Bound Free Fatty Acids in Glucoamylase-Digested Starches of Corn and Sweetpotato

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ABSTRACT

The raw starches from corn and sweetpotato were digested by glucoamylase. The lipids remaining in the residual starch granules, especially free fatty acids (FFA), were investigated. FFA were determined by high-performance liquid chromatography after fluorescence labeling of the FFA. This method enabled the determination of the FFA content with samples of 20–100 mg without requiring any procedures for concentration. The total FFA content of corn starch (18.41 μmol/g of starch) was much larger than that of sweetpotato starch (1.07 μmol/g of starch), which is in agreement with previous reports. At the initial stage of digestion, the FFA in both starches were rapidly released. During the later stages, linoleic acid in corn starch decreased in proportion to the extent of the digestion, but the FFA in sweetpotato were hardly released. Gel-permeation chromatography analysis of both starches showed no significant difference in the mode of enzyme action on the starch components. On the other hand, observation by scanning electron microscopy of the residual starches revealed differences in their appearances, suggesting that the manner in which they are digested is different.

Starch granules usually contain lipids, the content and composition of which varies from plant to plant (Morrison 1988). In general, the lipids are classified into two groups on the basis of extractability from granules with common fat solvents. Morrison (1981) has also classified the lipids experimentally into three categories: nonstarch lipids, starch surface lipids, and internal starch lipids. In both classifications, location and existing state of the respective lipids has become a theme of research. We have divided the lipids into two groups based on extractability from granules with diethyl ether. Ether-extractable lipids are considered to be adsorbed on the surface of starch granules. The other lipids that cannot be extracted with diethyl ether, but can be extracted with hot aqueous alcohol, exist firmly in starch granules. We call these lipids bound lipids. Most of the bound lipids, mainly free fatty acids (FFA) and lysophospholipids, are considered to be complexed with amylose in granules. There is a good correlation between amylose and lipid contents in maize starches of various mutants (South et al 1991). Recently, further study, using a solid-state nuclear magnetic resonance technique, has proved the existence of the complex in intact starch (Morrison et al 1993).

Previously, we reported (Kitahara et al 1993) that when FFA were artificially introduced into three kinds of starches (gajutsu [*Curcuma zedoaria* Roscoe], teppô-yuri [*Lilium longiflorum* Thunb.], and potato starches), there were differences in the quantity of FFA introduced. Also, when FFA was introduced into the Nägeli-amylodextrins, which are regarded as a model of the crystalline portion, the quantities of introduced FFA decreased and became comparable. From the results, we speculated that the quantity of introduced FFA might depend upon differences in the structure of the amorphous portion of the starches. Thus, study of the lipids in starch granules may give useful information concerning, not only the nature of starch-lipid relationships, but also the granule structures of native starch.

In this study, the raw starches from corn and sweetpotato were digested by glucoamylase, and the lipids remaining in the residual starch granules, especially FFA, were investigated. To determine the FFA compositions with high sensitivity, we applied the method of fluorescence labeling of FFA (Nimura and Kinoshita 1980), followed by high-performance liquid chromatography (HPLC). Also, the residual starches were examined by gel-permeation chromatography (GPC), and scanning electron microscopy (SEM).

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MATERIALS AND METHODS

Starches and Reagents

Corn starch was obtained from Nihon Shokuhin Kako Co., Ltd. (Tokyo). Sweetpotato starch was obtained from Ei Agricultural Cooperatives (Kagoshima). Starch samples used in this study were washed with diethyl ether for 24 hr in a Soxhlet extractor to remove lipids adsorbed on the surface of the granules. All reagents and solvents, unless otherwise specified, were obtained from Wako Pure Chemical Industries (Osaka) and were of reagent grade.

