COMPARATIVE SUSCEPTIBILITY TO AMYLASES OF STARCH GRANULES OF SEVERAL SINGLE ENDOSPERM MUTANTS REPRESENTATIVE OF FLOURY-OPAQUE, STARCH-DEFICIENT, AND MODIFIED STARCH TYPES AND THEIR DOUBLE-MUTANT COMBINATIONS WITH OPAQUE-2 IN FOUR INBRED LINES OF MAIZE

Hidetsugu Fuwa, David V. Glover, Yoshimi Sugimoto, and Mie Tanaka

Department of Food and Nutrition, Osaka City University, Sugimoto-cho, Osaka 558, Japan
3Department of Agronomy, Purdue University, West Lafayette, IN 47907, U.S.A.
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Summary Starch granules were prepared from kernels of eight single endosperm mutants, brittle-1 (bt1), brittle-2 (bt2), floury-1, floury-2, soft starch, opaque-1 (o1), shrunken-2 (sh2), and sugary-2 (su2), and their double-mutant combinations with opaque-2 (o2) of four inbred lines of maize (Zea mays L.), B37, C103, Oh43 and W64A. We compared the susceptibility of various starch granules to Rhizopus glucoamylase and pancreatin. Starch granules of the su2 and su2o2 mutants were digested by amylases much faster than those of the normal counterparts. Starch granules of the bt1, bt2, o1 and sh2 mutants tended to be digested by amylases faster than those of normal maize. Starch granules of double-mutant combinations with the o2 gene were, in general, digested to an extent very comparable to their respective non-opaque single mutant counterparts in each of their four inbred backgrounds. We followed the relative digestion of starch granules by using scanning electron microscopy. Starch granules of endosperm mutants susceptible to amylases showed numerous pin holes on the surface layer and the pores penetrated into the inner layers of the granules during the attack by amylases. In some of the granules the inner portion, which appeared terraced or step-shaped, could be seen. This may be indicative of layered internal structures of the granules.

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不破英次，杉本温美，田中三恵
We reported that starch granules of the sugary-1 (su1) and sugary-2 (su2) mutants of the maize inbred Oh43 were digested by Rhizopus glucoamylase, pancreatin or bacterial α-amylase considerably faster than those of their normal counterpart (1, 3). Starch granules of the brittle-2 (bt2), shrunken-2 (sh2) and waxy (wx) mutants were digested by amylases more rapidly than those of normal maize, but not as rapidly as the su1 and su2 mutants. The amylase extender (ae) mutant starch granules from the Oh43 background were more resistant to the action of the enzymes than those of the normal counterparts. The opaque-2 (o2) mutant, which improves nutritional quality of endosperm proteins, in general, did not change the susceptibility of starch granules to degradation by amylases. Double-mutant combinations of starch modifying genes with o2 exhibited the same properties as their respective non-opaque single-mutant counterpart.

With the use of a scanning electron microscope (SEM) (2), we showed that starch granules of the ae and ae o2 mutants were resistant to the action of amylases and had similar shapes and surfaces to the native undegraded granules following the enzyme attack. On the other hand, those endosperm mutants susceptible to amylases showed numerous pin holes on the surface layer and the pores penetrated into the inner layers of the granules during the attack with amylases.

We confirmed these results for the o2, ae, ae o2, wx, wx o2, su1 and su1 o2 near-isogenic line mutant conversions and their normal counterparts with additional genetic backgrounds of maize by studying the enzyme degradation of starch granules prepared from kernels of the three inbreds B37, C103, and W64A in addition to Oh43 (3).

This paper deals with comparisons of the relative susceptibility to fungal glucoamylase and pancreatin of starch granules prepared from kernels of near-isogenic conversions of eight single endosperm mutants representative of floury-opaque, starch deficient, and modified starch types and their double-mutant combinations with o2. These observations were made in Oh43 and three additional inbreds, B37, C103, and W64A, representing four broad genetic relationship groups, in order to determine whether genetic background affects the susceptibility of the endosperm starch granules to amylases and to confirm and extend our previous observations (1–3).

**MATERIALS AND METHODS**

**Maize mutants.** Mature kernels of near isogenic conversions of eight single endosperm mutants of maize (Zea mays L.); brittle-1 (bt1), brittle-2 (bt2), floury-1 (fl1), floury-2 (fl2), soft starch (h), opaque-1 (o1), shrunken-2 (sh2), sugary-2 (su2), and four double-mutant combination with opaque-2 (o2); fl1o2, fl2o2, h o2 and su2o2 of four inbred lines, B37, C103, W64A and Oh43, sh2o2 of B37, W64A and Oh43, and bt1o2, bt2o2 and o1o2 of Oh43 were used. These inbred lines represent different broad genetic relationship groups. The materials were grown at the Purdue Agronomy farm. Kernels of the commercial dent maize were kindly supplied by Mr. T. Miwa,
Central Research Laboratories, Nihon Shokuhin Kako Co., Ltd., Mishima, Japan.

Starch granules. Starch granules of maize were prepared by the method reported previously (1).

