Biol. Pharm. Bull. 18(8) 1133—1137 (1995)

Characterization of Partially Purified α-Glucosidase in the Insoluble Fraction of Bovine Crystalline Lens

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Two fractions of neutral α-glucosidase were partially purified from the insoluble fraction of bovine lens. This is the first report of such an event to the best of our knowledge. The apparent native molecular weights of these fractions were 121 kDa (fraction-I) and 254 kDa (fraction-II). Both fractions contained three polypeptides with molecular weights of 21, 25 and 30 kDa, although the proportion of these peptides was different in both fractions. The optimal pH of fraction-I and fraction-II was pH 6.0 and 6.5, and the optimal temperature for both fractions was approximately 50 °C. The K_m values of fractions-I and -II for 4-methylumbelliferyl-α-glucopyranoside were 0.086, and 0.192 mM. The activities of these enzymes were inhibited strongly by $HgCl_2$ and slightly by D-iodoacetic acid, but not by D-turanose. From this, we suggest that the enzyme in the insoluble fraction of bovine lens may be a cytoplasmic neutral α-glucosidase which binds to the cell membrane.

Key words bovine lens; insoluble fraction; neutral α-glucosidase

α-Glucosidase (Glcdase) hydrolyzes the α-1,4 glucosidic bonds at the non-reducing terminal of maltose, glycogen and some oligosaccharides to yield free glucose.[1] In general, mammalian α-Glcdase is classified into two groups, neutral α-Glcdase (optimal pH, 6—7) [E.C. 3.2.1.20] and acidic α-Glcdase (optimal pH, 4—5) [E.C. 3.2.1.3], and these occur in various tissues.[2]

Regarding the crystalline lens of the eye, Dryfus *et al.*[3] reported the existence of α-Glcdase in the soluble fraction of human and bovine crystalline lens. They reported that the optimal pH of the enzyme is 6.5 and its activity is 256 ± 100 pmol/min/mg protein at 37 °C. The activity was higher in the epithelium (capsule) than in the cortex and nucleus. Furthermore, they suggested that the enzyme may play an important role in the lens: namely the carbohydrate moiety of the glycoprotein in the lens may undergo changes due to the action of α-Glcdase, and these changes may alter the function of lens proteins or crystallins to maintain the normal state of the lens. However, Carlin *et al.* reported that in the soluble fraction of human, rat and rabbit lenses β-Glcdase is present, but not α-Glcdase.[4] Howkins *et al.* reported also that activity of α-Glcdase in the soluble fraction of human and bovine lenses is very low being detectable.[5] Our recent study (unpublished) also demonstrated the existence of α-Glcdase in the soluble fraction of bovine lens, although the enzyme activity was 1.933 ± 0.43 pmol/min/mg protein and much lower than reported by Dryfus *et al.*[3] All the lens α-Glcdase of various animals reported up to now has been found only in the soluble fraction of the lens.

Recently we also found α-Glcdase activity in the insoluble fraction of the bovine lens. To the best of our knowledge, there have been no previous reports of α-Glcdase in the insoluble fraction of the lens. It is well established that the insoluble fraction or water-insoluble protein in the lens can be classified into several subgroups by the difference in molecular weight and/or crystallins species and is formed by aggregation of crystallins.[6] Many investigators have reported that the abnormal development of lens protein aggregation may induce lenticular opacity or cataract. Glycosylation of lens proteins with various sugars in the lens is now suspected to be one of the main causes of lens protein aggregation or insolubilization.[7] In a previous paper, we reported that senile cataract may be caused by stimulation of such glycosylation.[8] Because of those studies, we thought that it would be very important to clarify the nature of the enzyme and its role in the lens, since α-Glcdase in the insoluble fraction of the lens, in addition to other various glycosidases in the lens, may regulate the degree of glycosylation in the lens. Therefore, we attempted to characterize the enzyme.

The current paper reports the initial characterization of α-Glcdase partially purified from the insoluble fraction of bovine lens.

MATERIALS AND METHODS

Bovine Lens Bovine lenses were obtained from the eyes of animals killed at a local slaughterhouse. They were frozen and stored at −80 °C immediately after being removed from the eyes.

