Studies on Q-Enzyme of Rice Plant

Part X.* Purification and Physico-chemical Properties of Q-Enzyme**

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Purification of Q-enzyme prepared from the extract of rice seed at the milky stage employing precipitation by lead acetate, fractionation by ammonium sulfate and chromatography on hydroxylapatite was carried out. The highly purified preparation of Q-enzyme was nearly homogeneous in electrophoresis, chromatography and solubility, and was nearly free from amylase, maltase, isoamylase, phosphorylase and phosphatase activities. Some of the physico-chemical and enzymic properties of the preparation were also investigated.

Since Q-enzyme was isolated by Haworth et al.¹⁾ from potato juice, its purification has been studied by many workers on various sources.

As for the purification of potato Q-enzyme, Bourne et al.²⁾ have reported a method by kaolin treatment and ammonium sulfate fractionation. Thereafter, Barker et al.³⁻⁵⁾ have obtained a preparation substantially free from amylases and fatty acids by using a modified method involving precipitation as a lead complex, while, Gilbert and Patrick^{6,7)} have prepared potato Q-enzyme in crystalline form by ethanol-citrate fractionation at low temperature. Further improvements of the methods for purification have been developed by Baum & Gilbert⁸⁾ and Peat et al.⁹⁾

Q-enzyme of pulses, *Polytomella coeca*, green gram, corn and others have been partially purified in a similar manner.

As described above, we may credit the main achievement on the purification of Q-enzyme to Gilbert et al., but the properties of the crystalline preparation obtained by them have not yet been made clear.

Few reports appeared on the purification of Q-enzyme of rice plant. Recently, Nakamura et al.¹⁰⁾ have reported the partial purification of Q-enzyme in rice seed of waxy race at the perfect ripe stage. Our preliminary examinations¹¹⁾ with the Q-enzyme fraction prepared from rice seed at the milky stage by lead acetate precipitation and ammonium sulfate fractionation have shown that chromatography on hydroxylapatite is effective in separating Q-enzyme from other impurities.

Its further purification yielded a Q-enzyme preparation which appeared to be homogeneous by electrophoretic analysis.

The present report deals with the purification

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tion of Q-enzyme of rice plant and with the informations obtained during the process.

MATERIALS AND METHODS

Rice Plants. Rice plants of starchy race, such as variety Koshiziwase, Nihonkai and Yomohikari, were the materials mainly used. Rice seed was sampled at the milky stage (8 to 12 days after flowering), the highest activity of Q-enzyme during ripening.

Substrates. Amylose: Rice amylose which was used as the substrate of Q-enzyme throughout this study, was prepared from the defatted starch of variety Koshiziwase by the method of Schoch¹²⁾, and was recrystallized 5 times from water saturated with *n*-butanol.

Amylopectin: Rice amylopectin was isolated from the first mother liquor after removal of amylose fraction and purified by precipitation with methanol.

Beta-Limit Dextrin: Amylopectin β -limit dextrin was prepared by the prolonged action of crystalline β -amylase on rice amylopectin, followed by dialysis to be removed from maltose and inorganic substance and by precipitations twice with ethanol. The β -amylase was a preparation from sweet potato¹³⁾ recrystallized three times.

Maltose: Commercial maltose, Difco Laboratories, U.S.A., was used without further purification.

Glucose-1-Phosphate: Crystalline dipotassium salt was prepared by the enzymic method of Nakamura¹⁴⁾, followed by recrystallization twice from ethanol and acetone, respectively.

Beta-Glycerophosphate: Commercial sodium β -glycerophosphate, Kanto Chemicals Co., Japan (extra pure), was used without further purification.

Determination of Enzyme Activity. Q-Enzyme Activity: Q-Enzyme activity was determined by the iodine-staining method as reported previously¹⁵⁾.

The components of standard digest were as follows: 5 ml of 0.2% amylose solution, 3 ml of 0.2 M citrate buffer, pH 6.7, 1 ml of Q-enzyme solution and 1 ml of distilled water (total volume 10 ml). The amylose solution was prepared as follows: 200 mg (dry weight) of amylose was dissolved in 2 ml of 2.5 N sodium hydroxide solution and diluted with water to about 50 ml. After heating at 80°C for 10 minutes, the

solution was cooled, neutralized with diluted hydrochloric acid and made up with water to 100 ml. The digest solution was incubated at 27°C.