Determination of Bound FFA in Starch

In this study, the bound FFA were determined by two methods. Method 1: To determine the FFA composition, a starch sample (20 mg of corn starch or 100 mg of sweetpotato starch) containing 0.1–1.0 μmol of FFA was placed in a screw-cap tube and liquefied with 1 ml of 0.5% (v/v) Termamyl 120L (Novo-Nordisk A/S, Denmark) for 10 min at 95°C. Three milliliters of 1-propanol was added to the hydrolysate, and then the mixture was heated for 10 min at 95°C. After addition of behenic acid as an internal standard, insoluble substances were removed by centrifugation at 7,000 × *g* for 10 min. An aliquot of the supernatant was mixed with the same volume of 0.05% 9-anthryldiazomethane (Funakoshi Co. Ltd., Tokyo) in methanol to label FFA as fluorescent derivatives. After allowing to stand for 1 hr, 5 μl of the reaction mixture was injected to an HPLC (Tosoh Co. Ltd., Tokyo) using a LiChrospher RP-8 (E. Merck, Germany) column (0.46 × 25 cm), and eluted with 90% acetonitrile for 25 min and then 100% acetonitrile for 20 min at a flow rate of 1.1 ml/min. The fluorescence was measured at 412 nm, with excitation at 365 nm, using a fluorescence HPLC monitor (Shimadzu Co., Tokyo). Method 2: Starch sample (20 mg of corn starch or 100 mg of sweetpotato starch) containing 0.1–2 μmol of FFA was liquefied as described above. One milliliter of 1-propanol was added to the hydrolysate, and then the mixture was heated for 10 min at 95°C. After centrifugation at 4,500 × *g* for 10 min, the FFA in the supernatant of the aqueous propanol solution were determined as the total FFA using a NEFA-C test kit (Wako), which consisted of acyl-CoA synthetase, acyl-CoA oxidase, peroxidase, and a color-producing reagent.

Both of the determinations were performed in triplicate or quadruplicate. Coefficients of variation were <3%.

Digestion of Starch Granules by Glucoamylase

Starch granules were digested by glucoamylase (specific activity: 16.8 U/mg) from *Rhizopus niveus,* which was purchased from

Seikagaku Corporation (Tokyo). The starch granules (2 g, dw) were digested by 400 U of the enzyme in 20 ml of 40 m*M* acetate buffer (pH 5.0) containing 0.02% sodium azide with gentle stirring on a shaker for various times at 35°C. To obtain the more highly digested starch, the starch (2 g, dw) was digested by 800 U of the enzyme in 40 ml of the same buffer for 24 hr. After the reactions, the residual starch was collected by centrifugation at 4,500 × *g* for 10 min. The collected residues were filtered, washed with distilled water, dehydrated with a small amount of cold methanol, and then washed with ether for 24 hr in a Soxhlet extractor to remove lipids adhering to the surface of the granules. Solubilized starch in the supernatant was determined by the phenol-sulfuric acid method (Dubois et al 1956).

The glucoamylase we used contained a trace amount of α-amylase activity, but no lipase activity was detected by the rhodamine method (Hirayama and Matsuda 1972).

GPC

The defatted starch (25 mg, dw), prepared according to the method of Takeda et al (1986), was dissolved by adding, successively, 0.2 ml of ethanol, 1 ml of distilled water, and 1 ml of 2*M* sodium hydroxide at 4°C. The sample solution was increased to 10 ml by adding distilled water; 2 ml of the solution (5 mg of starch) was applied to a column (2.2 × 90 cm) packed with Toyopearl HW-75F (Tosoh). The column was kept at 30°C and eluted with 200 m*M* sodium hydroxide at a flow rate of 50 ml/hr. Each 5 ml of the eluate was neutralized with 1*M* hydrochloric acid, and total carbohydrate content was determined by measuring glucose according to the phenol-sulfuric acid method (Dubois et al 1956).

Debranching of starch samples was done by isoamylase (*Pseudomonas*) (Nacalai Tesque Inc., Kyoto) according to the method of Ikawa and Fuwa (1980). After stopping the reaction in boiling water, the solution was filtered through a 0.45-μm filter and injected onto an HPLC (Tosoh). Two sequentially linked columns of Superose 12 (1 × 30 cm, Pharmacia, Sweden) and Sephadex G25 (1 × 30 cm, Pharmacia), which were kept at 40°C, were eluted at a flow rate of 0.7 ml/min with 100 m*M* phosphate buffer (pH 6.2) containing 0.02% sodium azide. The eluate was monitored by an RI detector (Knauer Nr. 98.00). Both GPC analyses were determined in duplicate.

