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PURIFICATION OF A DEBRANCHING ENZYME (R-ENZYME) FROM MALTED BARLEY, AND THE ROLE OF THE ENZYME IN THE DIGESTION OF STARCH GRANULES DURING THE GERMINATION OF BARLEY SEEDS*

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ABSTRACT

A debranching enzyme (R-enzyme or pullulan-6-glucanohydrolase, EC 3.2.1.41), free from contaminating carbohydrases and homogeneous by poly(acrylamide) disc-gel electrophoresis, has been purified from malted barley. A partially purified preparation of this enzyme (3.1 units/mg of protein) accelerated the rate of digestion of barley-starch granules by the action of purified alpha and beta amylases to the same extent as was effected by the dialyzed, crude extract from malted barley. Contrary to expectation, the debranching enzyme, purified to homogeneity (10 units/mg of protein), had very little accelerating effect. These results indicate that a factor or factors, which may be maltase or α-D-glucosidase and were lost during the purification of the debranching enzyme, may play a role in the digestion of starch granules by the dialyzed, crude extract from malted barley *in vitro* and by enzymes in the endosperm of germinating barley seeds *in vivo*. The debranching enzymes, including barley-malt R-enzyme, *Aerobacter* pullulanase, and *Pseudomonas* isoamylase, did not digest starch granules to a detectable extent.

INTRODUCTION

During the germination of barley seeds, starch granules are digested by amylolytic enzymes in order to be used as energy and carbon sources in growing parts of the seedlings. In their studies on the mechanism and mode of digestion of barley-starch granules by the enzymes present in the germinating barley endosperm, Kiribuchi and Nakamura[1–4] found that the activities of alpha and beta-amylases were at their highest when barley seeds were germinated for 6 days at 20° in the dark. A debranching-enzyme activity, assayed by using alpha, beta-limit dextrin as the substrate, appeared a little later in the course of germination. The dialyzed, crude extract of

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

barley malt (germinated for six days) digested barley-starch granules at a reasonable rate, and barley-starch granules treated with this extract *in vitro* were indistinguishable from starch granules isolated from germinating barley-malt when examined under a scanning electron microscope. Therefore, the dialyzed crude extract obtained from germinating barley seeds were assumed to contain all of the enzymes necessary for the digestion of starch granules in the germinating barley endosperm.

In order to elucidate the mechanism whereby starch granules are digested in germinating barley seeds and to know what enzymes are responsible for the digestion of starch granules, Maeda *et al.*[5] purified beta and alpha amylases from barley and malted barley, respectively, and studied the mode of digestion of barley-starch granules by the action of the purified alpha and beta amylases, both alone and in combination. The results showed that purified alpha amylase digested starch granules at a rate about one half that with the dialyzed crude extract containing the same activity with respect to alpha amylase. Beta amylase had very little, if any, activity on starch granules but, when added to alpha amylase, it showed a synergistic effect in accelerating the rate of digestion of starch granules. However, the combined action of both enzymes was still inferior to the action of the crude extract. Moreover, paper chromatograms of the solubilized digestion-products showed that those obtained by the combined action of purified alpha and beta amylases contained oligosaccharides of higher molecular weight. These oligosaccharides may constitute branched oligosaccharides containing alpha, beta-limit dextrins and they were absent in the chromatogram of the solubilized digestion-products formed by the dialyzed, crude extract. These results indicate that the presence of a debranching enzyme may be responsible for the difference between the chromatograms of the two systems and that the debranching enzyme, as well as alpha and beta amylases is essential for the digestion of starch granules. It was of interest, therefore, to purify the debranching enzyme from malted barley and to examine the effect of added debranching enzyme on the digestion of starch granules by purified alpha and beta amylases. This paper deals with the purification of the debranching enzyme by using cyclohexaamylose–Sepharose as the affinity adsorbent and the action of the debranching enzyme on starch granules, both in the presence and absence of alpha and beta amylases.

MATERIALS AND METHODS

Materials. — Maltose, maltotriose, cyclohexa- and hepta-amylose, pullulan, *Aerobacter* pullulanase, and *Pseudomonas* isoamylase were obtained from Hayashibara Biochemical Labs., Inc., Okayama. Sephadex G-150 (superfine) and epoxy-activated Sepharose 6B were obtained from Pharmacia Fine Chemicals. Bovine serum albumin, tris(hydroxymethyl)aminomethane, and *Rhizopus niveus* glucoamylase were products of Armour, Sigma, and Nagase, respectively.

