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Purification and Kinetic Properties of Guinea Pig Liver β-Mannosidase

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β-Mannosidase was purified to electrophoretic homogeneity from the 20000*g* supernatant of guinea pig liver homogenate. A highly purified enzyme preparation was also obtained from the acetone powder. This enzyme had a pH optimum of 4.0 and molecular weights of *ca.* 120000 as determined by gel filtration and 110000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. It was free from other glycosidase activities, as far as was tested.

The enzymatic hydrolysis of *o*- and *p*-nitrophenyl β-mannosides exhibited an unusual relationship of rate to substrate concentration, indicative of the involvement of two molecules of substrate in the reaction. The rate of hydrolysis was enhanced markedly by several *p*-nitrophenyl compounds including *p*-nitrophenyl glycosides, and also by Triton X-100 and chlorinated pesticides such as aldrin.

Keywords——enzyme activation; chlorinated pesticide; enzyme kinetics; guinea pig liver; β-mannosidase; β-mannoside; modifier; non-Michaelian behavior

β-Mannosidase (β-mannoside mannohydrolase, EC 3.2.1.25) is known to be widely distributed in various biological sources,[2,3] though with low activity compared with that of α-mannosidase. Well-defined β-mannosidase preparations from snail viscera[2a] and hen oviduct[2b] are available for studies on the enzymatic cleavage of β-mannosidic linkages in oligosaccharide. Several β-mannosidases have been purified to electrophoretic homogeneity from sources in the vegetable kingdom[2c-f] but not from mammalian sources.[3] A partial purification was reported for the rat liver enzyme.[3d]

It has recently been reported that β-mannosidosis of goat, an inherited disorder of glycoprotein catabolism, arises from the deficiency of lysosomal β-mannosidase.[4] The goat liver enzyme was resolved by chromatography on concanavalin A bound to Sepharose into acidic (lysosomal) and neutral (nonlysosomal) forms and only the acidic form was able to hydrolyze a trisaccharide, Manβ GlcNAcβ[^{3}H]GlcNAc.[4b]

Houston *et al.*[5] studied the effect of *p*-nitrophenyl α-mannoside on the activity of β-mannosidase from malted barley using *p*-nitrophenyl β-mannoside as a substrate, and found that the corresponding anomer, *p*-nitrophenyl α-mannoside, activated the enzyme (*ca.* 1.6-fold) at low concentrations (0.1 to 2 mM) of the α-mannoside added but competitively inhibited it at higher concentration (3 mM). These findings stimulated our interest and led us to reexamine and extend this work using β-mannosidase from guinea pig liver.

This paper describes the purification of the enzyme and the kinetic properties of the purified enzyme, which showed deviation from Michaelis–Menten type kinetics and unexpected activation by a variety of compounds.

Materials and Methods

Materials——Nitrophenyl β-mannosides were prepared by condensation of dicyclohexylidene α-manno-

pyranose with phenols.[2f,6] Other glycosides were obtained from Boehringer or Sigma. 2-Acetamido-1-*N*-(ε-aminohexanoyl)-2-deoxy-β-D-glucopyranosylamine–Sepharose 4B (GlcNAc–agarose) was prepared according to the procedure of Lotan *et al.*[7]

Enzyme Assay——For routine assay, a reaction mixture containing 5 mM *p*-nitrophenyl β-mannoside, 0.01% bovine serum albumin and enzyme solution in a final volume of 0.4 ml of 0.05 M sodium phosphate-citrate buffer, pH 4.0, was incubated for 2—10 min at 37 °C. The reaction was stopped by the addition of 0.4 M glycine–sodium hydroxide buffer, pH 10.5 and released *p*-nitrophenol was measured according to the method of Levvy and Conchie.[8] One unit of enzyme activity releases 1 μmol of *p*-nitrophenol per min under the above conditions. Protein was determined by the method of Lowry *et al.*[9] using bovine serum albumin as a standard.

Analytical Gel Filtration——Molecular weight was estimated by Sephadex G-200 gel filtration according to Andrews.[10] The enzyme (*ca.* 5 units) was applied to a column (1.5 × 96 cm) and eluted with 0.05 M sodium phosphate-citrate buffer, pH 5.0, or 0.05 M Tris–hydrochloric acid buffer, pH 8.0. The column was calibrated with standard proteins (molecular weights in parentheses): bovine serum albumin (67000), aldolase from rabbit muscle (158000) and catalase from beef liver (240000).

