STUDIES ON THE GENUS *MONASCUS*.
I. PURIFICATION AND PROPERTIES OF TWO
FORMS OF GLUCOAMYLASE FROM
MONASCUS KAOLIANG NOV. SP. F-1¹

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Two forms of glucoamylase [α -D-(1 $\rightarrow$ 4)-glucan glucohydrolase, EC 3.2.1.3] were isolated and purified from the culture of *Monascus kaoliang* nov. sp. F-1 on wheat bran. These glucoamylases were separated from each other by polyacrylamide gel electrophoresis or by gel filtration on Sephadex G-150 column. They were designated as GA-I and GA-II in the order of elution from the column. The purified enzymes were homogeneous in polyacrylamide gel electrophoresis.

The pH optima of GA-I and -II were found to be 4.5 and 4.7, respectively. GA-I was more resistant than GA-II against treatment with urea or guanidine. The approximate molecular weights by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of GA-I and -II were estimated to be 4.8×10^4 and 6.8×10^4 , respectively.

Presence of glucoamylase [α -D-(1 $\rightarrow$ 4)-glucan glucohydrolase, EC 3.2.1.3] has been found in *Aspergillus niger* (1,2), *Asp. oryzae* (3), *Rhizopus delemar* (4), *Rh. javanicus* (5), *Endomycopsis capsularis* (6), etc. In addition, it has been reported that several fungi (e.g. *Asp. niger* (7), *Asp. awamori* var. *kawachi* (8), *Asp. cinnamomeus* (9), *Rhizopus* sp. (10,11), *Rh. niveus* (12), and *Mucor rouxianus* (13)) produce two or more forms of glucoamylase. In the case of *Monascus* species, Ang-Quack which is prepared from a culture of *Monascus* species has been widely used for red wine brewing and for coloring the drinks and foods in the Asia, and SATO (14) and KITAHARA and MURATA (15) have reported about amylase and maltase. No attempt, however, has yet been made to isolate glucoamylase from *Monascus* species. This might have been due to the weakness of the gluco-

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amylase activity in *Monascus* species known hitherto. In 1975, a culture of *Monascus kaoliang* nov. sp. F-1, identified by LIN and IIZUKA (16), was found to produce a comparatively high glucoamylase activity. Recently, we found that *M. kaoliang* nov. sp. F-1 produce two forms of glucoamylase and that their mixture can be separated by polyacrylamide gel electrophoresis, as well as by gel filtration on Sephadex G-150.

In this paper, the purification and some properties of two forms of glucoamylase from the culture of *M. kaoliang* nov. sp. F-1 on wheat bran will be presented.

MATERIALS AND METHODS

Microorganism. *Monascus kaoliang* nov. sp. F-1 (16) was isolated from *koji* of *kaoliang* liquor, Taiwan, China.

Cultivation method. A mixture of wheat bran and water (1 : 1) was sterilized at 120° for 20 min, inoculated with conidia and spores of the test microorganism, and incubated at 30° for 3 days.

Materials. Soluble starch was purchased from Kokusan Chemical Works, Ltd., Tokyo. Sephadex G-50 and G-150, and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, CM-cellulose from Brown Co., Berlin, New Hampshire, U.S.A., and carrier ampholyte (pH range 3.5-5) was from LKB Produkter AB, Sweden. Bovine serum albumin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., egg white albumin and lysozyme from egg white were from Seikagaku Kogyo Co. Ltd., Tokyo, Japan, and trypsin and Kieselguhr G were from E. Merck, Darmstadt, Germany.

Assay of glucoamylase activity. Glucoamylase activity was measured as follows: A reaction mixture containing 5 ml of 1% soluble starch in distilled water, 4 ml of 0.1 M acetate buffer, pH 4.7, and 1 ml of suitably diluted enzyme solution was incubated at 40° for 30 min. The amount of reducing sugar produced was determined by the method of SOMOGYI-NELSON (17,18). One unit of glucoamylase activity was defined as the amount of enzyme which liberates reducing sugar equivalent to 1 mg of glucose per 30 min under the above conditions.

