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 (FFAs), were investigated. FFAs were determined by high-performance liquid chromatography after fluorescence labeling of the FFAs. This method enabled the determination of the FFAs content with samples of 20–100 mg without requiring any procedures for concentration. The total FFAs 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.

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 2 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 FFAs were determined by two methods. Method 1: To determine the FFA composition, a starch sample (20 mg of 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 l-propanol was added to the hydrolysate, and then the mixture was heated for 10 min at 95°C. After addition of boric 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 (Toch 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 l-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 NEF-C test kit (Wako), which consisted of acetyl-CoA synthetase, acetyl-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

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Seikagaku Corporation (Tokyo). The starch granules (2 g, dw) were digested by 400 U of the enzyme in 20 ml of 40 mM 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 X 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 2M 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 X 90 cm) packed with Toyopearl HW-75F (Tosoh). The column was kept at 30°C and eluted with 200 mM sodium hydroxide at a flow rate of 50 ml/hr. Each 5 ml of the eluate was neutralized with 1M 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 X 30 cm, Pharmacia, Sweden) and Sephadex G25 (1 X 30 cm, Pharmacia), which were kept at 40°C, were eluted at a flow rate of 0.7 ml/min with 100 mM phosphate buffer (pH 6.2) containing 0.02% sodium azide. The eluate was monitored by an RI detector (Knauer N. 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 1,000X.

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 FAA contents with samples of only 20–100 mg. As the HPLC analysis required only 0.1 nmol of the FAA for an injection, it will also be possible to make the experimental scale smaller without requiring any procedures for concentration.

Table I shows the total contents of five kinds of bound FAA 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). FAA contents of normal corn starch have been reported in the 0.3–0.5% range (Tan and Morrison 1979, Morrison et al 1984, Morrison 1985). Sweetpotato starch has been reported as 0.01–0.08% (Fujimoto et al 1971) using a GLC. The FAA contents vary with the cultivar, environmental condition of growth, or procedure of starch isolation. The predominant FAA were linoelic acid in corn starch and palmitic acid in sweetpotato starch.

Total bound FAA were determined colorimetrically (NEFA-C test kit, Wako). This kit can quantitatively saturated FAA with carbon numbers of 10–18, as well as unsaturated FAA. Preliminary experiments revealed that a final concentration of 50% aqueous 1-propanol (v/v) effectively extracted the FAA from liquefied starch without any influence on this enzyme assay. Table I also shows that the total FAA contents were 20.53 μmol/g and 1.21 μmol/g in the corn and sweetpotato starches, respectively.

The sum of five kinds of FAA determined by HPLC, including palmitic, stearic, oleic, linoelic, and linolenic acid, comprised 90 and 89%, respectively, of the total FAA in the starches. The other FAA 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 α-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 FAA in Residual Starch upon Digestion
Figure 1 shows changes in the bound FAA contents in the residual starches upon digestion. At the initial stage of digestion, greater decreases of palmitic acid and linoelic acid contents were observed in both starches. At the later stages, the changes in FAA 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 FAA varied from 31% in native starch to 45% in 76% digested starch. On the other hand, all FAA in sweetpotato starch increased in the same manner; the proportions of respective FAA were nearly constant. In Figure 1, the apparent increase of FAA contents upon digestion suggests that the FFA tend to remain in the residual starch.

Figure 2 shows repploting of the FAA contents based on the initial starch granules for palmitic acid and linoelic acid, which

| Table I: Bound Free Fatty Acid Contents in Starches |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Starch          | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | Total |
| Corn            | 5.697 | 0.649 | 2.559 | 8.997 | 0.505 | 18.407 |
| Sweetpotato     | 0.538 | 0.094 | 0.165 | 0.232 | 0.044 | 1.073 |
| ± 0.054         | ± 0.014 | ± 0.034 | ± 0.030 | ± 0.010 | (20.53)^b  |
| ± 0.005         | ± 0.001 | ± 0.001 | ± 0.014 | ± 0.001 | (1.21)^b  |
| ① Mean ± standard deviation, n = 4. ② Determined colorimetrically.

| Table II: Digestibility of Starches by Glucoamylase |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Reaction Time, hr | 0.5^a | 1^a | 4^a | 8^a | 16^a | 24^a |
| Corn            | 6.3    | 10.5 | 27.0 | 42.3 | 58.5 | 76.2 |
| Sweetpotato     | NP^a  | 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.
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

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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; Δ = stearic acid; • = oleic acid; ▲ = linoleic acid; ■ = linolenic acid.

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.
of corn and sweet potato 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 sweet potato 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 sweet potato were slightly pitted or scratched. The residues of sweet potato appeared less damaged than expected from 62% digested starch. Most corn starch granules seemed to be equally hydrolyzed by the enzyme, but those of sweet potato starch were hydrolyzed in an "all-or-none" mode. In sweet potato 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 sweet potato starch proceeds unequally among the respective granules still remains to be resolved.

**LITERATURE CITED**


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[Received December 8, 1993. Accepted June 1, 1994.]