Sources of enzymes. Sources of *Rhizopus* glucoamylase, pancreatin, glucose oxidase and peroxidase were described earlier (1).

Analytical methods. Susceptibility of starch granules to glucoamylase and pancreatin were studied by the methods reported previously (1). In the case of glucoamylase, the data were expressed as a percentage of glucose equivalent. Data on pancreatin digestion were expressed on the basis of a relative percentage of commercial normal control enzyme degradation determined as a percentage of glucose equivalent.

Preparation of starch granules attacked by amylases, specimen mounting and scanning electron microscopy (SEM). Starch granules were attacked by amylases under the conditions described previously (1). Procedures for specimen mounting and scanning electron microscopic observations were the same as those reported previously (3).

RESULTS AND DISCUSSION

Starch granules susceptibility to glucoamylase of the *h, ho2, fl1, fl1o2, fl2, fl2o2, su2*, and *su2o2* endosperm mutants and the normal maize

We found insignificant differences in susceptibility to either *Rhizopus* glucoamylase or pancreatin of starch granules prepared from the B37, C103, W64A, and Oh43 normal and those prepared from commercial normal dent maize. Therefore, we used starch granules of commercial normal maize as a control in this study. As shown in Fig. 1, starch granules of the *su2* and *su2o2* mutants were digested 2 to 3 times faster than those of the normal control. The *h, fl1*, and *fl2* mutants, in general, were digested to about the same degree as the normal control, or somewhat faster. Starch granules of the double-mutant combinations with *o2* were digested to an extent very comparable to their respective non-opaque single mutant counterparts.

Starch granules susceptibility to pancreatin of the *h, ho2, fl1, fl1o2, fl2, fl2o2, su2*, and *su2o2* endosperm mutants and the normal maize

The *su2* and *su2o2* starches were also digested by pancreatin about two times faster than those of the normal, depending upon the background genotype (Fig. 2). These data on the *su2* mutant support the findings of Sandstedt et al. (4) and observations on enzyme attack on *su2* in the Oh43 background (1). As was true in the case of enzyme attack by fungal glucoamylase, the *h, fl1*, and *fl2* mutants and their double-mutant combinations with *o2* were degraded by pancreatin to a similar extent as the normal maize or even to a greater degree.
Fig. 1. Digestion of starch granules of the soft starch (h), floury-1 (fl1), floury-2 (fl2) and sugary-2 (su2) mutants, their double-mutant combinations with opaque-2 (o2), ho2, fl1o2, fl2o2 and su2o2 mutants of four inbred lines of maize, B37, C103, Oh43 and W64A, and a commercial normal maize by *Rhizopus* glucoamylase. Dotted bar; normal maize. Open bar; single endosperm mutants. Shaded bar; double-mutant combinations with o2. Reaction mixture contained 40 mg (by dry weight) of starch granules, 0.32 M acetate buffer (pH 4.8), and 0.2% *Rhizopus* glucoamylase preparation in a total volume of 5.0 ml. Two blanks were run simultaneously, one under identical conditions except for the starch granules and the other identical except for the enzyme. Incubation was made at 37 °C. At 2 hr, 5.0 ml of 0.6 M perchloric acid was added to the mixture. After stirring and centrifugation, an aliquot of the supernatant was used for assay of glucose colorimetrically by the glucose oxidase-peroxidase method. Data were expressed as percentage glucose equivalent. Each value is the average of two determinations.

Fig. 2. Digestion of starch granules of the h, ho2, fl1, fl1o2, fl2, fl2o2, su2 and su2o2 mutants of four inbred lines of maize, B37, C103, Oh43 and W64A, and the normal maize by pancreatin. Reaction mixture contained 50 mg (by dry weight) of starch granules, 6.7 mM phosphate buffer (pH 7.2), 10 mM sodium chloride, 10 mM calcium acetate, and 0.5% pancreatin in a total volume of 1.5 ml. Incubation was made at 37 °C. At 1 hr, 5.0 ml of ice-cold water was added to the mixture. After stirring and centrifugation, an aliquot of the supernatant was used for assay of solubilized carbohydrates by the phenol-sulfuric acid method. Data were expressed on the basis of a relative percentage of commercial normal control enzyme degradation determined as a percentage glucose equivalent. Each value is the average of two determinations. See also legend to Fig. 1.
Starch granules susceptibility to glucoamylase and pancreatin of the o₁, o₁o₂, bt₁, bt₁o₂, bt₂, bt₂o₂, sh₂ and sh₂o₂ endosperm mutants and the normal maize

The o₁ starch granules were degraded by both fungal glucoamylase and pancreatin somewhat faster than those of the normal control (Figs. 3 and 4) and very similar to the other floury-opaque type mutants, h, fl₁, and fl₂ (Figs. 1 and 2). The starch granules from those mutants which were reduced substantially in starch content, namely, the bt₁, bt₂, and sh₂ endosperm mutants, tended to be digested by fungal glucoamylase some 20 to over 100% faster than normal (Fig. 3). Pancreatin digestion in these same endosperm mutants was some 10 to 50% greater than on the normal maize control (Fig. 4). Though there is one notable extreme difference in the case of pancreatin digestion of the W64A sh₁o₂ starch granules (Fig. 4), the results indicated that, in general, the double-mutant combinations with o₂ were digested by glucoamylase and pancreatin to the extent very similar to their respective non-opaque single mutant counterparts (Figs. 3 and 4). These results confirm our previous findings of the effect of o₂ on other endosperm mutants in the Oh43 background (1, 3).