Determination of protein. Protein concentration was determined by measuring the absorbancy at 280 nm with a Hitachi spectrophotometer, model EPS-3T or model 101, in a 1-cm cell.

Disc electrophoresis. Disc electrophoresis of the enzyme was carried out in 7.5% polyacrylamide gel with 0.04 M Tris-glycine buffer (pH 8.3), according to the method of DAVIS (19). After the run, the gel was stained for proteins with Coomassie Brilliant Blue in a mixture of methanol-acetic acid-distilled water (2:3:35, v/v) and destained diffusely in this mixed solution.

Molecular weight estimation. The molecular weight was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis according to the method of WEBER and OSBORN (20).

Thin-layer chromatography of sugars. Hydrolysis products of soluble starch was identified by thin-layer chromatography. Kieselguhr G was used for the thin layer, and solvent system was butanol-pyridine-water (6:3:1, v/v). The sugars were detected by spraying a mixture of 4% diphenylamine in acetone-4% aniline in acetone-phosphoric acid (5:5:1, v/v).

RESULTS

Purification of the enzyme

Unless otherwise specified, all of the operations were carried out at 5°.

Step 1. Preparation of crude enzyme. The mold wheat bran preparation was extracted with 5 volumes of tap water for 2 hr at room temperature. The crude extract was centrifuged at 12,000 rpm for 20 min. The supernatant solution was precipitated by the addition of solid ammonium sulfate to 0.8 saturation. The precipitate was collected by centrifugation at 10,000 rpm and lyophilized after addition of a small amount of kaoline.

Step 2. Acetone fractionation. To the crude enzyme (10 g) was added 15 volumes of distilled water and extracted with stirring for 1 hr. The precipitate was removed by centrifugation and the supernatant solution was concentrated by ultrafiltration under the pressure of 3–4 kg/cm² in nitrogen stream. The resultant precipitate was removed by centrifugation. To the supernatant solution, acetone previously chilled to –15° was added slowly with vigorous stirring to give a concentration of 80% by volume. The suspension was centrifuged and the precipitate was dissolved in a small amount of 0.01 M acetate buffer, pH 5.5.

Step 3. Rivanol treatment. To the enzyme solution (53 ml) obtained from Step 2 was added 5.5 ml of 2% aqueous solution of Rivanol (2-ethoxy-6,9-diamino-acridinium lactate). After 1 week, the precipitate was removed by centrifugation. The impurities could be removed considerably as the precipitate in this step, and the glucoamylase activity did not exist in the precipitate but did in the supernatant solution. The supernatant solution was dialyzed against a large quantity of 0.01 M acetate buffer, pH 4.1, for 48 hr.

Step 4. CM-cellulose column chromatography. The dialyzed solution (about 58 ml) obtained from Step 3 was applied to a CM-cellulose column (2.6×35 cm) previously equilibrated with 0.01 M acetate buffer, pH 4.1. The column was washed with the same buffer to remove the unadsorbed substances, and then linear gradient elution was started with a system from 0 to 0.5 M NaCl in the same buffer. Four-ml fractions were collected at a flow rate of 24 ml/hr (Fig. 1). The glucoamylase was eluted with the same buffer containing 0.07 M NaCl in tube numbers 86–122 and these were pooled.

Step 5. Sephadex G-150 column chromatography. The pooled glucoamylase preparation from Step 4 was concentrated by ultrafiltration and dialyzed overnight against a large quantity of 0.01 M acetate buffer, pH 5.5. The dialyzed

Fig. 1. Chromatography of glucoamylase preparation from Step 3 on CM-cellulose column.

For details of the procedure, see the text (Step 4). ●, absorbancy at 280 nm; ○, enzyme activity; ---, concentration of NaCl.

solution was applied to a Sephadex G-150 column (2.6×60 cm) pre-equilibrated with the same buffer. The column was eluted with the same buffer at a flow rate of 10 ml/hr and 5-ml fractions were collected (Fig. 2). Two active components of glucoamylase separated by this process were named glucoamylase-I (fraction numbers 34–45) (GA-I) and glucoamylase-II (fraction numbers 54–65) (GA-II), and were pooled respectively. Each component was concentrated to 15 ml by ultrafiltration. These solutions were dialyzed overnight against a large quantity of 0.01 M acetate buffer, pH 5.5.