SEM Observation

Samples were gold coated (10 nm thick) twice, using a fine coat JEOL JFC-1100E and observed with a JEOL JSM-5300 scanning electron microscope working at 10kV. Magnification was 1,000×.

RESULTS AND DISCUSSION

Determination of Bound FFA in Starches

In most previous work, bound FFA were determined as methyl esters by gas liquid chromatography, after extraction of lipids from starch granules with hot aqueous alcohol. However, these methods required more than 500 mg of starch and included tedious procedures such as extraction, evaporation, concentration, and methylation. Thus, after liquefaction of starch granules by a thermostable α-amylase, the lipids freed from granules were dissolved successively in a final concentration of 75% aqueous 1-propanol (v/v). To determine FFA content with high sensitivity, FFA were also fluorescence labeled and analyzed by HPLC. This method has enabled the determination of the FFA contents with samples of only 20–100 mg. As the HPLC analysis required only 0.1 nmol of the FFA for an injection, it will also be possible to make the experimental scale smaller without requiring any procedures for concentration.

Table I shows that the total contents of five kinds of bound FFA in corn and sweetpotato starches (18.41 μmol/g of starch [0.50%, w/w] and 1.07 μmol/g of starch [0.03%, w/w], respectively). FFA contents of normal corn starch have been reported in the 0.3–0.5% range (Tan and Morrison 1979, Morrison et al 1984, Morrison 1988). Sweetpotato starch has been reported as 0.01–0.08% (Fujimoto et al 1971) using a GLC. The FFA contents vary with the cultivar, environmental condition of growth, or procedure of starch isolation. The predominant FFA were linoleic acid in corn starch and palmitic acid in sweetpotato starch.

Total bound FFA were determined colorimetrically (NEFA-C test kit, Wako). This kit can quantify saturated FFA with carbon numbers of 10–18, as well as unsaturated FFA. Preliminary experiments revealed that a final concentration of 50% aqueous 1-propanol (v/v) effectively extracted the FFA from liquefied starch without any influence on this enzyme assay. Table I also shows that the total FFA contents were 20.53 μmol/g and 1.21 μmol/g in the corn and sweetpotato starches, respectively. The sum of five kinds of FFA determined by HPLC, including palmitic, stearic, oleic, linoleic, and linolenic acid, comprised 90 and 89%, respectively, of the total FFA in the starches. The other FFA were minor components, such as myristic acid and lauric acid reported in rye, oat (Acker and Becker 1971), rice (Ito et al 1979), and sweetpotato (Fujimoto et al 1971) starches.

Digestion of Starch Granules by Glucoamylase

Table II shows the extent of digestion by glucoamylase as a function of reaction time. After 16 hr, digestion of corn and sweetpotato starches by the enzyme (200 U/g of starch) reached 59 and 44% of the starches, respectively. To prepare more highly digested starch, it was necessary to subject the starches to prolonged treatment. However, a prolonged treatment was liable to cause unfavorable change in the lipids, such as autoxidation and hydrolysis of *o*-acyl ester bond. Thus, the starches were treated at a higher enzyme concentration (400 U/g of starch) for 24 hr. Digestion reached 76 and 62% of the starches, respectively.

Change in Bound FFA in Residual Starch upon Digestion

Figure 1 shows changes in the bound FFA contents in the residual starches upon digestion. At the initial stage of digestion, greater decreases of palmitic acid and linoleic acid contents were observed in both starches. At the later stages, the changes in FFA composition differed between corn and sweetpotato starches. In corn starch, only the content of palmitic acid gradually increased; the proportion of palmitic acid to the sum of the five FFA varied from 31% in native starch to 45% in 76%-digested starch. On the other hand, all FFA in sweetpotato starch increased in the same manner; the proportions of respective FFA were nearly constant. In Figure 1, the apparent increase of FFA contents upon digestion suggests that the FFA tend to remain in the residual starch.