Polyacrylamide Gel Disc Electrophoresis——Intact enzyme (5—20 μg protein) was run on a gel (0.5 × 8 cm) at 4 °C with a constant current of 2.5—4.0 mA per tube, using bromophenol blue or malachite green as a tracking dye. The buffer systems were acetate buffer, pH 4.3,[11] and Tris–hydrochloric acid buffer, pH 9.4.[12] The glycosidase activity was detected by incubating the gels in a substrate mixture containing 5 mM *p*-nitrophenyl β-mannoside and 0.01% bovine serum albumin in 0.1 M phosphate–citrate buffer, pH 4.0 at 37 °C for 30—60 min after the gel had been soaked for a short time in the buffer, pH 4.0. The gel was then washed with water and soaked in 0.4 M glycine buffer, pH 10.5, to give a yellow band.

For the determination of subunit molecular weight, sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the standard procedure of Weber *et al.*,[13] after incubation at 100 °C for 2 min in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol, the sample (*ca.* 10—20 μg) was run on 7.5% gel with malachite green as a tracking dye.

Subcellular Fractionation——The tissue was homogenized and fractionated by the method of de Duve *et al.*[14] β-Mannosidase activity in each fraction was assayed after treatment with 0.2% Triton X-100 at 4 °C for *ca.* 1.5 h, followed by dialysis against 0.05 M phosphate buffer, pH 6.0.

Purification of β-Mannosidase from the 20000 *g* Supernatant——The animals were killed by means of a blow on the head followed by decapitation. The liver was quickly removed and cooled to 4 °C. The liver (*ca.* 5 g) was immediately homogenized with 40 ml of 0.05 M Tris–hydrochloric acid buffer, pH 7.4 containing 0.25 M sucrose in a Potter–Elvehjem homogenizer at 1000 rpm. The homogenate from 433 g of wet liver was centrifuged at 2500 rpm for 15 min and further at 15000 rpm for 10 min. The supernatant (20000 *g* supernatant) was fractionated with ammonium sulfate. The precipitate formed at 70% saturation was dissolved in 400 ml of cold 0.05 M sodium phosphate–citrate buffer, pH 4.5, and dialyzed against 6 l of the same buffer overnight. The dialyzate was further fractionated with ammonium sulfate. The precipitate at 70% saturation was dissolved in 150 ml of cold 0.05 M sodium potassium phosphate buffer, pH 6.5, and dialyzed against 6 l of the same buffer overnight. The dialyzate, without concentration, was applied to a Con A-Sepharose column (1.5 × 30 cm) equilibrated with the buffer. The column was washed with the buffer and eluted with the same buffer but containing 0.5 M methyl α-D-mannoside (Fig. 1). The active fractions were pooled, concentrated to 12 ml by ultrafiltration and passed through a Sephadex G-200 column (2.6 × 100 cm). Elution was carried out with the same buffer (Fig. 2). Active fractions were pooled, concentrated to 21 ml, applied to a diethylaminoethyl (DEAE)–cellulose column (2.6 × 15 cm) and eluted with the same buffer (Fig. not shown). The activity was eluted without retardation. The active fractions were pooled, concentrated to 17 ml and passed through a GlcNAc–agarose column (1.5 × 16 cm). Elution was carried out with 0.05 M sodium potassium phosphate buffer, pH 6.0 (Fig. not shown). The β-mannosidase activity was eluted without retardation. Active fractions were pooled, concentrated to 17 ml and dialyzed against 0.01 M sodium potassium phosphate buffer, pH 7.0. The subsequent purification steps were carried out in the presence of 1 mM dithiothreitol. The solution was applied to a DEAE–Sephadex A-50 column (2.6 × 15 cm) equilibrated with 0.01 M phosphate buffer, pH 7.0. A large portion of the phosphatase activity was eluted with the same buffer, and the glycosidase activity was eluted with a linear gradient of 0.01—0.05 M phosphate buffer, pH 7.0 (Fig. 3). The active eluates were pooled, concentrated to 15 ml and dialyzed against 0.05 M phosphate–citrate buffer, pH 4.0. The enzyme solution was applied to a carboxymethyl (CM)–Sephadex C-50 column (1.5 × 25 cm) and the column was eluted with a linear gradient of 0.05—0.2 M phosphate–citrate buffer, pH 4.5 (Fig. 4). Fractions containing β-mannosidase were pooled, concentrated to 3 ml and stored at 4 °C.