Fig. 2. Gel filtration of glucoamylase preparation from Step 4 on a Sephadex G-150 column.

The experimental details are described in the text (Step 5). ●, absorbancy at 280 nm; ○, enzyme activity.

Step 6. Isoelectric focusing fractionation. Each dialyzed solution (20 ml) was separately applied to an ampholine column (column size, 110 ml), using the LKB apparatus as developed by VESTERBERG (21). The pH range of carrier ampholyte was 3.5–5 and electrophoresis was carried out for 44 hr at a constant voltage of 500 V. Two-ml fractions were collected and the pH of these fractions was measured with a Hitachi-Horiba pH-meter (model F-5). Next, 2 ml of 0.1 M acetate buffer (pH 5.5) was added to each fraction, and the absorbancy at 280 nm and glucoamylase activity were determined (Fig. 3, A and B). Each active fraction (GA-I, fraction numbers 15–26; and GA-II, fraction numbers 16–23) was dialyzed overnight against a large quantity of 0.01 M acetate buffer, pH 5.5, concentrated by

Fig. 3. Isoelectric focusing fractionation of GA-I (A) and GA-II (B) obtained from Step 5.

The details are given in the text (Step 6). —●—, pH value; ---●---, absorbancy at 280 nm; ○, enzyme activity.

ultrafiltration, and applied to a column (2.6×60 cm) of Sephadex G-50 equilibrated with the same buffer to remove the carrier ampholyte and accompanying saccharose. After this process, the preparation of GA-II gave a single band in disc electrophoresis on a polyacrylamide gel column (Fig. 6, b), while that of GA-I did not.

Step 7. DEAE-Sephadex A-50 column chromatography. The preparation of GA-I obtained by gel filtration on Sephadex G-50 was dialyzed overnight against a large quantity of Tris-HCl buffer (μ 0.01, pH 8.0), and was applied to a DEAE-Sephadex A-50 column (2.6×60 cm) previously equilibrated with the same buffer. The column was washed with a small amount of the same buffer, and then linear gradient elution was started with a system from 0 to 0.3 M NaCl in the same buffer, and 5-ml fractions were collected at a flow rate of 30 ml/hr. The glucoamylase activity was eluted in fraction numbers 193–209, as shown in Fig. 4. These frac-

Fig. 4. Chromatography of GA-I from Step 6 on a DEAE-Sephadex A-50 column.

The experimental details are described in the text (Step 7). ●, absorbancy at 280 nm; ○, enzyme activity; ---, concentration of NaCl.

tions were pooled. Since a very small quantity of coloring contamination, which may be higher molecular weight substance than GA-I, still existed in the resultant solution, gel filtration over Sephadex G-150 was repeated to remove it with a column of larger volume than in Step 5, as shown in the next step.

Step 8. Sephadex G-150 column chromatography. The pooled GA-I preparation from Step 7 was dialyzed overnight against 0.01 M acetate buffer, pH 5.5, and concentrated by ultrafiltration. The concentrated solution was applied to a Sephadex G-150 column (2.7×70 cm) previously equilibrated with the same buffer, and the column was eluted in the same manner as in Step 5 (Fig. 5). The coloring contamination was removed thoroughly in this step and the preparation of GA-I (fraction numbers 51–57) gave a single band in disc electrophoresis on a polyacrylamide gel column, and the mixture of GA-I and -II was separated from each other as shown in Fig. 6, a and c.

Fig. 5. Gel filtration of GA-I from Step 7 on a Sephadex G-150 column. The details are given in the text (Step 8). ●, absorbancy at 280 nm; ○, enzyme activity.

Fig. 6. Disc electrophoresis of purified glucoamylases. Tris-glycine buffer (pH 8.3) was used. a, GA-I (Step 8); b, GA-II (Step 6); c, mixture of GA-I (Step 8) and GA-II (Step 6).