Materials 4-Methylumbelliferone (4-MU) and 4-methylumbelliferyl-α-D-glucose (4-MU-α-Glc) were purchased from Wako Pure Chemical Industries Ltd., Japan. Detergents or surface-active agents for solubilizing the insoluble proteins were purchased from Dojindo Lab., Japan. Other chemicals were of analytical grade or of the highest purity available.

Preparation of Crude α-Glcdase from the Insoluble Fraction of Bovine Lens Twenty-five bovine lenses, 60 g, were homogenized in 102 ml 20 mM Tris–HCl buffer, pH 7.0, containing 2 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (buffer A), and centrifuged at 25000 × *g* for 30 min at 4 °C. The soluble fraction was exhaustively washed from the resulting pellet by resuspension in 240 ml of the same buffer and centrifugation. After washing the pellet 5 times, no protein was detected in the supernatant, by either colorimetry or absorbance at 280 nm. Then, the final pellet was suspended in 20 mM Tris–HCl buffer, pH 7.0, containing 2 mM EDTA, 500 mM NaCl, 30 mM *n*-heptyl-β-D-thioglucoside (*n*-heptyl-TG), and 0.2 mM PMSF (buffer B), and the suspension was

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stirred slowly at 4 °C for 12 h to solubilize the proteins. After that, the suspension was centrifuged at 25000 × *g* for 30 min at 4 °C. The supernatant was dialyzed against 2 mM Tris–HCl buffer, pH 7.0, containing 2 mM EDTA and 0.2 mM PMSF (buffer C) for 12 h, and the resulting solution in the dialysis bag was lyophilized. The final lyophilized material, 1010.56 mg, was dissolved in 100 ml 20 mM Tris–HCl buffer, pH 7.0, containing 2 mM EDTA, 30 mM *n*-heptyl-TG and 0.2 mM PMSF (buffer D), and used as the crude enzyme solution.

Column Chromatography The purification of α-Glcdase from the insoluble fraction was performed by column chromatography as described below.

Gel filtration chromatography was carried out on a Sephacryl S-300HR column (0.9 × 35 cm) using 2 mM Tris–HCl buffer, pH 7.0, containing 2 mM EDTA, 150 mM NaCl, 30 mM *n*-heptyl-TG and 0.2 mM PMSF (buffer E). The eluate (0.8 ml/tube) was collected using a fraction collector.

Anion exchange chromatography was performed on a DEAE-Sepharose CL6B column (3.5 × 15 cm) by linear gradient elution using 0% to 100% 1 M NaCl in buffer D. The eluate (10 ml/tube) was collected using a fraction collector.

Affinity chromatography was carried out on a Con A Sepharose column (1.6 × 20 cm). After the non-absorbed material was exhaustively washed from the column with buffer B, the absorbed materials were eluted with 0.5 M methyl-α-D-mannopyranoside in buffer B. The eluate (2 ml/tube) was collected using a fraction collector. Throughout the column chromatography, protein absorption was monitored at 280 nm.

Assay of α-Glcdase The enzyme activity was assayed using 4-MU-α-Glc as a substrate according to the method of Howkins *et al.*[5] Briefly, the reaction was performed as follows: the substrate solution, 150 μl 1.11 mM 4-MU-α-Glc and 150 μl 0.2 M phosphate–0.1 M citrate buffer, pH 6, was mixed, and the mixture was incubated for 5 min at 37 °C. Then, 45 μl enzyme solution was added to the mixture and incubated for 30 min at 37 °C. The reaction was terminated with 1 ml 0.2 M glycine–sodium hydroxide buffer, pH 10.7 and the fluorescence of liberated 4-MU at Em. = 450 nm (Ex. = 365 nm) was measured using a Shimadzu RF-5000 Spectrofluorophotometer with 4-MU as a standard.

Determination of Optimal pH and Temperature The optimal pH of the enzyme was determined using 0.2 M phosphate–0.1 M citrate buffer over a range of different pH values from pH 3 to 8.0 following 15 min incubations at 37 °C. The optimal temperature was determined using 0.2 M phosphate–0.1 M citrate buffer, pH 6.0, following 15 min-incubation at various temperatures.