One ml aliquots (equivalent to 1 mg of amylose) were removed at intervals and stained with 1 ml of 0.2% iodine solution (0.2 g of iodine and 2 g potassium iodide/100 ml) in a final volume of 25 ml. The optical densities were measured in 5 mm cuvettes using Hirma photoelectric colorimeter fitted with a 655 $m\mu$ filter. On unit of Q-enzyme activity was defined as the amount of enzyme causing a decrease of 1% in the optical density of amylose-iodine complex in the digest solution per minute under the above conditions, as reported previously¹⁶⁾.

As separated runs, 2 ml aliquots of the digest solution were subjected to Somogyi determination of reducing sugar¹⁷⁾. The reducing sugar liberated by the action of the enzyme on amylose was determined as the apparent conversion into maltose.

Amylase Activity: The test procedure for amylase activity was essentially the same as that reported previously¹⁸⁾. One ml of the enzyme solution was added at 40°C to a mixture of 5 ml of 0.2% amylose solution and 3 ml of 0.2 M acetate buffer, pH 5.5, and the mixture was made up to 10 ml with distilled water. The digest solution was incubated at 40°C and then 2 ml aliquots were taken to be used for determining the reducing sugar by the method of Somogyi; the degree of hydrolysis was calculated in terms of percentage of maltose formed from the theoretical maltose value of amylose. On the other hand, amylopectin β -limit dextrin in place of amylose in the above digest solution was used as the substrate, and the color reduction of iodine complex of amylopectin β -limit dextrin was determined by the iodine-staining method described above.

Maltase Activity: The composition of the digest solution was almost the same as that reported previously¹⁹⁾. Five ml of 0.2% maltose solution, 3 ml of 0.2 M acetate buffer, pH 4.4, 1 ml of enzyme solution and 1 ml of distilled water were mixed together to make the total volume of 10 ml and incubated at 40°C.

The glucose formed was determined by the method of Somogyi as described above and the degree of

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conversion of maltose to glucose was calculated in percentage.

Isoamylase Activity: The determination of isoamylase activity was made as reported previously²⁰. The test was carried out at 40°C on the digest solution containing 5 ml of 0.5% amylopectin, 3 ml of 0.2 M acetate buffer, pH 5.5, 1 ml of enzyme solution and 1 ml of distilled water, in a total volume of 10 ml. One ml samples were taken out at intervals, and the color increment of amylopectin-iodine complex was determined as described above.

Phosphorylase Activity: Phosphorylase activity was determined under the same conditions reported previously²⁰. A solution consisting of 0.5 ml of 5% soluble starch, 1 ml of 0.2 M citrate buffer, pH 6.7, 0.5 ml of 0.35 M sodium fluoride and 0.5 ml of enzyme solution was brought to 37°C and then 1 ml of 0.1 M dipotassium glucose-1-phosphate was added (total volume of 3.5 ml). At intervals, 0.5 ml samples were taken out and the test for liberated inorganic phosphate was made by the method of Nakamura¹⁴.

Phosphatase Activity: The test of phosphatase activity was carried out by the same procedure as for phosphorylase activity, using sodium β -glycerophosphate or dipotassium glucose-1-phosphate as substrate, excluding soluble starch and sodium fluoride which were used in the test solution for phosphorylase. The digest solution was incubated at pH 4.8 and 37°C.

Determination of Protein. Protein nitrogen was determined by a micro-Kjeldahl method. As for the determination of protein concentration on chromatography, the method of Folin-ciocalteu modified by Lowry et al.²² was used.

EXPERIMENTAL AND RESULTS

Purification of Q-Enzyme from Rice Seed at the Milky Stage.

I) Preparation of Original Extract: One and a half kg (wet weight) of rice seed at the milky stage were soaked for twenty minutes in 0.5% sodium hydrosulphite solution. The seeds were drained, washed, pounded with a small amount of distilled water and pressed. The turbid green juice was adjusted with 0.05 N sodium hydroxide to pH 7.2 with vigorous stirring and was centrifuged (3,000 r.p.m.,

for ten minutes) to remove starch and other precipitated materials.