A summary of the purification procedures for the two active components of glucoamylase is shown in Table 1. From the starting crude enzyme, GA-I and -II were purified 9.2- and 12.8-fold, respectively, and recovery of each activity was 16.8 and 11.3%, respectively.

Properties of GA-I and GA-II

Effect of pH on enzyme activity. Figure 7A shows the effect of pH on the enzyme activities. The pH optima of GA-I and GA-II were observed at 4.5 and

Table 1. Summary of the purification of glucoamylases.

No.	Step Procedure	Total volume (ml)	OD ₂₅₀	Total activity (unit)	Specific activity unit/(ml·OD ₂₅₀)	Recovery (%)
	Crude enzyme	10 g				
1.	Extraction with distilled water	153	14.0	49,500	23.1	100
2.	Acetone fractionation	50	16.4	38,000	46.4	76.8
3.	Rivanol treatment	58	14.0	37,000	45.6	74.7
4.	CM-cellulose column chromatography	157	1.52	32,000	134	64.6
5.	Sephadex G-150 gel filtration	I 57 II 57	2.32 0.698	19,500 9,150	147 230	39.4 18.5
6.	Isoelectric focusing	I 32.5 II 37	2.50 0.512	14,100 5600	174 295	28.5 11.3
7.	DEAE-Sephadex A-50 column chromatography	I 75	0.610	10,500	231	21.2
8.	Sephadex G-150 gel filtration	I 36	1.09	8310	212	16.8

Fig. 7. Effect of pH on the enzyme activity of GA-I and GA-II.

A, optimum pH: Reaction mixture of 2.5 ml of 1% soluble starch, 2.0 ml of McIlvaine buffer or borate buffer, and 0.5 ml of enzyme solution (0.77 unit) was incubated at indicated pH for 30 min at 40°. B, pH stability: After the enzyme (0.77 unit) in 0.1 ml of 0.05 M McIlvaine buffer or borate buffer was kept at 30° for 24 hr, 2.4 ml of 0.1 M acetate buffer (pH 4.7) and 2.5 ml of 1% soluble starch were added, and the reaction mixture was incubated at 40° for 30 min. ●, GA-I; ○, GA-II.

4.7, respectively, for soluble starch.

Effect of temperature on enzyme activity. The enzyme reaction was carried out at various temperatures at pH 4.7 for 30 min. The temperature optima of GA-I and GA-II were observed at 50° and 60°, respectively, as shown in Fig. 8A.

Fig. 8. Effect of temperature on the enzyme activity of GA-I and GA-II.

A, optimum temperature: Reaction mixture of 2.5 ml of 1% soluble starch, 2.0 ml of 0.1 M acetate buffer (pH 4.7), and 0.5 ml of enzyme solution (0.77 unit) was incubated at indicated temperature for 30 min. **B, thermostability:** A mixture of 2.0 ml of 0.1 M acetate buffer (pH 4.7) and 0.5 ml of enzyme solution (0.77 unit) was kept at the indicated temperature for 10 min. To the solution was added 2.5 ml of 1% soluble starch, and the reaction mixture was incubated at 40° for 30 min. ●, GA-I; ○, GA-II.

pH stability of the enzymes. As shown in Fig. 7B, GA-I and GA-II were stable over the pH range of 4.0–8.5 and 4.0–7.5, respectively, for 24 hr at 30°.

Thermostability of the enzymes. The enzyme solution (pH 4.7) was incubated at various temperatures for 10 min and the remaining activities were checked under the standard assay conditions. The results shown in Fig. 8B indicated that both GA-I and GA-II were stable at temperature below 50° but, while about 30% of the activity of GA-I remained at 55° and completely lost at 60°, about 85% of the activity of GA-II remained at 55°, about 55% at 60°, and completely lost at 70°.

Estimation of molecular weight. The molecular weights of GA-I and GA-II were estimated from the R_f values on SDS-polyacrylamide gel electrophoresis. As shown in Fig. 9, the molecular weights of GA-I and GAII were found to be approximately 48,000 and 68,000, respectively.