Figure 2 shows replotting of the FFA contents based on the initial starch granules for palmitic acid and linoleic acid, which

TABLE I
Bound Free Fatty Acid Contents in Starches

Starch	Fatty Acids, μmol/g of starch[a]					
	16:0	18:0	18:1	18:2	18:3	Total
Corn	5.697 ± 0.054	0.649 ± 0.014	2.559 ± 0.034	8.997 ± 0.030	0.505 ± 0.010	18.407 (20.53)[b]
Sweetpotato	0.538 ± 0.005	0.094 ± 0.001	0.165 ± 0.001	0.232 ± 0.014	0.044 ± 0.001	1.073 (1.21)[b]

[a]Mean ± standard deviation, $n = 4$.
[b]Determined colorimetrically.

TABLE II
Digestibility of Starches by Glucoamylase

	Digestibility, %					
	Reaction Time, hr					
	0.5[a]	1[a]	4[a]	8[a]	16[a]	24[b]
Corn	6.3	10.5	27.0	42.3	58.5	76.2
Sweetpotato	NP[c]	7.9	22.5	35.2	44.1	61.6

[a]200 U of enzyme/g of starch.
[b]400 U of enzyme/g of starch.
[c]Not prepared.

was calculated by multiplying the FFA contents in the residual starch by the yield of the residue. The greater losses of the FFA at the initial stage of digestion suggest that these starches have abundant FFA at the outer layer of the starch granules, since we consider that enzymatic digestion begins at the surface of the granules at such an initial stage. According to the classification of the lipids in starch by Morrison (1981), the abundant FFA at the outer layer correspond to the starch surface lipids.

During the later stages, it was obvious that the FFA in corn starch were more easily released from granules than were those in sweetpotato starch. In corn starch, linoleic acid, especially, decreased in proportion to the extent of digestion. When the lines were extrapolated to find the point of 100% hydrolysis (dotted lines in Fig 2), the amount of linoleic acid in corn starch was estimated at about zero, which indicated that linoleic acid in corn starch was not resistant to release from granules by the enzyme digestion. The other FFA resisted to some extent, because the extrapolated amounts were positive, not zero.