II) Treatment by lead Acetate Solution: To 1 l of the supernatant, 300 ml of lead acetate solution, pH 7.2²³, was added with stirring, and the green lead-protein precipitate was obtained by centrifugation (3,000 r.p.m., for twenty minutes). The precipitate was stirred with 300 ml of 0.2 N sodium hydrogen carbonate for ten minutes, and a stream of carbon dioxide was then passed through the suspension for 2.5 minutes. The insoluble residue removed by centrifugation (3,000 r.p.m., for twenty minutes) was discarded.

III) Ammonium Sulfate Fractionation: Solid ammonium sulfate (18 g) was added to 100 ml of the supernatant which was maintained at pH 7.0 by addition of dilute aqueous ammonia.

The precipitate was collected by filtration and dissolved in 100 ml of distilled water. The ammonium sulfate precipitation was repeated twice.

All these operations were performed in a cold room.

IV) Freeze-drying: The precipitate was dissolved in 50 ml of 0.2 M sodium phosphate buffer, pH 7.0. The solution was frozen in an acetone-carbon dioxide mixture and was dried in vacuo, 0.05~0.01 mmHg.

V) First Chromatography by Hydroxylapatite: As previously reported¹³, the author found that Q-enzyme adsorbed by hydroxylapatite at low concentrations of sodium phosphate buffer, pH 6.8, can be eluted quantitatively with 0.2 M concentration of the same buffer, giving reproducible chromatographic pattern. Further purification was achieved as follows. The freeze-dried material was dissolved in 20 ml of distilled water and was dialyzed with stirring against three changes of 0.05 M sodium phosphate buffer, pH 6.8, for thirty hours in a cold room.

The resulting precipitate was removed by centrifugation. The whole dialyzed enzyme solution was applied to a column 16×160 mm of hydroxylapatite, which was previously equilibrated with 0.05 M sodium phosphate buffer of pH 6.8. The stepwise elution with increasing concentrations of sodium phosphate buffer was applied at a flow rate of about 30 ml per hour, and 5 ml fractions were collected. The chromatographic operations were performed in a room at 10~15°C.

As shown in Fig. 1, the fraction with Q-enzyme activity appeared at 0.2 M concentration. The Q-

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FIG. 1. Chromatography of the Freeze-dried Q-Enzyme Preparation (Enzyme IV). Column, 16×160 mm.

—○— Optical density (720 mμ) as a measure of protein concn (Lowry method).
 ---×--- Q-Enzyme activity as percentage decrease in optical density (655 mμ) of iodine stain.

FIG. 2. Separation of Q-Enzyme by Chromatography on Hydroxylapatite.

Column, 12×150 mm. —○— Protein content (Lowry method); ---×--- Q-Enzyme activity.

enzyme fractions exclusive of those with low specific activity were pooled and precipitated by ammonium sulfate saturation.

VI) Second Chromatography by Hydroxylapatite: As revealed by chromatographic pattern in Fig. 1, the distributions of protein and the enzyme activity in each fraction are not always coincident with each other. Namely, the peak of Q-enzyme activity appears a little later than the protein peak. It may be con-

sidered that the protein fraction eluted earlier is the impurity with no activity against substrate. Further attempts to remove this impurity from the Q-enzyme fraction were performed as follows. Preliminary experiments¹¹⁾ have indicated that Q-enzyme activity was scarcely recovered from the hydroxylapatite column with 0.2 M sodium phosphate buffer of pH 6.0. Whereas, by the chromatography with 0.2 M concentration of buffer, pH 6.2, almost all

FIG. 3. Rechromatography of the Separated Q-Enzyme Fraction (Enzyme VI).
Column, 12×130 mm.

—○— Protein content (Lowry method)
---x--- Q-Enzyme activity

impurity and a small amount of Q-enzyme activity were obtained by the following elution with the same concentration of pH 6.8. Thus, the whole of the precipitate obtained by the first chromatography was dissolved in 0.05 M sodium phosphate buffer of pH 6.2 and was dialyzed against the same buffer.