Effect of urea and guanidine. Figure 10A and B show the effect of urea and guanidine hydrochloride on the enzyme activities. GA-II was more resistant against urea and guanidine. About 78 and 60% of the activity of GA-II remained even after treatment with 8 M urea and with 2.4 M guanidine, respectively, but the activity of GA-I was largely lost on treatment with 8 M urea, and was completely lost on treatment with 2.4 M guanidine.

Effect of salts and other chemicals. Effect of various salts and other chemical reagents on the glucoamylase activity is summarized in Tables 2 and 3. GA-I was somewhat inhibited by $HgCl_2$ and $ThCl_4$, and GA-II by $FeCl_3$ and $ThCl_4$.

Fig. 9. Estimation of molecular weights of GA-I and GA-II by SDS-polyacrylamide gel electrophoresis.

Electrophoresis was carried out in 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS under a constant current of 8 mA per tube for 5 hr. Proteins were stained in the same manner as in disc electrophoresis. Relative mobility expresses the distance of migration relative to that of bromophenol blue. a, GA-II (mol. wt., 68,000); b, bovine serum albumin (mol. wt., 67,000); c, GA-I (mol. wt., 48,000); d, egg white albumin (mol. wt., 44,500); e, trypsin (mol. wt., 24,000); f, lysozyme (mol. wt., 14,300).

Fig. 10. Effects of urea (A) and guanidine (B) on the enzyme activity of GA-I and GA-II.

To the enzyme solution (0.1 ml), the reagent in 1 ml of 0.1 M acetate buffer (pH 4.7) was added to give the final concentration as indicated. This solution was incubated at 30° for 30 min, and the remaining activity was measured. ●, GA-I; ○, GA-II.

Other salts, EDTA, and sulfhydryl reagents, such as *p*-chloromercuribenzoate (*p*-CMB) and monoiodoacetic acid, showed no effect on the activities of GA-I and GA-II. L-Cysteine and 2-mercaptoethanol also did not inhibit the activities of these glucoamylases. These enzymes were completely inactivated by KMnO_4 .

Thin-layer chromatography of enzymic products. Figure 11 shows the thin-layer chromatogram of hydrolysate from soluble starch by GA-I (a, b, and c) and GA-II

Table 2. Effect of salts on glucoamylases.

Salt (1×10^{-3} M)	Relative activity (%)	
	GA-I	GA-II
None	100	100
LiCl	102	94
KCl	108	102
MgCl ₂	109	102
CaCl ₂	108	105
SrCl ₂	104	106
BaCl ₂	103	99
CuCl ₂	106	105
AgNO ₃	97	101
ZnCl ₂	102	104
HgCl ₂	90	96
PbCl ₂	100	95
CrCl ₃	100	98
MnCl ₂	98	95
FeCl ₃	96	75
CoCl ₂	100	103
NiCl ₂	104	96
LaCl ₃	104	100
ThCl ₄ (0.1 %)	88	63

A mixture of 0.01 ml of enzyme solution (0.77 unit), 0.01 ml of 4 mM salt solution, and 0.02 ml of 0.1M acetate buffer (pH 4.7) was incubated at 30° for 30 min and the remaining activity was measured.

Table 3. Effect of chemical reagents on glucoamylases.

Chemicals	Final concentration (mM)	Activity (%)	
		GA-I	GA-II
None		100	100
Monoiodoacetic acid	1.0	98	100
p-CMB	5×10^{-2}	97	97
EDTA · Na ₂	1.0	101	107
L-Cysteine	1.0	98	106
2-Mercaptoethanol	1.0	99	101
KMnO ₄	0.5	0	0

The enzymes were incubated at 30° for 30 min with various chemical reagents at pH 4.7, and the remaining activities were measured.

(d, e, and f). Glucose was the only liberated product and no other oligosaccharide could be found in all samples tested after 30, 60, and 180 min of hydrolysis. Furthermore, both GA-I and GA-II did not shift the λ_{max} of iodine-starch complex during the course of the reaction.

Michaelis constants. The reaction was found to follow the normal Michaelis-Menten kinetics, and the Lineweaver-Burk plots indicated the K_m values of GA-I

Fig. 11. Thin-layer chromatogram of enzymic products from GA-I (a, b and c) and GA-II (d, e and f).