Other Analyses Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out on 12.5% gels by the method of Laemmli.[9] The protein concentration was measured by the method of Bradford.[10] Bovine serum albumin (BSA), 1.39 mg/ml, was used as a standard. The protein concentration on acrylamide gels was measured by densitometry using a TIAS-100 TEFCO Image Analysis System, TEFCO, Japan.

RESULTS

Examination of Detergents for Solubilization of Enzyme First, 9 different detergents were examined to determine their ability to solubilize the enzyme from the insoluble fraction. The detergents examined were 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (8 mM), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid (8 mM), *n*-octyl-β-D-glucoside (25 mM), *n*-octanoyl-*N*-methylglucamide (25 mM), *n*-decanoyl-*N*-methylglucamide (7 mM), sucrose monocaprate (2.5 mM), sucrose monolaurate (0.4 mM), *n*-octyl-β-D-thioglucoside (9 mM), and *n*-heptyl-β-D-thioglucoside (*n*-heptyl-β-TG) (30 mM). Values in parentheses represent the critical micelle concentration (CMC).

Each detergent was dissolved at its CMC concentration in 0.02 M Tris–HCl buffer, pH 7.0, containing 0.05 M NaCl. The solubilization experiment was carried out as follows. The detergent solution, 900 μl, was mixed with 100 μl of the insoluble lens fraction, and stirred slowly for 12 h. After that, the suspension was centrifuged at 25000 × *g* for 30 min at 4 °C. The α-Glcdase activity in the resulting supernatant was measured in order to select the most effective detergent. The enzyme activity of the sample solubilized with *n*-heptyl-TG was 25 ± 3.2 pmol/min/mg protein. These activity with other detergents was between 7.5 and 12 pmol/min/mg protein much lower than that with *n*-heptyl-TG demonstrating clearly that *n*-heptyl-TG was the most effective detergent. Therefore, *n*-heptyl-TG was used to solubilize α-Glcdase from the insoluble lens fraction in this experiment.

Then, the effect of time on solubilization was investigated. The activity of the enzyme solubilized increased up to 12 h reaching a maximum of 24.41 pmol/min/mg of protein at 12 h. After that, the activity declined and the amount of protein solubilized reached a constant level (data not shown). From this, it was decided that the solubilization of α-Glcdase from the insoluble lens fraction should involve treatment with *n*-heptyl-TG for 12 h at 37 °C.

Solubilization of α-Glcdase from the Insoluble Lens Fraction The insoluble fraction, 4.28 g, obtained from 25 bovine lenses was treated with *n*-heptyl-TG under the conditions described above and 1.01 g of solubilized protein was obtained.

Purification of α-Glcdase by Column Chromatography The separations obtained by column chromatography are shown in Fig. 1. First, the solubilized fraction obtained above was as described submitted to ion exchange chromatography on a DEAE-Sepharose CL6B column as shown in Fig. 1A. Fraction nos. 53—57 (Fraction A) with α-Glcdase activity were collected and dialyzed against buffer B. The solution in the dialysis bag was subsequently chromatographed on a Con A Sepharose affinity column. α-Glcdase activity was found in two different fractions, fraction nos. 18—22 (Fraction-1) and fraction nos. 27—34 (Fraction-2), respectively (Fig. 1B). These fractions were collected and concentrated by ultrafiltration through a Millipore filter (MW = 10000 cut). These fractions were purified further by gel filtration column chromatography on a Sephacryl S-300 column. Figure 1C-1 and Fig. 1C-2