Barley seeds and malted barley, both of variety "New Golden", were kind gifts from Sapporo Brewery & Co., Tokyo. Starch granules were prepared from barley

seeds essentially by the method of Banks *et al.*[6] and as described by Taniguchi *et al.*[7]. Alpha amylase from malted barley and beta amylase from barley grains were purified as described by Maeda *et al.*[5]. Other reagents were commercial products of the highest quality.

Analytical methods. — Protein was determined by the method of Lowry *et al.*[8] and reducing sugars were determined by the method of Somogyi[9] and Nelson[10]. Total sugars solubilized in the digestion mixtures were determined by the anthrone method[11]. Paper chromatography of the solubilized digestion-products of starch granules by enzymes was performed as follows: aliquots of the centrifuged, enzymic-digestion mixtures were spotted on a sheet of Toyo filter paper No. 50 (20 × 20 cm) and the paper was irrigated with 65% 1-propanol in water by the double-ascending technique at 70°. The dried paper was treated with glucoamylase[12] to hydrolyze the oligosaccharides *in situ* in order to intensify the color, and the products were detected by the alkaline acetone–silver nitrate dip-method[13].

Assay of the debranching-enzyme activity. — The mixture contained 0.4 ml of 0.25% pullulan solution in 25mM acetate buffer (pH 5.0) and 0.1 ml of the enzyme solution. The mixture was incubated at 30°. At time intervals, 0.1-ml aliquots of the mixture were withdrawn and the reducing sugar determined by the method of Somogyi and Nelson with maltotriose as the standard. Crude enzyme-preparations contain auxiliary enzymes that hydrolyze maltotriose, the reaction product from pullulan by the action of the debranching enzyme, into D-glucose and maltose and may give erroneous results in this assay procedure.

One unit of the debranching-enzyme activity is defined as the amount of enzyme that liberates 1 μmol of maltotriose in one min under the conditions just given. Specific activity is given as units of enzyme activity per mg of protein.

Preparation of the affinity adsorbent. — Cyclohexaamylose–Sepharose 6B was prepared by coupling cyclohexaamylose to epoxy-activated Sepharose 6B by the procedure recommended by the manufacturer.

Disc-gel electrophoresis. — Disc-gel electrophoresis was carried out in 7.5% poly(acrylamide) gel at pH 8.3 (Tris–glycine buffer) by the method of Ornstein[14] and Davis[15]. Other conditions were: ~40 μg of enzyme protein per tube, 2 mA per tube, and 150 min at room temperature. After the electrophoresis, the gel was stained overnight with 1% Amido Black 10B and then destained in 7% acetic acid by electrophoresis (10 mA per tube).

Digestion of starch granules. — A mixture containing 100 mg of barley-starch granules, 0.25 ml of 0.5M acetate buffer (pH 4.80), and 1.75 ml of the enzyme solution in 0.2% calcium acetate solution, was incubated at 30° in a shaker at 90 r.p.m. The amounts of enzymes used were: alpha amylase, 1,800 units: beta amylase, 120 units; and the debranching enzyme, 1 unit. At time intervals, 0.2 ml of the mixture was withdrawn and diluted with 1.8 ml of water. The solubilized total sugars in the centrifuged reaction-digest were determined by the anthrone method.

RESULTS

Purification of the debranching enzyme from malted barley. — Finely powdered malted barley (1 kg) was extracted with 3 liters of 5% sodium chloride solution for 3 h at 30° with occasional stirring. The extract was centrifuged at 10,000*g* for 30 min and the sediment was discarded. The supernatant was fractionated with ammonium sulfate and the protein fraction precipitating between 30 and 60% saturation of ammonium sulfate was recovered by centrifugation. The precipitate was dissolved in 10mM Tris–HCl buffer (pH 7.50) and dialyzed overnight in a cold room against the same buffer.