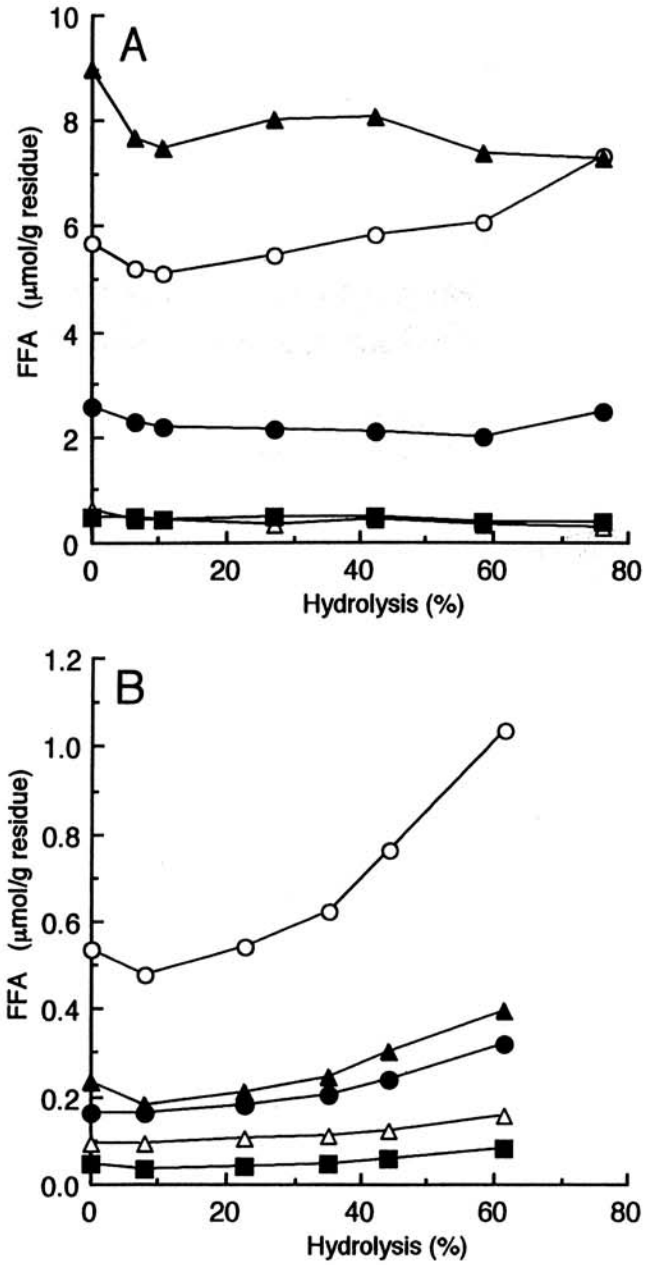

Fig. 1. Change in the bound free fatty acids (FFA) contents in the residual starches upon digestion. **A,** corn starch. **B,** sweetpotato starch. ○ = palmitic acid; △: stearic acid; ● = oleic acid; ▲ = linoleic acid; ■ = linolenic acid.

The amylose-lipid complexes, which are prepared from isolated amylose and lipids such as FFA or monoglyceride, are resistant to the amylolytic enzymes (Vieweg and de Fekete 1976, de Fekete and Vieweg 1978, Seneviratne and Biliaderis 1991). Even the complexed fatty acids are unsaturated (Eliasson and Krog 1985). If the bound FFA exist as a complex with amylose in the native granules as in the model experiment above, the FFA would be resistant to being released from granules. Thus, we note that linoleic acid in native corn starch shows no resistance to enzyme digestion.

Properties of Residual Starch

To study whether there is preferential degradation of starch components by glucoamylase, and whether there is localized attack of the enzyme on sections of a granule, we examined the residual starches by GPC analyses and SEM observation.

Figure 3 shows the GPC profiles of native and residual starches on a Toyopearl HW-75F column. After 76 and 62% digestion