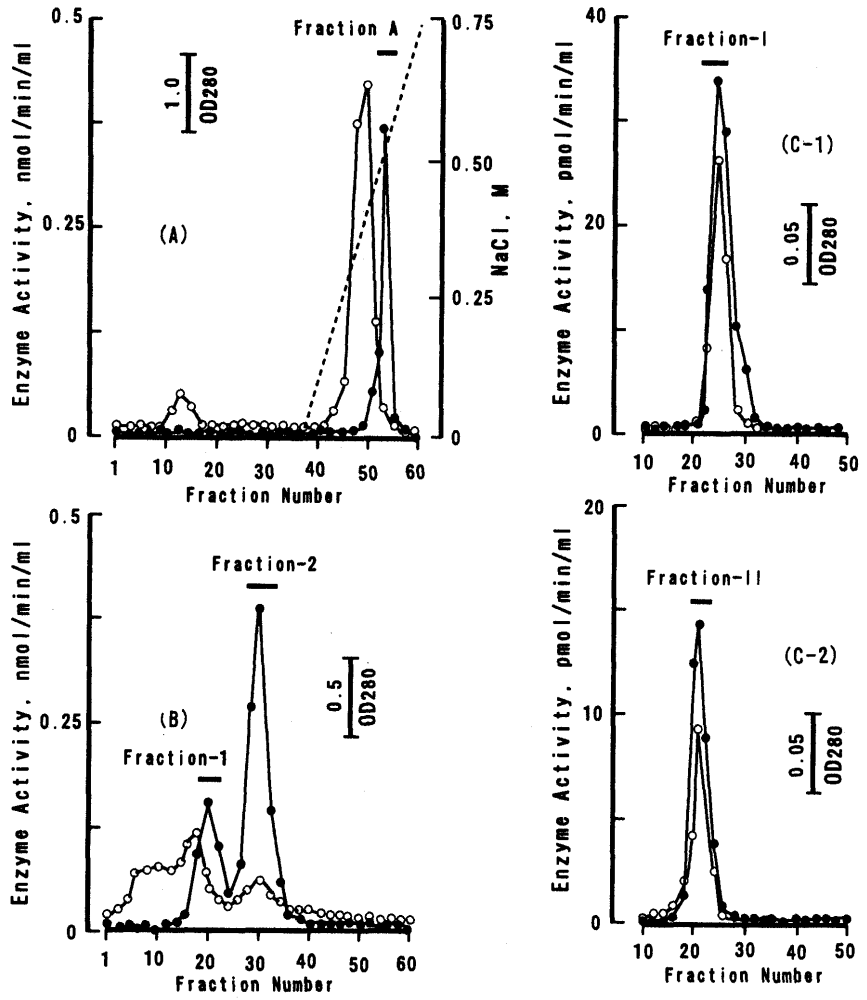

Fig. 1. Column Chromatography on DEAE Sepharose (A), Con A Sepharose (B) and Sephacryl S-300 (C-1 and C-2) Columns

The details are given under Materials and Methods. Enzyme activity was measured with 4-MU-α-Glc as substrate as described under Materials and Methods. —●—, enzyme activity; —○—, absorbance at 280 nm. (A) DEAE-Sepharose column chromatograms of the sample from the insoluble lens fraction solubilized with *n*-heptyl-thioglucoside from the insoluble fraction of the lens. Fractions 53—57 were collected as Fraction-A; (B) Con A Sepharose column chromatograms of Fraction-A. Fractions 19—21 and 28—31 were collected as Fraction-I, and Fraction-II, respectively; (C-1) rechromatograms of Fraction-I (fractions 22—24) on a Sephacryl S-300 column; (C-2) rechromatograms of Fraction-II (fractions 19—21) on a Sephacryl S-300 column.

Table 1. Purification of Neutral Lens α-Glucosidase

Fraction	Activity (pmol/min)	Protein (mg)	Specific activity (pmol/min/mg protein)	Yields (%)
Homogenate	15689.7	24250	0.64	100
Precipitate	10908.9	4278	2.25	17.6
Heptyl-TG	8725.7	1010	18.53	4.2
DEAE-Sepharose CL6B	5110.0	62.5	81.71	0.2
Con A-Sepharose				
Fraction-1	2062.1	13.4	153.88	0.05
Fraction-2	2184.8	12.6	173.39	0.05
Sephacryl-S300				
Fraction-I	1223.2	3.85	317.71	0.0015
Fraction-II	837.4	2.21	378.91	0.0009

