Purification and Some Properties of Acid-stable α -Amylases from *Shochu koji* (*Aspergillus kawachii*)

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Aspergillus kawachii α -amylase [EC 3.2.1.1] I and II were purified from *shochu koji* extract by DEAE Bio-Gel A ion exchange chromatography, Sephacryl S-300 gel chromatography (pH 3.6), ω -amino dodecyl agarose column chromatography and Sephacryl S-200 gel chromatography. By gel chromatography on a Sephacryl S-300 column, the molecular weights of the purified α -amylase I and II were estimated to be 104,000 and 66,000, respectively. The isoelectric points of α -amylase I and II were 4.25 and 4.20, respectively. The optimal pH range of α -amylase I was 4.0 to 5.0, and the optimum pH of α -amylase II was 5.0. The optimum temperatures of both α -amylases were around 70°C at pH 5.0. Both α -amylases were stable from pH 2.5 to 6.0 and up to 55°C, retaining more than 90% of the original activities. Heavy metal ions such as Hg^{2+} and Pb^{2+} were potent inhibitors for both α -amylases.

Usually *shochu koji* is prepared with Kawachi white *koji* mold (*Aspergillus kawachii*) as *tane koji*, seed of the *koji* mold for *shochu* making in the south Kyushu district that is the main area of *shochu* production in Japan. The dissolution and saccharification of the raw material and fermentation are done in the strongly acidic conditions (pH 3.2 to 4.3) of *shochu moromi* mash. The enzymes such as α -amylase, glucoamylase, etc. produced by *A. kawachii* seem to be responsible for this. On the other hand, it is well-known¹⁸⁾ that the α -amylase of *sake koji* (*A. oryzae*) is scarcely active in such hard conditions. To elucidate the activities of various enzymes of *shochu koji* in *shochu* making, we have tried to purify some enzymes from *A. kawachii*.¹⁾ This paper describes the purification and some properties of *A. kawachii* α -amylase (1,4- α -D-glucan glucanohydrolase [EC 3.2.1.1]) I and II.

MATERIALS AND METHODS

Materials. Rice *koji* of *Aspergillus kawachii* was prepared by the conventional method²⁾ using Kawachi white

koji mold (Kawachi Gen-ichiro Shoten Co.) as *tane koji*. A Labo-module CL-10 cartridge was purchased from Asahi Chemical Industry Co., Ltd.. A DIAFLO membrane PM-10 filter was from Amicon Far East Ltd.. Bio-Gel P-2 and DEAE Bio-Gel A were products of Bio-Rad Laboratories. Sephacryl S-200 Superfine, Sephacryl S-300 Superfine, high and low molecular weight gel filtration calibration kits, and Pharmalyte 2.5~5 were purchased from Pharmacia Fine Chemicals AB. An isoelectric point calibration kit Electran range 2.4~5.65 from BDH Chemicals Ltd. was used. All other materials were obtained from commercial sources.

Assay of α -amylase activity. The activity of α -amylase was assayed by the official method of analysis of the National Tax Administration Agency of Japan,³⁾ and a slight modification was included as described in our previous paper.⁴⁾ It was based on the conventional starch-iodine color method using soluble starch as the substrate. The enzyme activity was expressed by the Wohlgemuth value.⁵⁾

Estimation of molecular weight. The molecular weight of the purified enzyme was estimated by Sephacryl S-300 gel chromatography according to the method of Andrews.⁶⁾ Sephacryl S-300 Superfine was packed into a 1.5 cm glass column 95 cm high. Chromatography was done with a constant flow (15 ml/hr) of 50 mM phosphate buffer (pH 6.0) containing 0.1 M NaCl. Elution was monitored by UV absorption at 280 nm, and fractions of 2 ml were collected.

The column was calibrated with several marker proteins. The void volume was measured with Blue Dextran 2000. Standard proteins for molecular weight estimation are described in the legend to Fig. 2.