There was a tendency for a more differential response among the different inbred backgrounds to enzyme degradation by amylases of the starch granules of

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Fig. 3. Digestion of starch granules of the opaque-1 (o₁), brittle-1 (bt₁), brittle-2 (bt₂) and shrunk-2 (sh₂) mutants, their double-mutant combinations with o₂, o₁o₂, bt₁o₂, bt₂o₂ and sh₂o₂ mutants of four inbred lines of maize, B37, C103, Oh43 and W64A, and the normal maize by Rhizopus glucoamylase. See legend to Fig. 1.

Fig. 4. Digestion of starch granules of the o₁, o₁o₂, bt₁, bt₁o₂, bt₂, bt₂o₂, sh₁ and sh₁o₂ mutants of four inbred lines of maize, B37, C103, Oh43 and W64A, and the normal maize by pancreatin. See legend to Fig. 2.
bt₁, bt₂, and sh₂ and those of their combinations with o₂. This was particularly true of the latter two mutant types. The four different inbreds were more uniformly similar in their response to amylase digestion of the starch granules of su₂, h₃, f₁₁, f₁₂ and o₁ mutants and their o₂ combinations.

**SEM observations of starch granules attacked by amylases**

With the use of SEM, we followed the degradation of starch granules of this series of single mutants and their double-mutant combinations with o₂ following amylase attack. However, because of the limited space, we showed photoelectron-micrographs of starch granules of the h and h o₂ mutants only (Figs. 5 and 6). Since the original or native starch granules of the h and h o₂ mutants are round shaped, have smooth surfaces and show wide variations in size, they are more or less representative of most of the mutants reported in this paper, all of which are readily attacked by either *Rhizopus* glucoamylase or pancreatin. The starch granules of the bt₁, bt₂, sh₂, and su₂ mutants and their o₂ combinations are however smaller and it is difficult to follow the degradation following amylase attack particularly in the case of su₂. Although each mutant type has its own peculiar degradation characteristics, in general, they may be characterized by the SEM observations shown by the h and h o₂ endosperm mutants. Numerous pin holes could be seen on the surfaces of starch granules attacked by glucoamylase and pancreatin (Figs. 5a–d and 6a–d). This agrees with observations of Evers et al. (5) by using wheat starch and glucoamylase of *Aspergillus niger*, Shetty et al. (6), Smith and Lineback (7) using wheat and maize starches and glucoamylases of *Aspergillus niger* and *Rhizopus niveus*, and Fuwa et al. (2, 3, 8). We also observed that the pores penetrated into the inner layers of granules during the enzyme action and some of the granules exhibited a terraced or step-shaped appearance in their inner portions (Figs. 5c and 6a–d). This was quite typical of previous observations we have made on other endosperm mutants of maize (2, 3, 8), and agrees with observations of others (7, 11, 14) on normal maize and other starch species. We used partially purified glucoamylase of *Rhizopus amagasakiensis* which contained some α-amylase activity detected by the method of Marshall and Whelan (9). This perhaps accounted for some of the small differences which we observed in patterns of the enzyme attack on granules as

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**Fig. 5.** Scanning electron photomicrographs (SEM) of the soft starch (h) and soft starch opaque-2 (h o₂) mutants of maize inbred B37 and C103 attacked by *Rhizopus* glucoamylase.

- a. Granules of h starches of B37 (percent degradation, 28.4%).
- b. Granules of h o₂ starches of B37 (percent degradation, 25.4%).
- c. Granules of h starches of C103 (percent degradation, 22.1%).
- d. Granules of h o₂ starches of C103 (percent degradation, 24.3%).

**Fig. 6.** SEM of starch granules of the h and h o₂ mutants of maize inbred Oh43 and W64A attacked by pancreatin.

- a. Granules of h starches of Oh43 (percent degradation, 37.8%).
- b. Granules of h o₂ starches of Oh43 (percent degradation, 41.4%).
- c. Granules of h starches of W64A (percent degradation, 38.7%).
- d. Granules of h o₂ starches of W64A (percent degradation, 38.4%).
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compared to those patterns of enzyme attack of crystalline glucoamylase of *Rhizopus niveus* reported by Shetty et al. (6). Further investigations on degradation of starch granules by several glucoamylases from different origin are in progress (8, 10).

These internal terraced or step-shaped characteristics are most likely indicative of layered internal structures of the granules as shown by others using native (17), and enzymatically (2, 8, 11–16, 18), chemically (19), and physically (17) modified starch granules as observed by SEM.

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