The dialyzed enzyme solution was chromatographed on a column of 12×150 mm with a flow rate of about 20 ml per hour and 2 ml fractions were collected. The column was stepwise eluted with 0.2 M buffer of pH 6.2, and was followed by the same concentration of pH 6.8. As shown in Fig. 2, a major amount of Q-enzyme activity appeared at 0.2 M concentration of buffer, pH 6.8. Since the impurity was removed by these procedures, the small loss of Q-enzyme activity, as may be seen in Table I, was not disadvantageous. The peak of Q-enzyme activity in the main fraction was fairly coincident with the protein peak, and the specific activity in each fraction was nearly constant. By this chromatographic procedure, the specific activity of Q-enzyme fraction rose more than 2-folds that of the first chromatographic preparation, and 77% of the starting activity was recovered.

When Q-enzyme fraction in this step was rechromatographed by the same procedure, a single symmetrical curve was obtained at the same concentration of buffer, as shown in Fig. 3. The Q-enzyme fractions (main fractions) were pooled and precipitated by ammonium sulfate saturation.

The precipitate was freeze-dried in 25 ml of 0.2 M citrate buffer, pH 7.0 by the same procedure as in step IV and was stored in a cold room. In Table I

the recovery data at each step in the purification procedure were summarized. In the steps before step III, it was difficult to determine the significant Q-enzyme activity to be compared, owing to the considerable interference by the contaminating enzymes.

Therefore, the recovery data in the steps after step III was described in the table.

TABLE I. ACTIVITIES AND YIELDS OF Q-ENZYME AT EACH STEP OF PURIFICATION

Step	Purification procedure	Volume ml	Units/ml	Units/mg protein	Yield %
III	Ammonium sulfate fractionation	50	12.2	8.7	100
IV	Freeze-drying	28	19.7	9.5	90
V	1st. chromatography	100	4.8	65.2	78
VI	2nd. chromatography	25	14.9	155.0	61

Electrophoresis of Q-Enzyme Preparations at Various Steps of Purification.

The enzyme preparations, enzyme IV, V and VI obtained by the purification procedures summarized in Table I were subjected to electrophoretic experiments.

Each enzyme solution was dialyzed for twenty four hours with stirring in a cold room against phosphate buffer of ionic strength 0.1 at pH 7.8, making the protein concentration of 1~1.35%. The measurements were made in a Tiselius apparatus of Hitachi Model HTD-1 with current of 6 mA at 3~7°C. From the patterns in Fig. 4b, it was found that enzyme V was composed of two components, as expected from the chromatographic behavior. The electrophoretic

FIG. 4. Electrophoretic Patterns of Q-Enzyme Preparations at Various Steps of Purification.

Solvent, phosphate buffer of pH 7.8, ionic strength, 0.1; electrophoresis, at 6 v/cm, 6 mA in Tiselius apparatus.

- (a) Electrophoresis of enzyme IV (protein concn., 1.35%, at 7°C)
- (b) Electrophoresis of enzyme V (protein concn., 1%, at 7°C)
- (c) Electrophoresis of enzyme VI (protein concn., 1%, at 3°C)

FIG. 5. Ultracentrifugal Patterns of Purified Q-Enzyme Preparation (Enzyme VI).

Solvent, phosphate buffer of pH 7.8, ionic strength, 0.1; protein concn., 0.9%; rotar speed, 56,100 r.p.m.; temperature, 5°C.

mobility of both components was calculated as -5.9×10^{-5} and $-5.1 \times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$, respectively.

As shown in Fig. 4c, the electrophoretic behavior of enzyme VI was considered to be nearly homogeneous. The similar results were obtained at pH 4.0 (in acetate buffer), 6.0 and 7.0 (in phosphate buffer). As the Q-enzyme solution is unstable at pH 4.0⁽¹¹⁾, it is anticipated that the activity may be considerably lost during the experiments. However, no appreciable coagulation of the enzyme protein was observed either during the preliminary dialysis or electrophoresis at low temperature.