Enzyme activity was measured with 4-MU-α-Glc as described under Materials and Methods.

show the elution patterns of Fraction-1 and Fraction-2, respectively. Fraction nos. 21—25 (Fraction I) of Fraction-1, and fraction nos. 19—23 (Fraction II) of Fraction-2 were collected and subsequently rechromatographed. The final fractions, Fraction-I (22—24) and Fraction-II (19—21) obtained by rechromatography, were used as the partially purified enzyme solution. The enzyme preparation represented more than a 1000-fold purification compared with the starting homogenate and contained about 2% (Fraction-I + Fraction-II) of the activity initially present (Table 1).

Purity and Physiological Properties of Fraction-I and Fraction-II The degree of purification of each fraction in each step was examined by SDS-PAGE. Fraction-I and Fraction-II were subjected to electrophoresis on 12.5% polyacrylamide gel at pH 7.2 (Fig. 2). SDS-PAGE showed that both fractions consisted of three polypeptides with molecular weights of 21, 25 and 30 kDa, although the proportion of each fraction was apparently different. Protein bands on the gel were stained with 0.2% Coomassie brilliant blue, and the protein concentration of each band stained was measeured by densitometry. As a result, the proportion of 21, 25 and 30 kDa polypeptides in Fraction-I was 1.0 : 0.66 : 1.04, and that in Fraction-II was 1.0 : 0.75 : 0.66. Then, the apparent native molecular weights of Fraction-I and Fraction-II were estimated from the peak position on Sephacryl S-300 gel filtration chromatography: the molecular weight of Fraction-I was 254 kDa, and that of Fraction-II was 121 kDa.

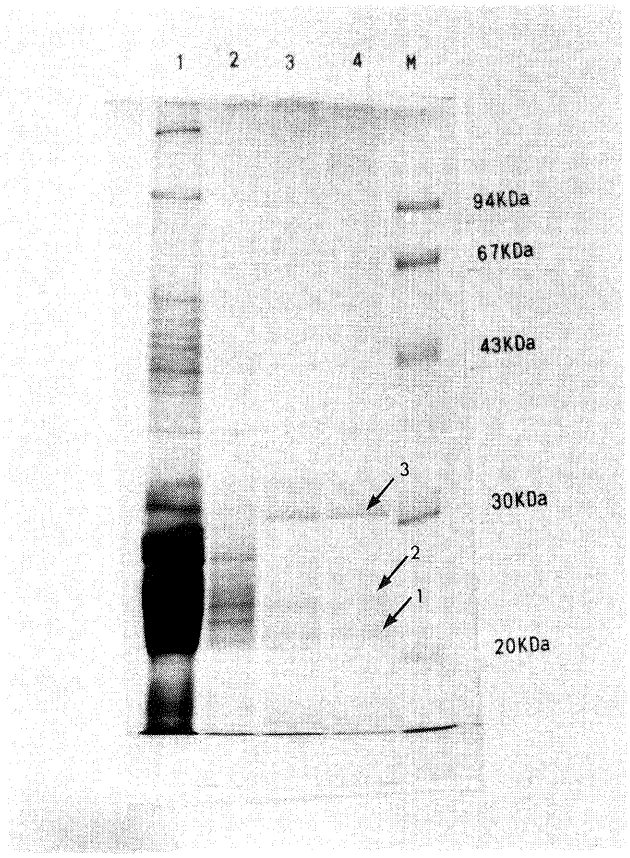

Fig. 2. SDS–PAGE of Fractions-I and II

The details of SDS–PAGE were given under Materials and Methods. M, marker, lane 1, insoluble fraction of the bovine lens; lane 2, fraction solubilized with *n*-heptyl-thioglucoside; lane 3, Fraction-I; lane 4, Fraction-II. Arrows 1, 2 and 3 represent the protein bands with molecular weight of 21, 25 and 30 kDa, respectively.

Optimal pH, Optimal Temperature and Stability The physiological properties of Fraction-I and Fraction-II are summarized in Fig. 3. The optimal pH of Fraction-I and Fraction-II on 4-MU-α-Glc was pH 6.0 and 6.5 respectively (Fig. 3A), and the optimal temperature for both fractions was approximately 50 °C as shown (Fig. 3B).