Any precipitate that formed was centrifuged off and the supernatant solution was subjected to chromatography on a column (4.8 × 18 cm) of DEAE-cellulose (Whatman DE 52) equilibrated with 10mM Tris–HCl buffer (pH 7.50) containing 0.1M sodium chloride. After the column had been washed with the same buffer, the adsorbed debranching-enzyme was eluted by a linear gradient of sodium chloride from 0.1 to 0.5M in the same buffer. Fractions containing pullulan-hydrolyzing activity were combined, and concentrated by an ultrafiltration apparatus (Amicon Corp.) with a PM-30 membrane. The enzyme solution was dialyzed overnight against 50mM acetate buffer (pH 5.0) and, after centrifuging off any precipitate formed, the supernatant was again concentrated.

The enzyme solution was divided into 2 parts and subjected to chromatography on columns of Sephadex G-150 (superfine, 2.6 × 90 cm) equilibrated with 50mM acetate buffer, pH 5.0. The elution pattern from the column by the same buffer is shown in Fig. 1. A debranching-enzyme preparation was obtained having a specific activity of 0.4 unit/mg of protein. Rechromatography of this preparation on the same column gave a preparation having a specific activity of 3.1 units/mg of protein; it was contaminated by a weak beta-amylase activity, as evidenced by the formation of maltose from amylopectin in the paper chromatogram of the digestion products.

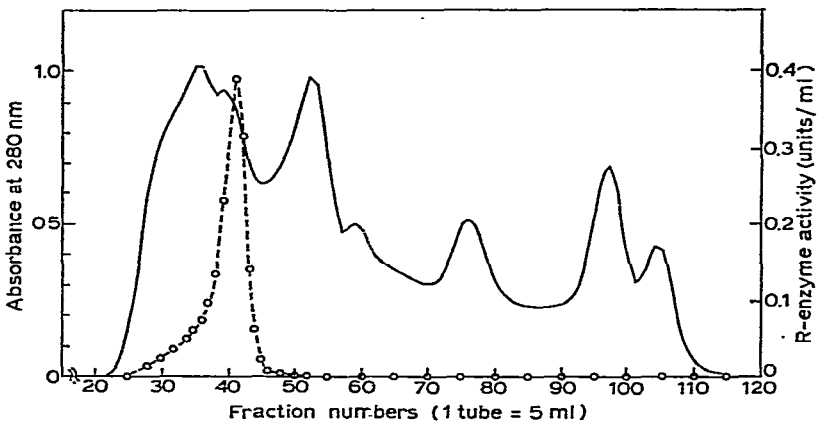

Fig. 1. Chromatography of malted-barley R-enzyme on Sephadex G-150 (superfine). Continuous line, absorbance at 280 nm; broken line, R-enzyme activity.

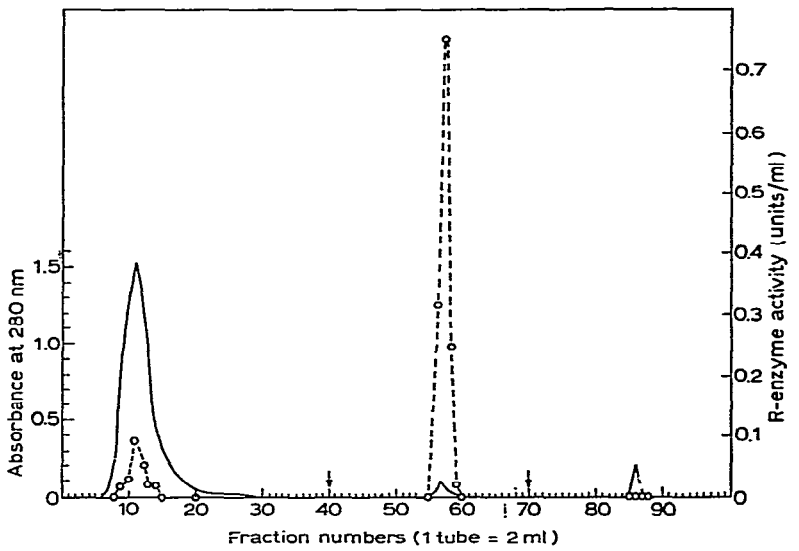

Fig. 2. Affinity chromatography of R-enzyme on a column of cyclohexaamylose–Sepharose 6B. Continuous line, absorbance at 280 nm; broken line, R-enzyme activity; first arrow, cycloheptaamylose (11.75 μg/ml) in 50mM acetate buffer (pH 5.0); second arrow, cycloheptaamylose (10 mg/ml) in the same buffer. A small peak at fraction 88 contained beta-amylase activity.