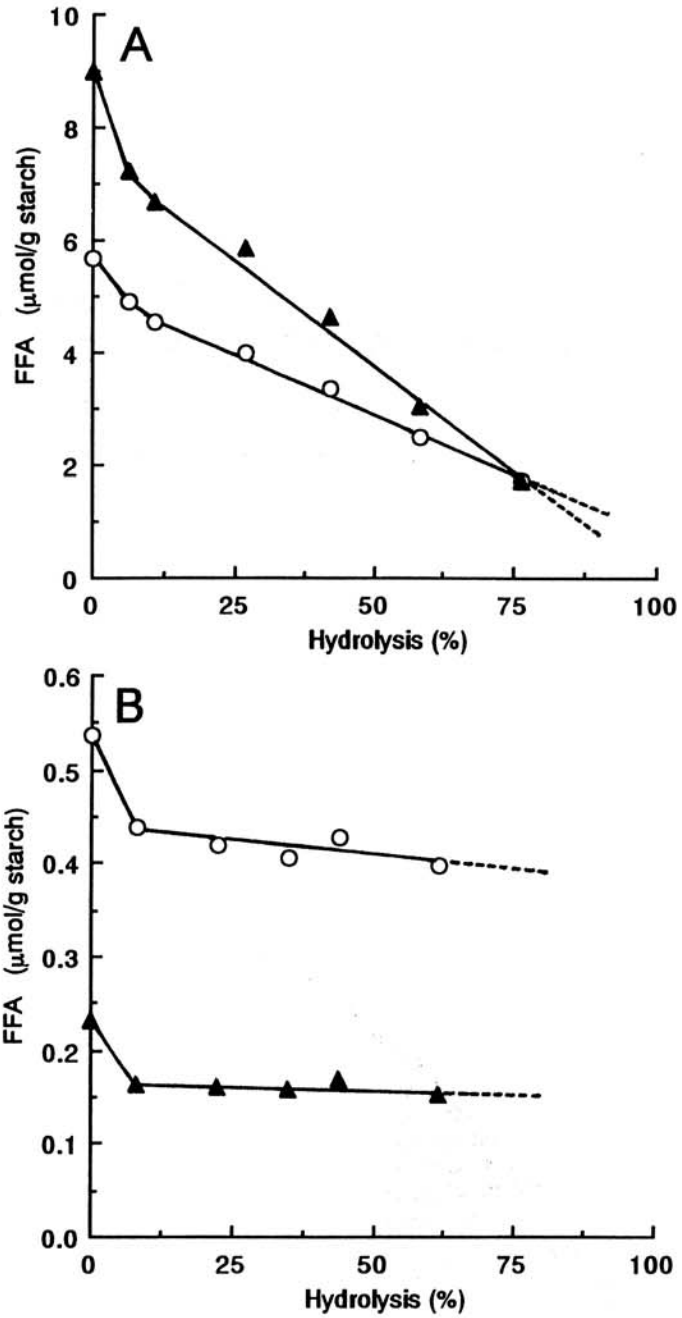

Fig. 2. Losses of the bound free fatty acids (FFA) palmitic acid (○) and linoleic acid (▲) in the starches upon digestion. **A,** corn starch. **B,** sweetpotato starch.

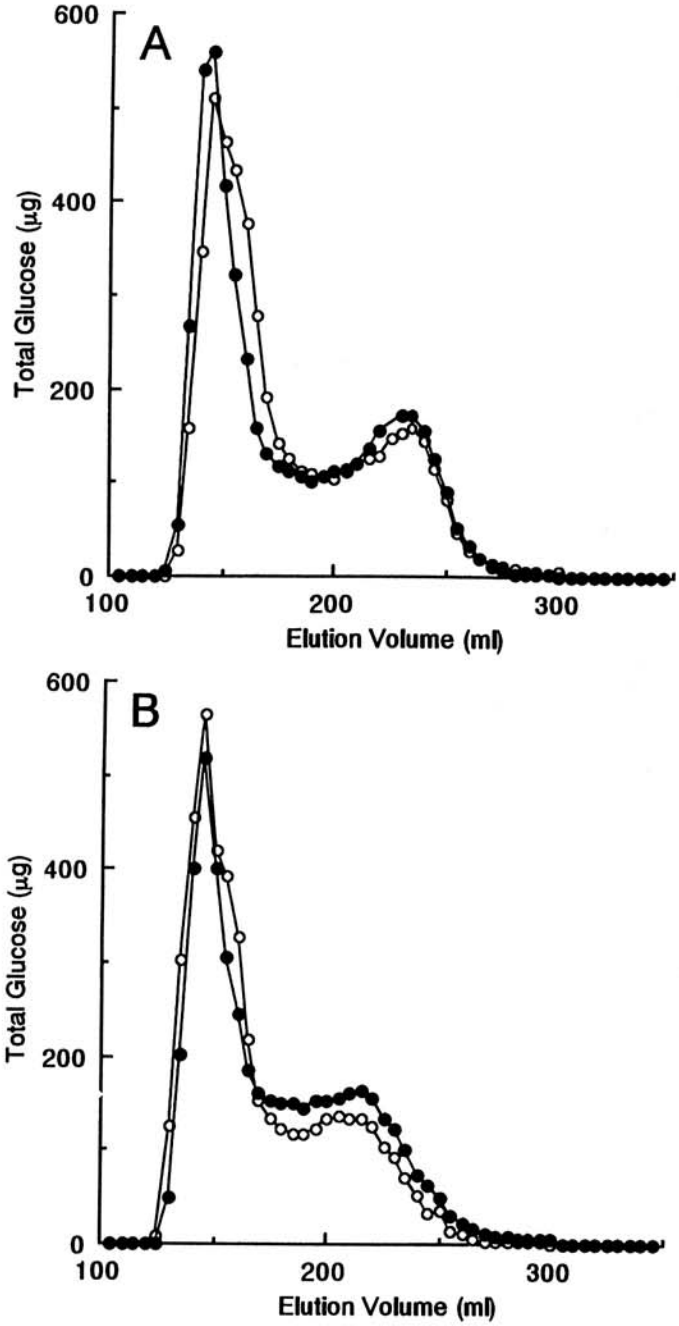

Fig. 3. Elution profiles of the native (○) and glucoamylase-digested (●) starches. **A,** native corn starch and 76% digested starch. **B,** native sweetpotato starch and 62% digested starch. Total glucose was determined by the phenol-sulfuric acid method.