Purification of β-Mannosidase from Acetone Powder——Guinea pig liver (80 g) was homogenized twice with 360 ml each of acetone for 2 min in a Waring blender. The homogenate was filtered, and the residue was washed with 160 ml of cold acetone and dried under reduced pressure. The acetone powder from 1 kg of wet liver was suspended in 3 l of cold 0.05 M sodium phosphate–citrate buffer, pH 4.5, containing 0.2 M sodium chloride. After standing overnight at 4 °C, the extract was filtered and the filtrate was fractionated with ammonium sulfate. The precipitate formed at 60% saturation was dissolved in cold 0.05 M sodium potassium phosphate buffer, pH 6.5, and dialyzed against the

same buffer (7 l × 2). The dialyzate was applied to a column of Con A–Sepharose (2.6 × 20 cm) equilibrated with the same buffer, and the column was washed with the buffer. β-Mannosidase activity was eluted with 0.4 l of 0.5 M methyl α-D-mannoside in the same buffer. The active fractions were pooled, concentrated to *ca.* 10 ml and applied to a Sephadex G-200 column (2.6 × 99 cm) equilibrated with 0.05 M sodium potassium phosphate buffer, pH 6.5. Elution was carried out with the same buffer. Active fractions were pooled (88 ml) and applied to a DEAE–cellulose column (2.6 × 20 cm) equilibrated with 0.05 M phosphate buffer, pH 6.5. On elution with the same buffer, β-mannosidase activity appeared without retardation. The active fractions were pooled (130 ml), concentrated to 10 ml and applied to a GlcNAc–agarose column (1.9 × 27 cm). The activity was eluted with the same buffer without retardation and the active fractions were pooled (45 ml). Subsequent purification steps were carried out with buffers containing 1 mM dithiothreitol. A 14-ml portion of the above enzyme solution was dialyzed against 0.01 M phosphate buffer, pH 7.0 and applied to a DEAE–Sephadex A-50 column (2.6 × 20 cm) equilibrated with the same buffer. A large amount of phosphatase activity was eluted with the same buffer (265 ml), then β-mannosidase activity was eluted with a linear gradient of 0.01—0.05 M phosphate buffer, pH 7.0. β-Mannosidase appeared at *ca.* 130 ml eluate after buffer concentration reached 0.04 M. Fractions containing β-mannosidase were pooled and concentrated to 10 ml. A 5-ml portion of the enzyme solution was dialyzed against 0.05 M phosphate–citrate buffer, pH 4.0, and applied to a CM–Sephadex C-50 column (1.5 × 12.5 cm) equilibrated with the same buffer. When the column was eluted with a linear gradient of 0.05—0.2 M phosphate–citrate buffer, pH 4.5, β-mannosidase activity appeared in the next *ca.* 150 ml of the 0.2 M buffer after the gradient was over. Active fractions were pooled, concentrated to 3.5 ml and stored at 4 °C.

Kinetic Measurements——All the experiments were carried out at 37 °C in the presence of 0.01% bovine serum albumin as described in "Enzyme Assay". Initial velocity was determined from five points in the linear part of the time course within 25 min. The enzyme activity was proportional to enzyme concentration in the range of 0.00125—0.089 units per ml of incubation mixture.

Results

Although identification of each fraction by estimation of marker enzyme activities was not done, the subcellular distribution of β-mannosidase was roughly as follows: nucleus, 10% mitochondria (containing lysosomes), 24.6%; microsomes, 5.4%; cytosol, 60%.

