Studies on Amylases of *Aspergillus oryzae* Cultured on Rice

Part I. Isolation and Purification of Glucoamylase

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Four fractions of glucoamylase, different chromatographically from one another, were isolated and purified from a culture of *Aspergillus oryzae* on steamed rice. All these fractions were found to be homogeneous in sedimentation analysis, and had almost the same enzymatic activities in the hydrolysis of soluble starch. The limit of hydrolysis of starch was about 60%, which was very similar to those of Taka-amylase B of Okazaki and Taka-amylase B₂ of Sawasaki.

INTRODUCTION

Since Tokuoka¹⁾ demonstrated the presence of a saccharifying amylase, later called glucoamylase, in cultures of *Aspergillus oryzae*, a number of investigations on this enzyme have been performed because of its importance in Saké-brewing as well as of glucose production from starch. Furthermore, it has been recently pointed out that glucoamylase-protein would be a major source of turbidity of Saké,^{2,3)} but it should not be completely confirmed until this enzyme would be highly purified. In connection with this, some highly purified or crystalline glucoamylases of *Aspergillus oryzae* have been obtained,^{4,5)} but all of these preparations were obtained from Taka-diastase, which was originally produced from a culture of the mold on steamed wheat bran, while almost no report on the purification of the enzyme from a culture on steamed rice, the material of Saké-brewing. Such a difference of culture might be of importance for elucidating the nature and the structure

of the enzyme, because some reports have been presented on the difference in properties of microbial amylases by different conditions of cultivation.^{6~8)} Moreover, Taka-diastase contains much more activities of proteolytic enzymes than the early culture on highly polished rice, and this fact suggests that the former material would not be a necessarily suitable material for preparing the unmodified glucoamylase.

In the present paper, we describe the isolation and purification of glucoamylase from a culture of *Aspergillus oryzae* on steamed rice in order to elucidate the characteristics of this enzyme.

MATERIAL AND METHODS

"Koji", a Culture of *Aspergillus oryzae* on Rice

The mold culture used in this experiment was kindly supplied by Okura Shuzo Co., Kyoto. This material was prepared by inoculation of a selected single strain of *Aspergillus oryzae* on steamed rice grains highly polished, and by incubating them for 34 hours at 32 to 39°C.

Measurement of Amylase Activity

In the course of isolation and purification of

1) Y. Tokuoka, *J. Brewing (Japan)*, **19**, 791 (1941).

2) O. Sugita and K. Kageyama, *J. Fermentation Technol.*, **36**, 157 (1958).

3) Y. Akiyama, *J. Agr. Chem. Soc. Japan*, **36**, 825, 830 (1962).

4) H. Okazaki, *ibid.*, **29**, 181 (1955).

5) T. Sawasaki, *Repts. Sci. Research Inst. (Tokyo)*, **36**, 584, 590 (1960).

6) T. Minoda, *J. Agr. Chem. Soc. Japan*, **35**, 479 (1961).

7) K. Yamada and T. Minoda, *J. Fermentation Assoc.*, **20**, 254 (1962).

8) G. B. Manning and L. L. Campbell, *J. Biol. Chem.*, **236**, 2952 (1961).

amylases, two methods were used to measure amylase activities. The first method was based on the estimation of reducing sugars produced from amylopectin with 3,5-dinitrosalicylate spectrophotometrically according to the method of Noelting and Bernfeld⁹⁾ with some modifications. One ml of 1% amylopectin solution buffered with 0.016 M acetate buffer, pH 4.8, was mixed with 1 ml of enzyme solution and incubated for 3 minutes at 35°C. After 2 ml of 3,5-dinitrosalicylate reagent was added to stop the reaction, the mixture was heated for 5 minutes in a boiling water bath, chilled with tap water, and diluted with 20 ml of water. The optical density of the solution at 540 m μ was measured by means of a Beckman Model DU spectrophotometer. Alternatively, an auto-analyzer, Technicon Ins. Co., was used to assay the amylase activity of effluents in chromatographic purifications, because the manual method was so troublesome for such a number of samples. The principle of measurement was the same as that of the manual method, and the flow diagram is shown in Fig. 1. The second method was based upon the measurement of iodine color reaction of residual amylose in digests spectrophotometrically in order to determine α -amylase activity according to the method of Fuwa¹⁰⁾ with some modifications. Two and a half

ml of soluble potato starch solution was mixed with 1 ml of 0.5 M acetate buffer, pH 5.0, and 1.5 ml of enzyme solution successively. After incubating the reaction mixture for 5 minutes at 35°C, 5 ml of 1 M acetic acid was added to stop the reaction, and it was mixed with 0.005% iodine solution, and then diluted with 20 ml of water. The optical density at 700 m μ was measured. Both Okazaki's and Sawasaki's methods^{3, 4)} were also used in order to compare the specific activities of the final preparations with those in literatures.