Then, the effect of pH on the stability of the enzyme was examined for a period of 3 d at 4 °C in buffers ranging from pH 3 to 10. Between pH 5.5 to 7.5, the activity of both fractions was remained stable, whereas at lower and higher pHs it progressively decreased. Furthermore, the activity was also examined as a function of temperature. Both enzyme fractions were stable up to 50 °C for 1 h, but Fraction-II was more rapidly inactivated than Fraction-I at a higher temperature (Fig. 3B).

Michaelis Constant and Maximum Velocity for 4-MU-α-Glc First, the initial rates of the enzyme-catalyzed reaction for 4-MU-α-Glc were measured (Fig. 3C). The initial rate of Fraction-I was 7.42 pmol/min and that of Fraction-II was 14.97 pmol/min. Then, the substrate dependence on activity was determined and the double-reciproal or Lineweaver–Burk plots are shown in Fig. 3D. The Michaelis constant (K_m) and maximum velocity (V_{max}) for 4-MU-α-Glc was estimated from the Lineweaver–Burk plots. The K_m of Fraction-I was 0.086 mM and that of Fraction-II was 0.192 mM. The V_{max} value of Fraction-I was 0.666 nmol/min/mg of protein and that of Fraction-II was 2.353 nmol/min/mg of protein.

Inhibitors Inhibition by specific chemical agents of the enzyme activity of the two fractions was examined (Table 2). Bivalent metal ions inhibited the activity of both fractions of the enzyme, especially Hg^{2+}, inhibited it completely. SDS and 2-mercaptoethanol exhibited strong inhibition. However, iodoacetic acid produced only slight inhibition, and D-turanose was devoid any activity.

DISCUSSION

Our results demonstrate that neutral α-Glcdase can be purified from the insoluble fraction of bovine crystalline lens by ion exchange column chromatography, gel filtration chromatography and affinity chromatography. We obtained finally two purified fractions, Fraction-I and Fraction-II, which exhibited α-Glcdase activity. Both fractions consisted of three polypeptides of molecular weight 21, 25 and 30 kDa, although the proportion of the polypeptides in these fractions differed significantly. Comparing Fraction-I and Fraction-II their optimal pH, optimal temperature and temperature stability were slightly different. Furthermore, affinity for a Con A column was considerably different. This fact demonstrates that both enzyme fractions may have sugar chains and that these chains may differ in the nature of their recurring monosaccharide unit, length and degree of branching. As far as the isozymes of α-Glcdase are concerned, it has been reported that neutral α-Glcdase purified from human placenta contains three isozymes with molecular weights of 92, 150 and 350 kDa.[11] This led us to suppose that the α-Glcdase in the insoluble lens fraction may contain two isozymes with molecular weights of 121 and 254 kDa. In this study, we could not determine exactly whether all the polypeptides detected on SDS–PAGE gels were subunits of the enzyme. Furthermore, we attempted to estimate the proportion of subunits in Fraction-I and Fraction-II, respectively, and however, we were unsuccessful.

It has been reported that there are two types of α-Glcdase in animal tissues.[12] One is acidic α-Glcdase, which is a lysozomal enzyme with an optimal pH of 4 to 5. The other is neutral α-Glcdase, which is a cytosolic enzyme with an optimal pH of 6 to 7. The two fractions of α-Glcdase purified in the present study involve an apparently neutral and cytoplasmic enzyme, because the optimal pHs are pH 6.0 and 6.5, and the enzyme activities were not inhibited by D-turanose which inhibits completely acidic α-Glcdase.[13] We also believe that the enzymes obtained in this study may bind to cell membranes, because they are neutral α-Glcdase and cytoplasmic enzyme in nature, and are solubilized only by detergents.

Using the purification method reported here, a pure enzyme can be obtained for more physicochemical studies detailed analysis of the molecular structure, characterization of the two components, and comparison studies of α-Glcdase between in the soluble and insoluble fractions of the lens need to be performed. However, we believe firmly that it is necessary to report the present study because our data will be useful for further research into crystalline lens composition and cataract formation.