of corn and sweetpotato starches, respectively, high and low molecular weight fractions (mainly amylopectin and amylose fractions) (Takeda et al 1984) remained in both starches without marked change. This result was also confirmed by GPC of the debranched starches using an HPLC. In both starches, the elution profiles of the debranched native starches were not altered by the enzyme digestion (data not shown). As a result, we considered that there was no preferential degradation of starch components by the enzyme, and we could not observe a difference in the mode of enzyme action on the starch components between corn and sweetpotato starches.

However, SEM observation of the residual starches revealed significant differences in their appearances. As seen in Figure 4, the residues of corn starch were highly degraded granules with pores, while the residues of sweetpotato were slightly pitted or scratched. The residues of sweetpotato appeared less damaged

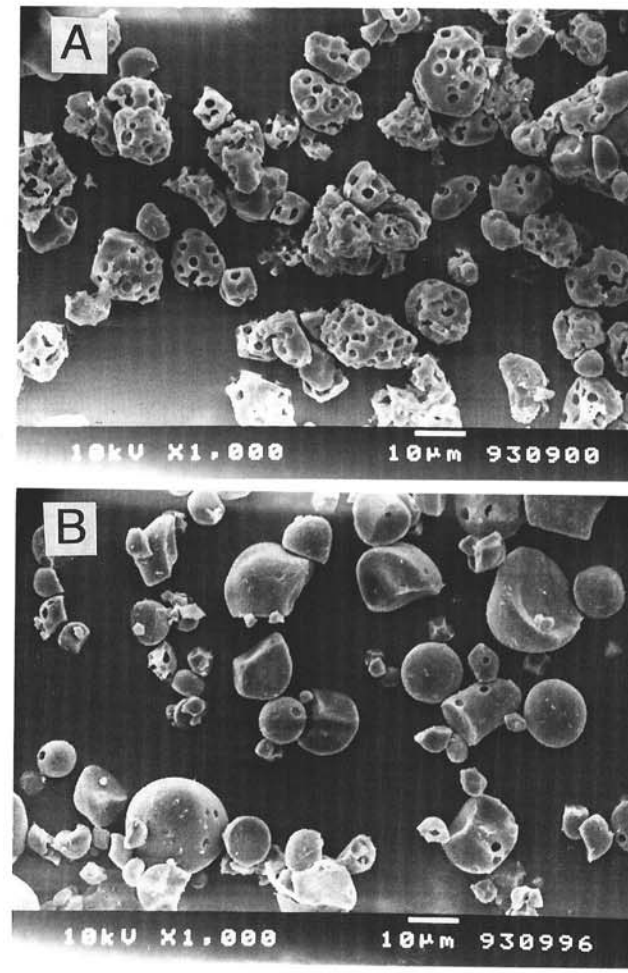

Fig. 4. Scanning electron micrographs of the glucoamylase-digested starches. **A,** 76% digested corn starch. **B,** 62% digested sweetpotato starch.

than expected from 62% digested starch. Most corn starch granules seemed to be equally hydrolyzed by the enzyme, but those of sweetpotato starch were hydrolyzed in an "all-or-none" mode. In sweetpotato starch, therefore, the mode of digestion might contribute to the apparent strong resistance of the FFA to release.

In this study, we established an improved method for determination of bound FFA in starch and estimated the mode of loss of the FFA upon glucoamylase digestion. Contrary to our prediction that the amylose-lipids complex resisted amylolysis, linoleic acid in corn starch, which was the major component of the bound FFA, decreased in proportion to the extent of the digestion.

Further work is in progress to study the existing state of bound FFA in native granules, and the reason why the digestion of sweetpotato starch proceeds unequally among the respective granules still remains to be resolved.

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