The electrophoretic mobility of enzyme VI was calculated as $-5.3 \times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$, at pH 7.8. Judging from these electrophoretic mobilities, it is concluded that the component with smaller mobility in Fig. 4b and the component with smaller mobility than that of the highest peak in Fig. 4a, correspond to Q-enzyme.

Ultracentrifugation of Enzyme VI.

The ultracentrifugal experiment was carried out in a Spinco Model E analytical centrifuge at 56,100 r.p.m. Prior to the analysis, the enzyme solution was dialyzed for twenty four hours with stirring in a cold room against phosphate buffer, pH 7.8, ionic strength 0.1. The protein concentration of 0.9% was used. As shown in Fig. 5, the protein of enzyme VI was not completely homogeneous in ultracentrifugal analysis.

Solubility of Enzyme VI.

Enzyme VI was dissolved in 0.02 M citrate buffer of pH 7.0 and was dialyzed for forty eight hours against the same buffer solution at 5°C. To 1 ml aliquots of this solution in small centrifuge tubes were added varying amounts of ammonium sulfate solution

(pH 7.0, 40 g/100 ml, in 0.02 M citrate buffer). The tube contents were mixed with a small stirring rod, allowed to stand for ten hours at 5°C and centrifuged (10,000 r.p.m., for ten minutes). One portion of the clear supernatant liquid was analyzed for protein by a micro-Kjeldahl method, and the other was used for determining the activity of Q-enzyme by the method described above.

The results of this solubility-activity test are graphically presented in Fig. 6. According to Fig. 6, the solubility behavior of enzyme VI against ammonium sulfate solution is presumed to indicate the homogeneity of the preparation.

FIG. 6. Solubility Behavior of Purified Q-Enzyme Preparation (Enzyme VI).

—○— Protein in supernatant
—×— Q-Enzyme activity in supernatant

Isoelectric Point of Enzyme VI.

The electrophoretic analysis of enzyme VI was conducted at pH 4.0 (in acetate buffer), 6.0, 7.0 and

FIG. 7. Electrophoretic Mobility of Purified Q-Enzyme Preparation (Enzyme VI).

Solvent, acetate buffer of pH 4.0, phosphate buffers of pH 6.0, 7.0 and 7.8, ionic strength, 0.1; electrophoresis, at 6 mA, 3~5°C in Tiselius apparatus.

7.8 (in phosphate buffer), respectively in a Tiselius apparatus under the conditions of ionic strength 0.1, current 6 mA, 3~5°C.

From the results of electrophoresis at various pH values shown in Fig. 7, the isoelectric point of enzyme VI is estimated to be about pH 5.8.

FIG. 8. Ultraviolet Absorption Spectrum of Purified Q-Enzyme Preparation (Enzyme VI).

Solvent, 0.02 M citrate buffer, pH 7.0.

Ultraviolet Absorption Spectrum of Enzyme VI.

The ultraviolet absorption spectrum of enzyme VI was obtained by the use of a Shimadzu Beckman type Model QB-50 spectrophotometer. The spectrum of enzyme VI shown in Fig. 8 indicates a typical absorption pattern of protein with the maximum absorption at 278 mμ.

Protein Nitrogen of Enzyme VI.

A solution of enzyme VI was dialyzed three days with stirring against distilled water at 4°C. An aliquot from the dialyzate was precipitated with trichloroacetic acid and was used for determining the nitrogen by a micro-Kjeldahl method.

Another aliquot was used for determining ash with the usual method. From the analytical results obtained, the nitrogen content of enzyme VI protein was found to be 14.9%.

Action of Enzyme VI on Amylose.

As shown in Table I, the purified Q-enzyme preparation (enzyme VI) yielded a specific activity of 155 units per mg protein; namely, 0.01 mg of enzyme preparation caused a decrease of 1.55% in the optical density (655 mμ) of amylose-iodine complex per minute under the optimal conditions of pH 6.7, 27°C.

The apparent velocity constant (k) characterized by the first-order reaction as described in the previous paper¹⁵⁾ was $1.84 \text{ min}^{-1} \text{mg}^{-1}$ at 25°C. Samples of Q-enzyme rapidly converted amylose into a polysaccharide stained red with iodine.