Thin-layer Chromatography of Sugars

The identification of the hydrolysis products of starch was performed by the thin-layer chromatography according to the method of Weill and Hanke.¹¹⁾ The enzymatic hydrolyzate corresponding to 5 μ g of glucose was applied to the plate after boiling and chromatographed using a solvent system of butanol-2,6-lutidine-water. The detection of sugars was performed by spraying anisaldehyde reagent of Stahl and Kaltenbach.¹²⁾

Sedimentation Analysis

Ultracentrifugal studies were performed with Spinco Model E ultracentrifuge.

FIG. 1. Flow Diagram of Auto-analyzer for Assaying Amylase Activity.

9) G. Noelting and P. Bernfeld, *Helv. Chim. Acta*, **31**, 286 (1948).

10) H. Fuwa, *J. Biochem. (Tokyo)*, **41**, 583 (1954).

11) C. E. Weill and P. Hanke, *Anal. Chem.*, **34**, 1736 (1962).

12) E. Stahl and U. Kaltenbach, *J. Chromatog.*, **5**, 351 (1961).

RESULTS

1. Isolation and Purification of Amylases

a) Extraction and fractionation with ammonium sulfate

Two hundred and forty kg of molded rice was extracted with 1,070 l of water. The extract was mixed with 1.8 kg of diethylaminoethyl (DEAE)-cellulose with stirring for 1 hour, more than 90% of amylase activity in extract being adsorbed on DEAE-cellulose. The amylases adsorbed on DEAE-cellulose were separated by decantation from the supernatant fluid, washed with water by filtration, and then eluted with 0.5 M sodium chloride solution in 0.1 M acetate buffer, pH 4.6. The eluate, 10.7 l, was fractionated with ammonium sulfate by adding 5.8 kg (0.8 saturation). The supernatant was separated by centrifugation, and designated as Fraction A. The precipitate, 177 g, was again dissolved in the equal volume of water and refractionated with ammonium sulfate into two fractions: Fraction B (0.6~0.8 saturation-precipitates) and Fraction C

(0.4~0.6 saturation-precipitates).

b) Purification of Fraction A

Fraction A, 12.8 l, was first desalted by gel filtration on Sephadex G-75 M columns, 6.5×40 cm. The desalted solution, 18.6 l, was divided into two halves, and each of them was passed through a DEAE-cellulose column, 6.5×30 cm, equilibrated with 0.05 M acetate buffer, pH 5.6, to concentrate amylase. The elution was performed by increasing linearly the concentration of sodium chloride from 0 to 1.0 M in 0.05 M acetate buffer, pH 5.0. The eluates were treated with ammonium sulfate to 0.83 saturation, and the precipitate was collected by ultracentrifugation, $30,000 \times g$ for 45 minutes, and dissolved in a small amount of water. The solution, 135 ml, was desalted by gel filtration on Sephadex G-25 M column, 3×43 cm, and then chromatographed on DEAE-Sephadex A-25 M columns, as follows. The desalted protein solution was mixed with 1 M acetate buffer, pH 5.6, to make the buffer concentration of 0.05 M, and was applied to

FIG. 2. Chromatography of Fraction A on a 3×47 cm Column of DEAE-Sephadex A-25 M.

Stepwise increases in the concentration of sodium chloride are indicated at the top of the figure. Solid and broken lines indicate the concentration of protein and the enzymatic activity, respectively.

columns equilibrated with the same buffer. The enzyme protein on the column was eluted by increasing stepwise the concentration of sodium chloride in 0.05 M acetate buffer, pH 5.0. A typical elution pattern is shown in Fig. 2. Three major fractions exhibiting strong amylase activities were obtained. All of these fractions, A-1, A-2 and A-3, were glucoamylase as shown later. The yields of them were 2.0, 1.4 and 1.7 g in dry basis, respectively, from 240 kg of the molded rice. All fractions were diluted 15-fold with water or desalted by Sephadex, and then they were concentrated by DEAE-cellulose as described before.