Active fractions from the Sephadex G-150 (superfine) column (specific activity, 0.4 unit/mg of protein) were further resolved on a column (9 × 390 mm) of cyclohexaamylose–Sepharose 6B. After the column had been washed with 50mM acetate buffer (pH 5.0), the debranching enzyme was eluted with the same buffer containing 11.75 μg of cycloheptaamylose per ml. A protein peak, which coincided with the pullulan-hydrolyzing activity, was obtained (Fig. 2). Active fractions were combined and concentrated to give a preparation of the debranching enzyme having a specific activity of 10 units/mg of protein. A summary of the purification procedure is given in Table I. The final preparation gave a single band in poly(acrylamide) disc-gel electrophoresis (Fig. 3).

Test for contaminating carbohydrases. — Mixtures containing 1 mg each of amylose, amylopectin, pullulan, or maltose in 0.2 ml of water, 0.03 ml of 0.5M acetate buffer (pH 5.0), and 0.02 ml of the purified solution of debranching enzyme (0.01 unit) were incubated at 30° for 22 h, and 10 μl aliquots were spotted onto a filter paper and subjected to paper chromatography. The chromatograms showed that amylose gave no hydrolysis product; amylopectin gave a series of higher oligosaccharides, produced presumably by hydrolysis of the α-D-(1 → 6) branching linkages; pullulan was hydrolyzed to maltotriose; and maltose was not hydrolyzed to D-glucose. It is concluded from these results that the purified enzyme preparation hydrolyzes only the α-D-(1 → 6), and not the α-D-(1 → 4), glucosidic linkages in the amylaceous saccharides.

Role of the debranching enzyme in the digestion of starch granules. — As reported

TABLE I

PURIFICATION OF R-ENZYME[a]

Step	*Total volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (U/mg)*	*Yields (%)*
Crude extract	2005	25463	(262.25)	(0.010)	(100)
Ammonium sulfate (30–60% saturated)	278	7122	24.27	0.003	9.3
DEAE-cellulose (Whatman DE-52)	24	316	13.44	0.043	5.1
Sephadex G-150 superfine	14.8	27	11.02	0.408	4.2
Cyclohexaamylose–Sepharose	1	0.49	4.92	10.0	1.9

[a]The determination of R-enzyme activity in the crude extract by the method described in the Methods section may give larger values than the real ones, because contaminating carbohydrases may hydrolyze maltotriose, the product of R-enzyme action. Therefore, total and specific activities of the crude extract, given in Table I, may be a little larger than real values; however, the percentage recovery at each purification step was calculated on this basis. Also, in the determination of R-enzyme activity of the affinity-column eluate, cycloheptaamylose interferes with the determination. However, the enzyme solution was diluted five times in the assay mixture and the degree of inhibition in this instance was ~20%.

in the previous paper[5], beta amylase had very little activity on starch granules (0.8% digestion after 3 days), whereas alpha amylase digested starch granules to the extent of 42.7%; by the combined action of alpha and beta amylases, starch granules were digested further to the extent of 52.3%. On the other hand, the dialyzed, crude extract from malted barley, which contained the same activities with respect to alpha and beta amylases, digested starch granules to the extent of 85.9%. Values of the extent of digestion given here are those obtained after incubation of starch granules with the respective enzyme or enzymes for 3 days at 30°, as described in the Methods section. Moreover, the paper chromatogram of the solubilized products of digestion of starch granules by alpha and beta amylases showed the presence of higher oligosaccharides, presumably limit dextrins, in addition to spots corresponding to maltooligosaccharides ranging from D-glucose to maltohexaose. In the case of the dialyzed crude extract, no spots corresponding to limit dextrins were observed in the chromatogram of the solubilized digestion-products. This fact indicated that the debranching enzyme was operative in the digestion of starch granules when the dialyzed, crude extract from malted barley was used as the enzyme.

