with a concomitant decrease in the size of the granules. This suggests that the action of the enzyme initially breaks the granules into two fragments of approximately equal size. Thereafter, the granules do not significantly change in number or size, indicating that the reaction is taking place within the granules. With shoti and potato starches there is only a slight increase in the number of granules and the numbers and size of the granules do not significantly change after 32 h of reaction with 100X amount of enzyme. Gallant et al. [14,15] and Valetudie et al. [16] also have observed that alpha-amylases enter starch granules and hydrolyze the interior of the granule, leaving various shells and internal layers.

It might be considered that the variance in the susceptibility of the starch granules to glucoamylase hydrolysis is related to the relative ease of gelatinization and solubilization of the individual types of starches. This too, however, is not consistent with the relative susceptibilities found in this study or in studies with alpha-amylases. Potato starch, which is readily gelatinized with a low gelatinization temperature of 60°C, was the least susceptible to glucoamylase hydrolysis and tapioca starch, which also readily gelatinizes and has a gelatinization temperature of 60°C, was much more susceptible to glucoamylase hydrolysis than was potato starch. Maize starch, which is much more difficult to gelatinize with a gelatinization temperature of 70°C, had about the same degree of susceptibility to glucoamylase hydrolysis as did tapioca starch, which is relatively easy to gelatinize. Further, waxy maize starch that has a gelatinization temperature of 65°C was the most susceptible. Amylomaize-7 starch, which is the most difficult to gelatinize with a gelatinization temperature of 90°C, was in the least susceptible category toward glucoamylase hydrolysis, but it was somewhat more susceptible than potato starch.

It has been speculated [4,14–17] that amylases hydrolyze native starch granules by entering the granule through pores or a loose, sponge-like structure that permits the enzyme molecules to penetrate into the granule and hydrolyze the starch chains. These pores might be inherent properties of the various starches and that the number may vary

according to the type of starch. Hall and Sayre [17,18] are credited with first observing the presence of pores in starch granules by scanning electron microscopy. The presence of pores has more recently been observed by Fannon et al. [19] for maize, sorghum, and millet starches and by Baldwin et al. [20] for potato, rice, and wheat starches. Fannon et al. [19] state that the pores are normal anatomical features of the native starch granule and that they were not artifacts produced by the isolation or observation techniques. Baldwin et al. [20] stated that potato starch has pores at the hilum. In this study, the observation of numerous holes in the granules as the reaction of glucoamylase progresses inferentially argues for the presence of pores in the original granules. The pores are enlarged by enzyme hydrolysis, making them visible. Differences in the numbers, depth, and locations of pores for the different varieties of starches could explain the observations made in this study on the wide variation of the rate and extent of reaction of the various starches to glucoamylase hydrolysis.

Potato starches that were acid-hydrolyzed in different alcohols had an increase in the number of holes and fissures in the granules and were more susceptible to glucoamylase hydrolysis. The increase, however, was not dramatic. It increased from 1.9% for native starch to 3.2% for methanol–acid hydrolyzed starch by 1X amount of enzyme and from 12.6% for native starch to 13.3% for 100X amount of enzyme. The increase for the 1-butanol–acid hydrolyzed starch (a material that had the highest degree of modification) was from 1.9 to 15.3% for 1X amount of enzyme and from 12.6 to 27.1% for 100X amount of enzyme. In none of the cases of alcohol–acid modified starches were the conversions into D-glucose close to the conversions observed for the most susceptible starch, waxy maize, or for the intermediately susceptible starches of barley, maize, or tapioca. While the modest increase shows that the number of pores and fissures is important, it also shows that the inherent internal structure of the starch chains in the various types of granules is more important in determining the overall extent to which the granules undergo reaction with amylases.

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