c) Purification of Fraction B

Fraction B, wet 70 g, precipitated by 0.8 saturation of ammonium sulfate, was dissolved in 30 ml of water and deionized by passing twice through columns of Sephadex G-75 M, 6.5×45 cm. The deionized solution was chromatographed on a DEAE-Sephadex A-25 M column, 3×53 cm, by the same procedure described on Fraction A. An elution pattern is shown in Fig. 3. Four major fractions exhibiting strong amylase activities, B-1, B-2, B-3 and B-4, were obtained, the yields of

them being 0.4, 0.7, 0.3 and 0.1 g, respectively, from 240 kg of the molded rice. Fractions B-1 and B-2 were both glucoamylase, while B-3 and B-4 were α -amylase, as described later. Fraction B-1 must be identical with Fraction A-3, because the elution patterns of both fractions from DEAE-Sephadex were identical and, moreover, B-1 had a solubility of 1.7 mg per ml of ammonium sulfate solution of 0.8 saturation at 4°C and pH 5.

d) Preliminary experiment on purification of Fraction C

Fraction C, wet 90 g, was desalted by gel filtration on Sephadex G-25 M columns, and an aliquot of desalted solution, containing about 75 mg of protein, was chromatographed on a DEAE-Sephadex column, 0.9×27 cm, by the same procedure described before. The elution pattern is shown in Fig. 4. Only one fraction exhibiting strong amylase activity could be seen in Fraction C. However as this fraction contained only α -amylase but not glucoamylase, no further purification was carried out in the present experiment. Further purification of α -amylase will be presented in a succeeding paper.

FIG. 3. Chromatography of Fraction B on a 3×53 cm Column of DEAE-Sephadex A-25 M.

Symbols are the same as in Fig. 1.

FIG. 4. Chromatography of Fraction C on a 0.9×27 cm Column of DEAE-Sephadex A-25 M.

Symbols are the same in Fig. 1.

2. Re-chromatography of Glucoamylase Fractions

All of the four chromatographically different fractions, A-1, A-2, A-3 and B-2, showed glucoamylase activities. These fractions were

re-chromatographed in order to elucidate whether or not they would differ chromatographically from each other. Each fraction was again deionized by gel filtration and an aliquot was put on a column of DEAE-Sephadex A-25 M column, 0.9×22 cm, equilibrated with 0.05 M acetate buffer, pH 5.6. The elution was performed by linearly increasing the concentration of sodium chloride from 0 to 0.2 M in 0.05 M acetate buffer, pH 5.0. The chromatograms of four fractions are shown in Fig. 5. As seen from this figure, the peaks of four fractions were slightly different from each other, though A-1 and A-2 would be still heterogeneous. This fact suggests that these fractions had either different electric charges or different molecular dimensions, which will be supported by ultracentrifugal and electrophoretic experiments as described later.

3. Ultracentrifugal Homogeneity of Glucoamylase Fractions

Homogeneity of four fractions, A-1, A-2, A-3 and B-2, was investigated by sedimentation analysis with a Spinco Model E ultracentrifuge. Lyophilized preparations of desalted proteins were dissolved in 0.1 M acetate buffer, pH 5.0, and adjusted its ionic strength to 0.1

FIG. 5. Re-chromatography of Fractions A-1, A-2, A-3 and B-2 on 0.9×22 cm Columns of DEAE-Sephadex A-25.

Concentration of sodium chloride was increased linearly from 0 to 0.2 M in 0.05 M acetate buffer, pH 5.0. The observed pH values of effluents are indicated at the top of the figures.

FIG. 6. Sedimentation Patterns of Glucoamylase Fractions A-1, A-2, A-3 and B-2.

Solutions contained 10mg of protein per ml of 0.1M acetate buffer, pH 5.0 and ionic strength 0.1. The photographs were taken 64 minutes after reaching final speed (59,780 r.p.m.). Temperatures were A-1: 17.9°C, A-2: 20.3°C, A-3: 18.3°C, and B-2: 18.2°C.

by adding sodium chloride. Typical photographs of patterns at 64 minutes after reaching at maximal speed 59,780 r.p.m. are shown in Fig. 6. All patterns exhibited a single peak, which revealed that all of these glucoamylase fractions were homogeneous in sedimentation

analysis. Making the usual corrections for temperature and viscosity, the sedimentation coefficients, $s_{20,w}$, were evaluated according to the method of Schachman.¹³⁾ The values for 1% of protein concentrations were about 4S, as described in detail in the succeeding paper.