The effect of added debranching enzyme on the digestion of starch granules by the action of purified alpha and beta amylases was next investigated. As shown in Fig. 4, starch granules were digested to the extent of 84.8% when a partially purified preparation of the debranching enzyme [obtained by rechromatography on a column of Sephadex G-150 (superfine); specific activity, 3.1 units/mg of protein] was added to a mixture of alpha and beta amylases. This extent of digestion is approximately the same as that obtained by the dialyzed crude extract (85.9%). However, the purified

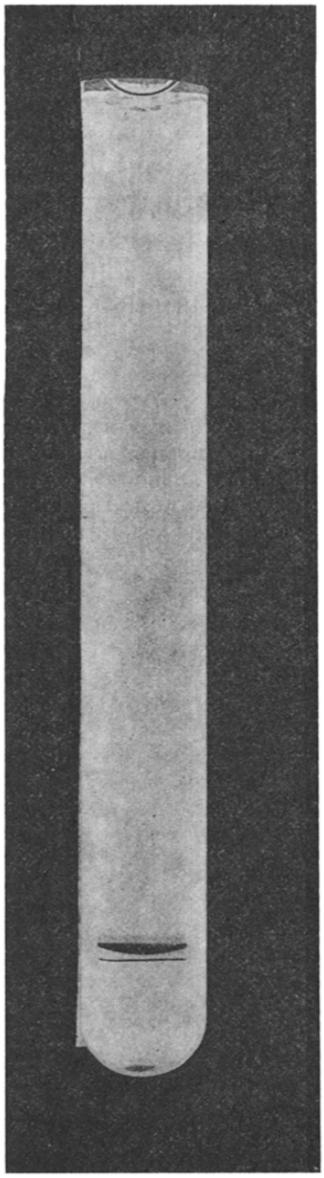

Fig. 3. Poly(acrylamide) disc-gel electrophoresis of the purified R-enzyme from malted barley. See the Methods section for experimental details.

debranching enzyme (obtained by affinity chromatography; specific activity, 10 units/mg of protein) had a lower accelerating effect on alpha and beta amylases, and the extent of digestion of starch granules was 74.1%. The combined action of alpha amylase and the purified debranching enzyme gave 51.4% digestion, a value similar to that obtained by the combined action of alpha and beta amylases (52.3%). When

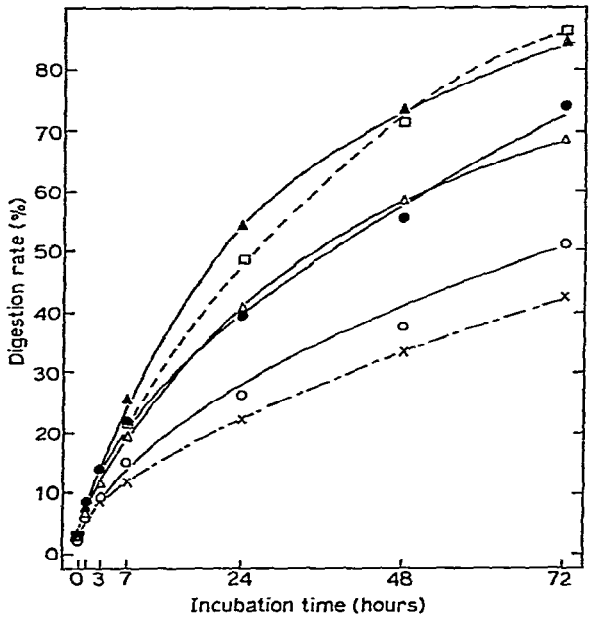

Fig. 4. Time-course of digestion of barley-starch granules by alpha and beta amylases in the presence of R-enzyme. See the Methods section for experimental details. ×, alpha amylase; ○, alpha amylase + purified R-enzyme; △, alpha amylase + partially purified R-enzyme; ●, alpha amylase + beta amylase + purified R-enzyme; ▲, alpha amylase + beta amylase + partially purified R-enzyme; □, dialyzed, crude malt-extract containing 1800 units of alpha amylase and 120 units of beta amylase activities. The specific activity and the amount added of each enzyme are: alpha amylase (10,900 units/mg of protein), 1800 units; beta amylase (218 units/mg of protein), 120 units; purified R-enzyme (10 units/mg of protein), 1 unit; partially purified R-enzyme (3.1 units/mg of protein), 1 unit. The amounts of alpha and beta amylases used in these experiments were based on the activities of these enzymes extracted from malted barley containing 100 mg of starch granules. The amount of the debranching enzyme used was arbitrary. Alpha amylase activity was determined, and the activity unit was defined by the method of International Association for Cereal Chemistry, as described by Perten[32]. Beta amylase activity was determined essentially by the method of Bernfeld[33], but at 30°. Beta amylase activity in the dialyzed, crude malt-extract was estimated by subtracting the reducing power (determined as maltose) arising from the alpha amylase component from the total reducing power.

the partially purified debranching enzyme was used in combination with the alpha amylase, the extent of digestion was 68.8%.