Reaction mixture of 2.5 ml of 1% soluble starch, 2.0 ml of 0.1 M acetate buffer (pH 4.7), and 0.5 ml of enzyme solution (7.7 unit) was incubated at 40° for 30 min (a, d), 1 hr (b, e) or 3 hr (c, f). Hydrolysis degree (%): a, 26; b, 42; c, 51; d, 26; e, 42; f, 55. s, corn syrup DE 15; G, glucose; M, maltose; MT, maltotriose.

Fig. 12. Lineweaver-Burk plot for GA-I and GA-II.

To the mixture of enzyme solution (0.5 ml) and 2.0 ml of 0.1 M acetate buffer (pH 4.7), 2.5 ml of soluble starch solution was added to give the final concentration as indicated. The reaction mixture was incubated for 10 min (GA-I) or 5 min (GA-II) at 40°. V, (glucose) mg/(ml·min); S, %; ●, GA-I; ○, GA-II.

and GA-II toward soluble starch to be $4.1 \times 10^{-1}\%$ and $7.5 \times 10^{-3}\%$, respectively (Fig. 12).

DISCUSSION

In the present series of work, we prepared two forms of glucoamylase from a culture of *Monascus kaoliang* nov. sp. F-1 on wheat bran by the following procedures: Ammonium sulfate fractionation, acetone fractionation, Rivanol treat-

ment, ion-exchange chromatography, Sephadex G-150 gel filtration, and isoelectric focusing. The enzymes isolated had a high degree of purity as indicated by polyacrylamide gel electrophoresis. By using these purified enzymes, some enzymic properties were examined.

Although these glucoamylases showed almost the same behavior in ion-exchange chromatography and isoelectric focusing, their behavior in Sephadex G-150 gel filtration, polyacrylamide gel electrophoresis, and SDS-polyacrylamide gel electrophoresis was different. The molecular weights of GA-I and GA-II were 4.8×10^4 and 6.8×10^4 , respectively, by SDS-polyacrylamide gel electrophoresis; the elution volume of GA-II in Sephadex G-150 gel filtration was larger than that of GA-I, however, and that of each enzyme in Sephadex G-100 gel filtration with 0.1 M acetate buffer showed the same tendency. From these facts, we suggest that this discrepancy might be due to the interaction, similar to the relation between the enzyme and substrate, of GA-II with Sephadex G-150 gel rather than by electrostatic adsorption between them.

The molecular weight of GA-II is similar to that of the glucoamylase from *Cephalosporium charticola* (22) and *Asp. oryzae* (3), but is less than the value of 97,000 daltons reported for the glucoamylase of *Asp. niger* (2). Some glucoamylases have smaller molecular weights; *e. g.*, glucoamylases from *Endomyces* sp. (23) and *Asp. phoenicis* (24) are reported to have molecular weights of 55,000 and 63,600, respectively, and the molecular weight of GA-I is similar to that of glucoamylase II from *Mucor rouxianus* (13).

The isoelectric point of GA-I and GA-II was 4.0, this value is similar to those (3.5 and 4.0) of the glucoamylases from *Asp. niger* (25), and is very low compared with pI of glucoamylase (7.8–8.0) from *Rh. delemar* (26), and microheterogeneous components of glucoamylase from *Rhizopus* (11,27) and *Asp. niger* (25) have different pI values, but, in the case of glucoamylases from *M. kaoliang* nov. sp. F-1, their pI values were identical.

Although thermal properties of GA-I and GA-II were different from each other, these properties, pH optima, and effect of chemical reagents on the enzyme activities were not so different from those of glucoamylases from other genera. The pH stabilities of GA-I and GA-II were low on acid side compared with those of glucoamylases from *Rh. delemar* (28) and *Asp. niger* (28), but GA-I was somewhat stronger than those on the alkaline side. While glucoamylase from *Rh. delemar* and *Asp. niger* was inactivated completely at pH 10, GA-I was completely inactivated at pH 11, at 30° for 24 hr. K_m values of GA-I and GA-II were markedly different for soluble starch, and such a tendency is different from microheterogeneous components of glucoamylase from *Rhizopus* sp. (11).