Purification from 20000 *g* Supernatant

β-Mannosidase was purified to electrophoretic homogeneity by the procedures summarized in Table I. In Con A–Sepharose chromatography, a large amount of protein was eluted without adsorption, accompanied by high arylphosphatase activity, and β-mannosidase activity was eluted with eluent containing 0.5 M methyl α-mannoside together with arylphosphatase activity (Fig. 1); the specific activity was increased *ca.* 9-fold with an excellent recovery. Subsequent gel filtration effectively removed proteins with high molecular weight (Fig. 2). DEAE-cellulose chromatography enhanced the specific activity 9-fold,

Table I. Purification of Guinea Pig Liver β-Mannosidase from 20000 *g* Supernatant

Procedure[a]	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)
20000 *g* supernatant from 433 g wet liver	36.2	46442	0.0008	100
Ammonium sulfate precipitation (pH 7.4)	35.4	18013	0.0020	97.8
Ammonium sulfate precipitation (pH 4.5)	31.0	9540	0.0033	85.6
Con A–Sepharose chromatography	44.1	1513	0.029	121.8[b]
Sephadex G-200 chromatography	41.3	886	0.047	114.1
DEAE–cellulose chromatography	36.2	96	0.38	100
GlcNAc–agarose chromatography	33.0	43	0.76	91.2
DEAE–Sephadex A-50 chromatography	17.4	5.7	3.05	48.1
CM–Sephadex C-50 chromatography	10.1	1.6	6.35	27.9

a) All the procedures were carried out at 4 °C.
b) The 20000 *g* supernatant contained a high concentration of sucrose, an effective inhibitor of this enzyme. The apparently unreasonable yield seems due to complete removal of sucrose at this stage.

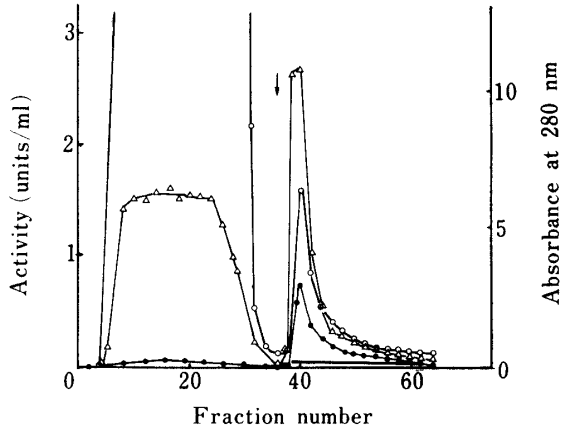

Fig. 1. Con A–Sepharose Column Chromatography

Stepwise elution was performed successively with 0.05 M phosphate buffer, pH 6.5, and the same buffer containing 0.5 M methyl α-mannoside (indicated by the arrow). Ten-milliliter fractions were collected and fractions under the line were pooled. ●, β-mannosidase activity; △, arylphosphatase activity; ○, absorbance at 280 nm.

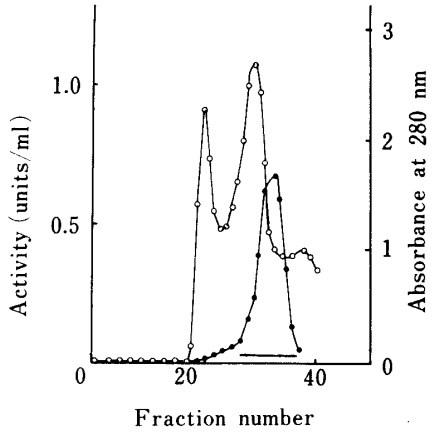

Fig. 2. Sephadex G-200 Column Chromatography

Ten-milliliter fractions were collected and fractions under the line were pooled. ●, β-mannosidase activity; ○, absorbance at 280 nm.

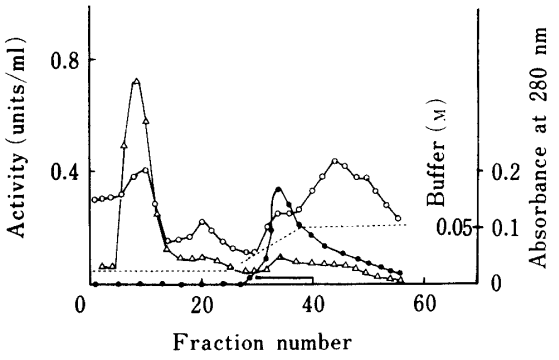

Fig. 3. DEAE–Sephadex A-50 Column Chromatography

The column was eluted with 0.01 M phosphate buffer, pH 7.0, and then with a continuous gradient formed from 0.01 M phosphate buffer, pH 7.0, and 0.05 M concentration of the same buffer. Ten-milliliter fractions were collected and fractions under the line were pooled. ●, β-mannosidase activity; △, arylphosphatase activity; ○, absorbance at 280 nm; – – –, concentration of phosphate buffer.