Similar experiments were carried out with *Aerobacter* pullulanase or *Pseudomonas* isoamylase in place of the barley-malt debranching enzyme. As shown in Fig. 5, the action of these enzymes is similar to that of the purified barley-malt debranching enzyme, and the extent of digestion of starch granules obtained by *Aerobacter* pullulanase or *Pseudomonas* isoamylase in the presence of alpha amylase was 48.3 and 54.0%, respectively; in the presence of both alpha and beta amylase the extents were 60.9 and 67.7%, respectively.

To see whether the debranching enzymes had any direct activity on starch granules, mixtures containing 500 mg of starch granules, 5 units each of the debranching

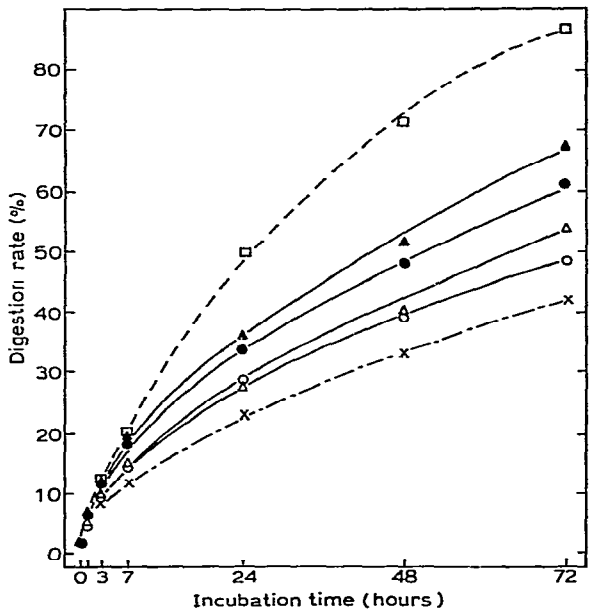

Fig. 5. Time-course of digestion of barley-starch granules by alpha and beta amylase in the presence of *Aerobacter* pullulanase or *Pseudomonas* isoamylase. ○, alpha amylase + pullulanase; △, alpha amylase + isoamylase; ●, alpha amylase + beta amylase + pullulanase; ▲, alpha amylase + beta amylase + isoamylase. The amount of pullulanase or isoamylase added was 1 unit. Other conditions are the same as in Fig. 4.

enzyme, 1.25 ml of 0.5M acetate buffer (pH 4.8), and 8.75 ml of 0.2% calcium acetate solution were incubated for 8 days at 30° under toluene. After the incubation, the starch granules were washed with water to remove the enzyme, dehydrated in alcohol, and dried to give debranching enzyme-treated starch granules. The treated starch-granules were then digested by alpha amylase in the presence or absence of beta amylase and the extents of digestion were determined. As shown in Fig. 6, the debranching enzymes had practically no modifying effect on starch granules with respect to the susceptibility to the action of alpha and beta amylases. These results show that the debranching enzyme, when used alone, has very little action on starch granules, but has an accelerating effect on alpha amylase in the digestion of starch granules.

DISCUSSION

Ever since Hobson *et al.*[16] found in potatoes and broad beans an enzyme hydrolyzing (1 → 6)-α-D-glucosidic linkages in amylopectin and named it R-enzyme, there have been many studies[17–22] on its mode of action, especially as regards the possible identity of this enzyme with limit dextrinase. According to Dunn *et al.*[22], the action of the plant debranching-enzyme on branched substrates is concentration-dependent, and the rate with amylopectin as the substrate decreases more rapidly