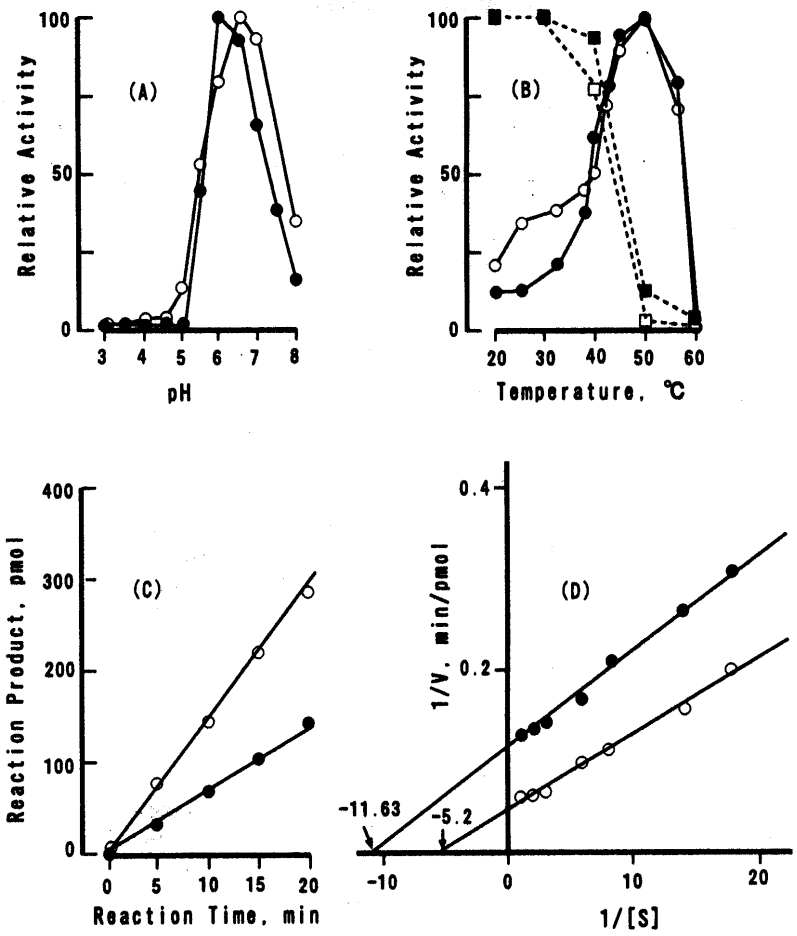

Fig. 3. Physiological Properties of Fractions-I and II

(A) Optimal pH. (B) Optimal temperature and temperature-stability curves. The enzyme was incubated for 1 h at various temperatures, then rapidly cooled and the activity determined. (C) Initial rate. (D) Lineweaver–Burk plots. —●—, Fraction-I; —○—, Fraction-II; ---■---, temperature stability of Fraction-I; ---□---, temperature-stability of Fraction-II. The details of each experiment are given under Materials and Methods.

Table 2. Effect of Inhibitors on Enzyme Activity

Metal ions and inhibitors	Concentration (mM)	Residual activity (%)	
		Fraction-I	Fraction-II
None	—	100	100
$MgCl_2$	1.0	65.7	95.5
$CaCl_2$	1.0	82.7	91.1
$CuCl_2$	1.0	67.3	72.5
$FeCl_2$	1.0	79.3	67.6
$MnCl_2$	1.0	84.9	92.2
$CoCl_2$	1.0	93.6	98.1
$HgCl_2$	1.0	0.4	0.6
EDTA	5.0	79.7	83.0
SDS	0.1%	0.4	0.4
Iodoacetic acid	1.0	92.2	86.4
2-Mercaptoethanol	5.0	46.9	52.8
D-Turanose	500	101.0	100.5

Enzyme activity was measured with 4-MU-α-Glcdase as described under Materials and Methods.

Further work along these lines is in progress.

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