Electrophoresis. Discontinuous polyacrylamide gel electrophoresis was done at pH 9.4 with 7.5% gel rods as described by Davis.⁷⁾ Polyacrylamide gel isoelectric focusing was done by the method of Wrigley⁸⁾ with 5% slab gels containing 2% carrier ampholyte Pharmalyte 2.5~5. The anode and cathode solutions were 1 M phosphoric acid and 0.1 M sodium hydroxide, respectively. Focusing was done at 4°C for 5 hr at a constant voltage of 200 V. The pH gradient of the gel was measured with isoelectric point marker proteins. After electrophoresis, the gel was immersed in 3.5% perchloric acid for fixation of protein, stained with 0.04% Brilliant Blue G, destained thoroughly with several changes of 7% acetic acid in 25% ethanol, and stored and photographed in 7% acetic acid.

High performance liquid chromatography. The saccharide composition of the hydrolysate of soluble starch with the purified enzyme was analyzed by high performance liquid chromatography with a Waters ALC/GPC 201 liquid chromatograph (Nihon Waters Ltd.) with a 680 automated gradient controller under the following conditions: column, radial pak cartridge of μ BONDAPAK NH₂; detector, R 401 differential refractometer; eluent, a mixture of distilled water and acetonitrile (35:65, v/v).

Measurement of optical rotation. The optical rotation of the starch hydrolysate was measured by the method of Hyun and Zeikus.⁹⁾ A reaction mixture consisting of 1% soluble starch in 40 mM acetate buffer (pH 5.0) and enzyme was incubated at 40°C. At various times, the optical rotation of the mixture was measured with a Schmidt Haensch polarimeter using the sodium line. After the enzyme reaction for 90 min or 120 min, the mutarotation of the hydrolysate was measured by adding about 10 mg of solid sodium carbonate per ml of the mixture.

RESULTS

Enzyme purification

All the procedures were at 4°C unless otherwise noted.

Preparation of a crude extract. Five kilograms of rice koji of *Aspergillus kawachii* were soaked in 20 l of 0.1 M acetate buffer (pH 5.0) containing 0.5% NaCl. The soaked suspension was left overnight at 4°C, and then it was filtered through a nylon filter bag. The filtrate was used as the crude extract and the starting material for the following purification.

Membrane ultrafiltration. The crude extract was concentrated by ultrafiltration with a Labo-module CL-10 cartridge and a DIAFLO membrane PM-10 filter. The concentrate was desalted by gel chromatography on a Bio-Gel P-2 column (2.6 $\times$ 45 cm) equilibrated with 10 mM acetate buffer (pH 5.0).

DEAE Bio-Gel A ion exchange chromatography. The desalted enzyme solution was put on a DEAE Bio-Gel A column (2.2 $\times$ 16 cm) previously equilibrated with 10 mM acetate buffer (pH 5.0). After the column was washed with the same buffer, the enzyme was eluted with a linear concentration gradient of NaCl (0~0.2 M) in the same buffer at a flow rate of 60 ml/hr. Fractions of 10 ml were collected. Alpha-amylase activity was eluted at 0.07 to 0.11 M NaCl. The active fractions were combined and concentrated by membrane ultrafiltration.

Sephacryl S-300 gel chromatography (pH 3.6). The concentrated enzyme solution was adjusted to exactly pH 3.6 and put on a Sephacryl S-300 column (2.6 $\times$ 100 cm) previously equilibrated with 10 mM acetate buffer (pH 3.6). Elution was done with the same buffer at a flow rate of 40 ml/hr. The α -amylase activity was separated into two peaks which were named F-I and F-II, respectively. The active fractions of F-I (fraction No. 35~39) and F-II (fraction No. 40~44) were separately combined and concentrated by membrane ultrafiltration. Then rechromatography on the Sephacryl S-300 column was done under the same conditions. Each rechromatographed enzyme solution of F-I and F-II was concentrated by membrane ultrafiltration and dialyzed against 50 mM acetate buffer (pH 5.0).