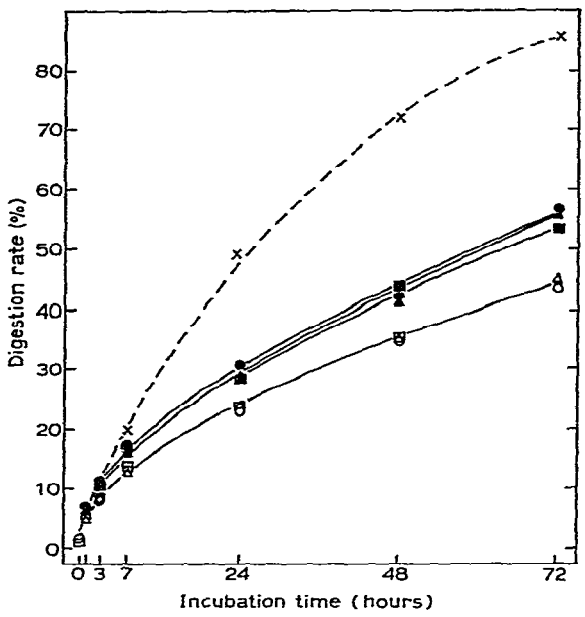

Fig. 6. Digestion of barley-starch granules, previously treated with debranching enzymes, by alpha and beta amylases. Pretreatment of starch granules: 1 unit of the debranching enzyme per 100 mg of starch granules for 8 days at 30° under toluene. Enzymes used in the pretreatment were: circle, R-enzyme; triangle, pullulanase; and square, isoamylase. Digestion of pretreated starch granules: open signs, alpha amylase; filled signs, alpha and beta amylases. Other conditions are the same as in Fig. 4.

than that with limit dextrin as the substrate with decreasing concentration of the enzyme.

The purification of the plant debranching-enzyme from various sources has been reported: oat (0.8 unit/mg of protein) and rice (0.6 unit/mg of protein)[23], broad bean (1 unit/mg of protein)[24], sorghum (1.08 units/mg of protein)[25], sweet corn (6.45 units/mg of protein)[26], and malted barley (3 units/mg of protein)[27]. No extensive purification of the plant debranching-enzyme has been achieved as yet.

The use of cyclohexaamylose, an inhibitor of beta amylase, coupled to epoxy-activated Sepharose 6B as the affinity adsorbent for sweet-potato beta amylase was reported by Vretbrad[28]. Hoschke *et al.*[29] reported a detailed examination of the reaction conditions and kinetics of the affinity chromatography of alpha amylase, beta amylase, and amyloglucosidase using this biospecific adsorbent. On the other hand, Marshall[30] reported that cycloamyloses are potent inhibitors of sweet-corn debranching enzyme, the degree of inhibition being 76% by 11.75 μg/ml of cycloheptaamylose and 54% by 58.8 μg/ml of cyclohexaamylose; barley beta amylase is inhibited 66% by 9.4 mg/ml of cyclohexaamylose. Taking advantage of these findings, and especially the difference between the affinities of cycloamyloses with amylases and the debranching enzyme, a purification procedure for barley malt-debranching enzyme was developed by using cyclohexaamylose coupled to epoxy-activated

Sepharose 6B as the affinity adsorbent. The final preparation obtained had the highest specific activity reported in the literature and gave a single band in poly(acrylamide) disc-gel electrophoresis.

Previous studies from this laboratory indicated that alpha amylase may play a major role in the digestion of starch granules during the germination of barley seeds. Although it has very little activity on starch granules by itself, beta amylase has a synergistic action on alpha amylase in accelerating the digestion of starch granules. The dialyzed, crude extract from malted barley was more potent in digesting starch granules than the combination of alpha and beta amylases, on the basis of the same enzyme activities with respect to alpha and beta amylases. As shown in this paper, a partially purified preparation of malted-barley debranching enzyme digested starch granules in the presence of alpha and beta amylases at a rate comparable with that of the dialyzed, crude extract. However, the debranching enzyme, purified extensively on an affinity column, had a lower synergistic effect with alpha and beta amylases on the digestion of starch granules. This indicates that an enzyme or enzymes, possibly containing α-D-glucosidase[31], might have been lost during the purification procedures. In the elution pattern of the affinity chromatogram, as shown in Fig. 2, there is, besides the main peak, a small peak that contains debranching-enzyme activity and was excluded unadsorbed from the affinity column. The enzymes contained in this fraction are now under investigation. As shown in this paper, the debranching enzyme by itself had little activity on starch granules, indicating that the major role of this enzyme *in vivo* may be the debranching of the dextrins solubilized by the action of alpha amylase on starch granules so that, in the presence of beta amylase and α-D-glucosidase, alpha amylase dextrins may be converted quantitatively into D-glucose at a reasonable rate in the germinating barley endosperms.

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