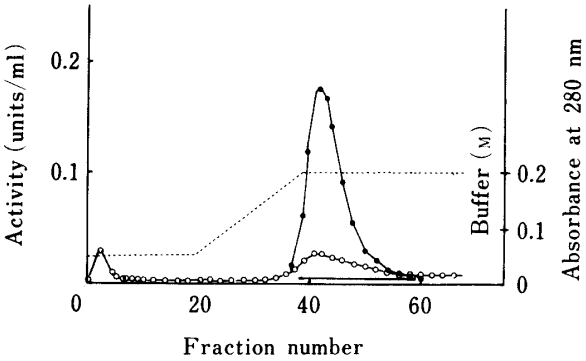

Fig. 4. CM–Sephadex C-50 Column Chromatography

The column was eluted with 0.05 M phosphate-citrate buffer, pH 4.0, then with a gradient of 0.05–0.2 M phosphate-citrate buffer, pH 4.5, and finally with 0.2 M concentration of the same buffer. Ten-milliliter fractions were collected and fractions under the line were pooled. ●, β-mannosidase activity; ○, absorbance at 280 nm; ------, concentration of phosphate-citrate buffer.

although the activity was eluted without retardation (not shown). An affinity column of GlcNAc–agarose was effective for the removal of β-acetylglucosaminidase activity; β-mannosidase activity was eluted without adsorption with 0.05 M phosphate buffer and *ca.* 97% of the β-acetylglucosaminidase activity was retained on the column. The enzyme solution, however, still exhibited high arylphosphatase activity. As shown in Fig. 3, the bulk of the arylphosphatase was removed by using a DEAE–Sephadex A-50 column, with a 4-fold increase in specific activity. Figure 4 shows the elution profile of β-mannosidase from a CM–Sephadex C-50 column. The enzyme activity was retained on the column until the buffer concentration reached 0.2 M.

In the above procedures, an 8000-fold purification was attained with a recovery of *ca.*

TABLE II. Purification of Guinea Pig Liver β-Mannosidase from Acetone Powder[a]

Procedure[b]	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)
Acetone powder extract	290	60160	0.0048	100
Ammonium sulfate precipitation	243	44600	0.0054	83.8
Con A–Sepharose and subsequent Sephadex G-200 chromatography	249	1210	0.21	85.9
DEAE–cellulose chromatography	235	—	—	81.0
GlcNAc–agarose chromatography	211	556	0.38	72.8
DEAE–Sephadex A-50 chromatography (65.6 units, 173 mg protein)	49.2	26.0	1.89	54.6
CM–Sephadex C-50 chromatography (24.6 units, 13 mg protein)	19.5	2.4	8.13	43.2

a) In the five steps to GlcNAc–agarose chromatography, values were per 1 kg wet liver. Only a portion of the enzyme solution was used in the last two steps.
b) All the procedures were carried out at 4 °C.

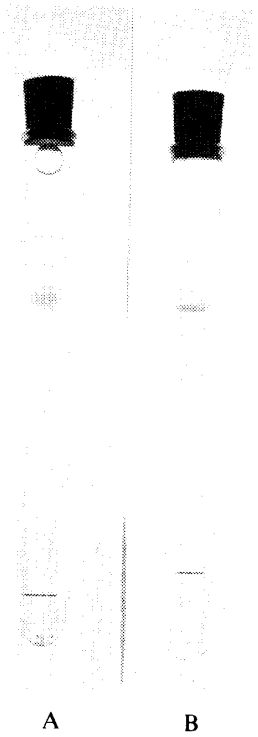

Fig. 5. Polyacrylamide Gel Disc Electrophoresis of β-Mannosidase Preparation from Acetone Powder

A, pH 9.4 gel; B, sodium dodecyl sulfate-polyacrylamide gel.