4. Enzymatic Hydrolysis of Starch

Typical time courses of hydrolysis of soluble starch by Fraction A-1 according to the method of Sawasaki and of Okazaki are shown in Fig. 7. All other fractions, A-2, A-3 and B-2, showed almost the same patterns. This fact suggests that the four fractions resemble Okazaki's Taka-amylase B or Sawasaki's Taka-amylase B₂ in hydrolytic properties, showing the hydrolysis degree of soluble starch being about 60%. The initial velocity of hydrolysis of soluble starch by Fraction A-1 was 47 mg glucose produced/min./mg enzyme under the condition of Okazaki's method, which was about 30% higher than that of the Okazaki's purest preparation. Sawasaki's Taka-amylase B₂ preparation still contained considerable amounts of non-nitrogenous contamination, so the specific activity was much lower in dry basis. Even with the same nitrogen amounts of the enzyme preparations, Fraction A-1 had

FIG. 7. Hydrolysis of Soluble Starch by Glucoamylase Fraction A-1.

A: by Sawasaki's method, B: by Okazaki's method. See literatures 4 and 5.

13) H.K. Schachman, in "Methods in Enzymology" edited by S.P. Colowick and N.O. Kaplan, Vol. IV, p. 55, Academic Press Inc., New York, 1957.

almost twice or more activity than the Taka-amylase B₂ preparation under the condition of Sawasaki's method.

The reaction products from soluble starch by enzymatic hydrolysis were followed by means of thin-layer chromatography. Fig. 8

FIG. 8. Thin-layer Chromatogram of Hydrolysis Product of Soluble Starch by Glucoamylase Fraction A-2.

G and M are authentic glucose and maltose, and 1 to 5 correspond to the products after 15, 30, 60, 180 and 360 minutes of hydrolyses, respectively.

shows a chromatogram with use of Fraction A-2. As seen from this figure, glucose was the only liberated product and no other oligosaccharide could be found in the course of the reaction. Fractions A-1, A-3 and B-2 also produced only glucose. Furthermore, all of these fractions did not turn the iodine color of starch even after the incubation of 72 hours. Hence, it could be concluded that all of these fractions were glucoamylase containing neither α -amylase nor glucosyltransferase. Moreover, all these fractions, unlike γ -amylase,¹⁴⁾ could hydrolyze maltose. Further enzymatic properties will be presented in a succeeding paper.

DISCUSSION

In the present paper, we prepared four

14) K. Kitahara, *J. Fermentation Technol.*, 27, 254 (1948).

glucoamylase fractions from a culture of *Aspergillus oryzae* on rice by ion-exchange chromatography and ammonium sulfate fractionation, all of which were found to be homogeneous in sedimentation analysis. Although these fractions showed almost the same enzymatic properties, their chromatographic behaviors were different from one another, and this fact suggested slight differences among four fractions in chemical or physical structure of the enzyme molecule. This suggestion will be revealed by the facts that these four fractions are slightly different from one another in sedimentation coefficients, electrophoretic mobilities, ultraviolet extinction coefficients, and also in chemical compositions such as nitrogen contents and carbohydrate contents, as presented in details in succeeding papers. Such a multiplicity of mold glucoamylase has been recently presented on *Aspergillus niger*,¹⁵⁾ but it is still unknown whether the multiplicity would be due to the true isozyme system or it would come from the modification of the enzyme protein by limited proteolysis or other conformational changes. However, the culture of mold in the present work was carried out using a selected single strain of *Aspergillus oryzae*, though not absolutely pure cultivation, and, moreover, much less proteolytic enzyme could be found in the culture. So, it seems likely that these components would be the isozymes.

The hydrolytic properties of these fractions toward soluble starch were very similar to those of Taka-amylase B of Okazaki or Taka-amylase B₂ of Sawasaki. The limits of hydrolysis of soluble starch were almost 60%. However, all of the glucoamylase fractions in the present experiment exhibited the sedimentation constants of around 4S at 1% concentration, and this means the molecular weights of about 70,000, if the molecules were assumed to be spheres with 30% hydration. Okazaki demonstrated that the molecular

15) H. Kamiyama, T. Hukinbara and G. Terui, presented at the annual meeting of the Society of Fermentation Technology, Osaka, Nov. 11, 1964.

weight of Taka-amylase B would be about molecular weight.

7,500, which was too small compared with the present value. Unless the Okazaki's Taka-amylase B would be an active core of glucoamylase produced by limited proteolysis, such a small value would be derived from misleading the method of determination of

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