ω -Amino dodecyl agarose column chromatography. Each dialyzed enzyme solution was put on a ω -amino dodecyl agarose column (2.6 $\times$ 40 cm) previously equilibrated with 50 mM acetate buffer (pH 5.0). After the column was washed with the same buffer, elution was done with a linear gradient of NaCl (0~0.4 M, total volume, 800 ml) in the same buffer at a flow rate of 40 ml/hr. The enzyme activity of F-I was eluted with 0.21 M

NaCl, while that of F-II was eluted with 0.28 M NaCl. The active fractions of F-I and F-II were separately combined and concentrated by membrane ultrafiltration.

Sephacryl S-200 gel chromatography. Finally, each resulting enzyme solution was chromatographed on a Sephacryl S-200 column (1.9 $\times$ 100 cm) equilibrated with 10 mM acetate buffer (pH 5.0) at a flow rate of 30 ml/hr. The active fractions of F-I and F-II were pooled as purified α -amylase I and α -amylase II, respectively.

A summary of the purification procedure is shown in Table I. The specific activities of the purified α -amylase I and α -amylase II were increased 211 and 190-fold over the initial crude extract, and their yields were 3 and 2%, respectively. The purity of the purified α -amylase I and α -amylase II was evaluated by disc electrophoresis on polyacrylamide gel. Each final purified enzyme preparation was considered to be homogeneous from the result shown in Fig. 1.

General properties

Molecular weight and isoelectric point. The molecular weights of the purified α -amylases of *A. kawachii* were estimated by gel chromatography on a Sephacryl S-300 column as described in MATERIALS AND METHODS. From the data in Fig. 2A, the molecular weights of α -amylase I and α -amylase II were estimated to be 104,000 and 66,000, respectively. By polyacrylamide gel isoelectric focusing, the isoelectric points of α -amylase I and α -amylase II were estimated to be 4.25 and 4.20, respectively (Fig. 2B).

Products of hydrolysis and mutarotation. The product distribution of hydrolysis of 40 mg soluble starch with both α -amylases was analyzed by high performance liquid chromatography. The results are shown in Fig. 3. Both of them took similar courses of starch hydrolysis. The early reaction products (at 1 hr) were predominantly maltotriose, maltotetraose, and maltopentaose. Production of maltose and maltohexaose was small. Then the amounts of maltotetraose, maltopentaose, and malto-

TABLE I. PURIFICATION OF *Aspergillus kawachi* α -AMYLASES

Procedure	Total protein (mg)	Total activity (kU)	Specific activity (U/mg)	Yield (%)
Crude extract	69,200	142.0	2.1	100
Ultrafiltration	2,110	96.1	46	68
DEAE Bio-Gel A	1,050	87.8	84	62
Sephacryl S-300 (pH 3.6)				
F-I	76	16.3	214	11
2nd F-II	46	12.8	278	9
Agarose-C ₁₂ NH ₂				
F-I	20	8.3	415	6
F-II	18	5.9	328	4
Sephacryl S-200				
F-I	9	4.0	444	3
F-II	8	3.2	400	2

Agarose-C₁₂ NH₂: ω -Amino dodecyl agarose.

Protein concentrations were measured spectrophotometrically by the absorbances at 280 nm using bovine serum albumin as a standard.

FIG. 1. Disc Polyacrylamide Gel Electrophoresis of *A. kawachii* α -Amylase I, II.

See MATERIALS AND METHODS for details. Samples: lane 1, purified α -amylase I; lane 2, purified α -amylase II.

FIG. 2. Molecular Weight and Isoelectric Point Estimation of *A. kawachii* α -Amylase I, II.