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REFERENCES

- 1) J. H. PAZUR and T. ANDO, *J. Biol. Chem.*, **235**, 297 (1960).
- 2) J. H. PAZUR and K. KLEPPE, *J. Biol. Chem.*, **237**, 1002 (1962).
- 3) M. OHGA, K. SHIMIZU, and Y. MORITA, *Agric. Biol. Chem.* (Tokyo), **30**, 967 (1966).
- 4) L. L. PHILLIPS and M. L. CALDWELL, *J. Am. Chem. Soc.*, **73**, 3559 (1951); *idem, ibid.*, **73**, 3563 (1951).
- 5) K. WATANABE and T. FUKIMBARA, *J. Ferment. Technol.* (Osaka), **46**, 992 (1968).
- 6) H. EVERTOVÀ, *Folia Microbiol.*, **11**, 422 (1966).
- 7) J. H. PAZUR and T. ANDO, *J. Biol. Chem.*, **234**, 1966 (1959).
- 8) S. HAYASHIDA, *Proc. 14th Symp. Enz. Chem. Japan*, p. 16 (1962), abstract in Japanese.
- 9) M. KURUSHIMA, J. SATO, and K. KITAHARA, *Nippon Nogeikagaku Kaishi*, **48**, 379 (1974), in Japanese.
- 10) S. UEDA, *Bull. Agric. Chem. Soc. Japan*, **21**, 379 (1957).
- 11) T. HANDA, S. MINEKI, and H. IIZUKA, *Eiyo To Shokuryo*, **29**, 439 (1976), in Japanese.
- 12) Y. TSUJISAKA, N. HAMADA, and S. TAKENISHI, *Proc. Annu. Meet. Agric. Chem. Soc. Japan*, p. 34 (1976), abstract in Japanese.
- 13) A. TSUBOI, Y. YAMASAKI, and Y. SUZUKI, *Agric. Biol. Chem.* (Tokyo), **38**, 543 (1974).
- 14) K. SATO, *Jozogaku Zasshi*, **11**, 189 (1933), in Japanese.
- 15) K. KITAHARA and U. MURATA, *Hakko Kogaku Zasshi*, **32**, 473 (1954), in Japanese.
- 16) C. F. LIN and H. IIZUKA, *Proc. Annu. Meet. Agric. Chem. Soc. Japan*, p. 268 (1975), abstract in Japanese.
- 17) N. NELSON, *J. Biol. Chem.*, **153**, 375 (1944).
- 18) M. SOMOGYI, *J. Biol. Chem.*, **160**, 61 (1945); *idem, ibid.*, **195**, 19 (1952).
- 19) B. J. DAVIS, *Annu. N. Y. Acad. Sci.*, **121**, Art. 2, 404 (1964).
- 20) K. WEBER and M. OSBORN, *J. Biol. Chem.*, **244**, 4406 (1969).
- 21) O. VESTERBERG, *In Methods in Enzymology*, ed. by W. B. JAKOBY, Academic Press, New York (1971) Vol. XXII, p. 389.
- 22) M. KRZECZOWSKA and H. URBANEK, *Appl. Microbiol.*, **30**, 163 (1975).
- 23) T. FUKUI and Z. NIKUNI, *Agric. Biol. Chem.* (Tokyo), **33**, 884 (1969).
- 24) D. R. LINEBACK and W. E. BAUMANN, *Carbohydr. Res.*, **14**, 341 (1970).
- 25) D. R. LINEBACK, I. J. RUSSELL, and C. RASMUSSEN, *Arch. Biochem. Biophys.*, **134**, 539 (1969).
- 26) Y. TSUJISAKA and J. FUKUMOTO, *Koso Kagaku Shinpojium*, **13**, 84 (1958), in Japanese.
- 27) Y. TSUJISAKA and S. TAKENISHI, *Proc. Annu. Meet. Agr. Chem. Soc. Japan*, p. 315 (1977), abstract in Japanese.
- 28) Y. TSUJISAKA, *Osaka-shiritsu Kogyo Kenkyujo Hokoku*, **28**, 1 (1960), in Japanese.