The changes in the iodine-staining properties and

FIG. 9. Action of Purified Q-Enzyme Preparation (Enzyme VI) on Amylose.

The digest contained 5 ml of 0.2% amylose solution, 3 ml of citrate buffer (pH 6.7), 1 ml of Q-enzyme solution (3.4 units) and 1 ml of distilled water.

Temperature, 27°C.

—○— Iodine-staining power
---x--- Reducing power as maltose

reducing power of the standard digest with 3.4 units of enzyme VI are shown in Fig. 9. It can be seen that the enzyme preparation causes a decrease of about 92% of the initial optical density ($655\text{ m}\mu$) of iodine stain after twenty four hours. Thereafter, there was no more decrease of this value during the next forty eight hours. Liberation of reducing sugar was only 1.7% as maltose after twenty four hours.

Enzymic Purity of Enzyme VI.

In the crude preparation of Q-enzyme, contamination of other enzymes concerned in synthesis and degradation of starch is found. The most persistent impurity is amylase, and its presence interferes with the measurements of activity of Q-enzyme.

Therefore, the purity of the purified Q-enzyme was examined by determining the activities of contaminating enzymes in the preparation of enzyme VI equivalent to 34 units (protein of 0.22 mg).

Amylase Activity: Incubation of enzyme VI with rice amylose under the optimal conditions of amylase action resulted in liberation of only 2.2% of reducing sugar as maltose after twenty four hours. The iodine-staining power of amylopectin β -limit dextrin incubated with enzyme VI under the same conditions remained constant after twenty four hours.

Maltase Activity: Enzyme VI was incubated with maltose under the optimal conditions of maltase action. The analytical result after twenty four hours showed an increase of only 1.4% of reducing sugar as glucose. Further, little liberation of glucose in paper chromatographic analysis was detected after twenty-four hours.

Isoamylase, Phosphorylase and Phosphatase Activity: No isoamylase, phosphorylase and phosphatase activity were detected in either case when enzyme VI was incubated with rice amylopectin, glucose-1-phosphate or β -glycerophosphate for twenty four hours.

From these results, it is apparent that the purified Q-enzyme preparation (enzyme VI) is nearly free from amylase, maltase, isoamylase, phosphorylase and phosphatase activity, and therefore, the color reduction of amylose stained with iodine is independent of the action of these contaminating enzymes.

DISCUSSION

Amylase bears an apparent resemblance to Q-enzyme in its action on amylose and it was a key problem in purification to prepare Q-enzyme free from amylases.

Q-Enzyme from rice seed at the milky stage was separated with considerable effectiveness from amylases, fatty acids and chlorophyll by precipitation as a lead complex according to the method of Barker et al.³⁻⁵), and by subsequent fractionation with ammonium sulfate. However, a trace amount of amylase, maltase and isoamylase was still present in the preparation obtained by the above procedure.

Further fractionation by precipitation with solvents or adsorption on starch and activated charcoal was not so effective in the attempt to remove contaminating enzymes.

Whereas, when the chromatography of these enzymes on hydroxylapatite was carried out by stepwise addition of sodium phosphate buffer, pH 6.8 of increasing molarity, it was observed that isoamylase was not adsorbed to the hydroxylapatite column even at low concentration of 10^{-3} M , while amylase, maltase and Q-enzyme was adsorbed at the same concentration. Amylase and maltase was subsequently eluted at concentrations below 0.1 M, and Q-enzyme was eluted at 0.2 M. The Q-enzyme fraction obtained was nearly free from contaminating enzymes, however, it was proved to be still heterogeneous in chromatographic and electrophoretic patterns.

Furthermore, by pH changes of the buffer to be used during chromatography, Q-enzyme was isolated to be electrophoretically homogeneous. This preparation was also considered to be nearly homogeneous by solubility, chromatographic and enzymic analyses, but the purified preparation of Q-enzyme was not completely homogeneous so far as ultracentrifugal analysis was concerned. Whether this heterogeneity is due to the contamination with other proteins or to the association of the enzyme protein is not clear. Attempts to crystallize the purified enzyme preparation were not successful. The highly purified Q-

enzyme preparation had a powerful activity in conversion of rice amylose into a polysaccharide stained red with iodine.

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