See MATERIALS AND METHODS for details. A: Molecular weight. Standard proteins: 1, horse spleen ferritin (mol. wt. 440,000); 2, bovine liver catalase (232,000); 3, rabbit muscle aldolase (158,000); 4, bovine serum albumin (67,000); 5, hen egg ovalbumin (43,000); 6, bovine pancreas chymotrypsinogen A (25,000); 7, bovine pancreas ribonuclease A (13,700). B: Isoelectric point. Standard proteins: 1, *P. aeruginosa* azurin (pI 5.65); 1*, reduced form of 1 (4.95); 2, cow's milk β -lactoglobulin (A 5.25, B 5.35); 3, *A. nidulans* C-phyco-cyanin (4.75, 4.85); 4, fungal glucose oxidase (4.25); 5, horse heart acetylated cytochrome *c* (3.95); 6, *Aspergillus* amyloglucosidase (3.65).

hexaose decreased with increasing reaction time, while maltose and maltotriose increased progressively. The production rate of glucose was quite slow, although the production continued throughout the reaction period. After 24 hr of incubation of the reaction mixtures, the amounts of glucose, maltose, and maltotriose were found to be 2.5, 17.3, and 18.5 mg for α -amylase I and 4.6, 15.2, and 14.6 mg for α -amylase II, respectively.

The optical rotation of hydrolysate was measured during hydrolysis of soluble starch, as shown in Fig. 4. From the mutarotation study which used Taka-amylase A as the control, the downward shift of optical rotation on addition of a base to the hydrolysates indicated that the products of starch hydrolysis with both purified enzymes had an α -anomeric configuration. The data presented above provided evidence that both amylases of *A. kawachii* were α -amylases.

Optimum pH and pH stability. The effects of pH on the activity and stability of α -amylase I and α -amylase II were examined at pHs from 2.5 to 8.0 with McIlvaine buffer. As shown in Fig. 5A, the optimal pH range of α -amylase I

was 4.0 to 5.0, while the optimum pH of α -amylase II was 5.0. The enzymes were incubated for 60 min at 30°C in the buffer of various pHs, and then the residual activities were measured under the standard assay conditions. Both α -amylases were stable from pH 2.5 to 6.0, retaining more than 90% of the original activities (Fig. 5B).

Optimum temperature and heat stability. The effects of temperature on the activities of both α -amylases were examined. The optimum temperatures of both enzymes were around 70°C (10 min) at pH 5.0, as shown in Fig. 6A. The enzymes were incubated in acetate buffer (pH 5.0) for 10 min at various temperatures, and then the residual activities were measured. Both of them were stable up to 55°C (Fig. 6B).

Effects of metal ions and inhibitors. The effects of various kinds of metal ions and inhibitors on the activities of both α -amylases were examined. The effects of metal ions are summarized in Table II. The relative activity was represented as the percentage of the control activity without metal ion. Among the metal ions tested, the heavy metal ions such as Hg^{2+} and Pb^{2+} were potent inhibitors for both

FIG. 3. High Performance Liquid Chromatograms of Hydrolysis Products of Soluble Starch with *A. kawachii* α -Amylase I, II.

A mixture (2 ml) consisting of 2% soluble starch in 40 mM acetate buffer (pH 5.0) and enzyme was incubated at 40°C. At proper intervals, the saccharide composition in the hydrolysate was analyzed by high performance liquid chromatography. (A), α -amylase I (18 units); (B), α -amylase II (16 units). Saccharides: G₁, glucose; G₂, maltose; G₃, maltotriose; G₄, maltotetraose; G₅, maltopentaose; G₆, maltohexaose.

FIG. 4. Optical Rotation of the Starch Hydrolysates with *A. kawachii* α -Amylase I, II and Taka-amylase A.

See MATERIALS AND METHODS for details. ○, α -amylase I; ●, α -amylase II; ⊙, Taka-amylase A. The dash line indicates the mutarotation on addition of sodium carbonate to the hydrolysate.

enzymes. Copper (II) ion and Fe^{3+} also caused partial inhibition. On the other hand, none of them stimulated the enzyme activities.

To investigate the effects of inhibitors, the enzymes were incubated for 30 min at 40°C with several kinds of inhibitors, and then the residual activities were measured. Neither α -amylase activity was affected by ethylenediaminetetraacetate (5 mM), phenylmethylsulfonyl fluoride (1 mM), monoiodoacetate (2 mM), *p*-chloromercuribenzoate (0.1 mM), *N*-ethylmaleimide (10 mM), or sodium dodecyl sulfate (1 mM) within the concentrations we used.

DISCUSSION

Aspergillus kawachii⁽¹⁰⁾ is one of the most

FIG. 5. Effects of pH on the Activity and Stability of *A. kawachii* α-Amylase I, II.

A: Activity. The enzyme activity was measured under the standard assay conditions except for McIlvaine buffer of various pHs. The relative activity was expressed as the percentage of the enzyme activity at pH 5.0. B: Stability. The enzyme was incubated for 60 min at 30°C in the buffer of various pHs, and then the residual activity was measured under the standard assay conditions. ○, α-amylase I; ●, α-amylase II.

FIG. 6. Effects of Temperature on the Activity and Stability of *A. kawachii* α-Amylase I, II.

A: Activity. The enzyme activity was measured under the standard assay conditions except for various temperatures. The relative activity was expressed as the percentage of the enzyme activity at 70°C. B: Stability. The enzyme was incubated in acetate buffer (pH 5.0) for 10 min at various temperatures, and then the residual activity was measured. ○, α-amylase I; ●, α-amylase II.

important industrial strains of *Aspergillus* for *shochu* making in Japan. Recently Murakami¹¹⁾ classified all of the strains of the Black *Aspergilli* into the *A. niger* group and the Black *Koji*-mold group, and divided the latter group into *A. aureus* series and *A. awamori* series. *A. kawachii* belongs to the *A. awamori* series. Alpha-amylases^{12~16)} have been purified from a few strains of the *Aspergilli* including *A. awamori*. Acid-stable α-amylase¹⁴⁾ also has

been purified from *A. niger*, but not yet from any strain of the Black *Koji*-mold group. Now we have purified and characterized two kinds of acid-stable α-amylases from *A. kawachii*.

The molecular weights of the purified α-amylase I and α-amylase II were estimated to be 104,000 and 66,000, respectively. The latter was comparable to the values reported for the enzymes from *A. oryzae* (53,000),¹⁷⁾ *A. niger* (acid-stable, 58,000; and-unstable, 61,000),¹⁹⁾

TABLE II. EFFECTS OF METAL IONS ON THE ACTIVITY OF *A. kawachii* α -AMYLASE I, II

Metal ion (2 mm)	Relative activity (%)	
	I	II
No addition	100	100
MgCl ₂	96	101
CaCl ₂	99	101
MnCl ₂	98	101
FeCl ₃	97	102
FeCl ₂	75	76
CoSO ₄	98	103
CuSO ₄	42	48
ZnSO ₄	96	100
HgCl ₂	14	11
Pb(CH ₃ COO) ₂	16	19

and *A. awamori* (54,000).¹⁶⁾ The isoelectric points of α -amylase I (pH 4.25) and α -amylase II (pH 4.20) were similar to the value for the enzyme from *A. awamori* (pH 4.2),¹⁶⁾ but higher than those for the enzymes from *A. niger* (acid-stable, pH 3.44; acid-unstable, pH 3.70).¹⁹⁾ Alpha-amylase I and α -amylase II were quite similar to the acid-stable α -amylase from *A. niger* in some enzymatic properties, i.e., optimal pH around 5.0; pH stability between 2.5 and 6.0; heat stability up to 55°C retaining more than 90% of the original activity.

We have investigated the correlation between various enzyme activities of *shochu koji* and fermentation characteristics of *shochu moromi* mash.²⁰⁾ It was found that α -amylase I and α -amylase II held respectively 80 to 90% and 60 to 70% of their full activities at pH 3.2 to 3.5 which was the pH range of *shochu* seed mash. We are going to study the role of both α -amylases in dissolution and saccharification of the raw material and fermentation in the *shochu moromi* mash using the purified enzymes.

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