28%. The final preparation migrated as a single protein band coincident with the band stained for β-mannosidase activity when tested by polyacrylamide gel electrophoresis at pH 9.4 and 4.3, though it exhibited some arylphosphatase activity. The arylphosphatase activity seems to be a side activity, since phenyl phosphate markedly inhibited the enzymatic hydrolysis of *p*-nitrophenyl β-mannoside, probably by competing with the glycoside for the same active site. The preparation was free from α-mannosidase, α- and β-glucosidase, α- and β-galactosidase, α-xylosidase, β-glucuronidase and β-acetylglucosaminidase activities, all of which were present in the liver extract, when tested by incubation for 16 min with a 5 mM concentration of the corresponding *p*-nitrophenyl glycosides at pH 4.0, 5.0 and 5.5.

Purification from Acetone Powder

The purification of β-mannosidase from acetone powder is summarized in Table II. The

purification steps were similar to those for the 20000 *g* supernatant except for the pH of the elution buffers, and the concentration of ammonium sulfate in the precipitation procedures. The final preparation exhibited a single protein band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and had a higher specific activity than the preparation from 20000 *g* supernatant, although a very faint band of contaminating protein was observed on disc gel electrophoresis (Fig. 5.). This preparation was free from glycosidase activities other than β-mannosidase activity.

Properties

The enzyme preparations from 20000 *g* supernatant and acetone powder were the same on the basis of the molecular weight, pH optimum, heat stability, behavior in electrophoresis

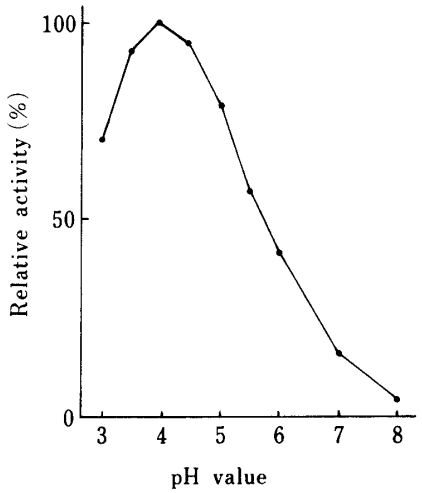

Fig. 6. The Effect of pH on the Enzyme Activity

p-Nitrophenyl β-mannoside was used as a substrate.

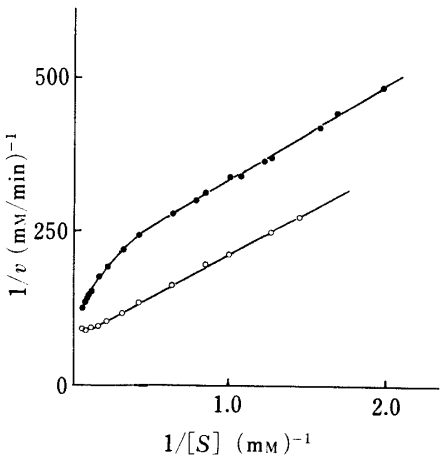

Fig. 7. Lineweaver–Burk Plots for *o*-Nitrophenyl β-Mannoside in the Absence and Presence of *p*-Nitrophenyl α-Mannoside

●, no additive; ○, 10 mM *p*-nitrophenyl α-mannoside. Enzyme concentration, 0.0017 units/0.4 ml of incubation mixture.

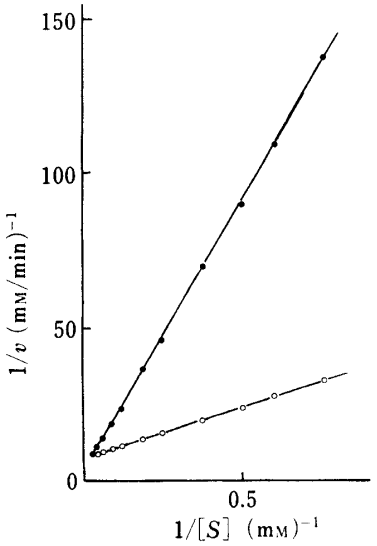

Fig. 8. Lineweaver–Burk Plots for *m*-Nitrophenyl β-Mannoside in the Absence and Presence of *p*-Nitrophenyl α-Mannoside

●, no additive; ○, 10 mM *p*-nitrophenyl α-mannoside. Enzyme concentration, 0.015 units/0.4 ml of incubation mixture.

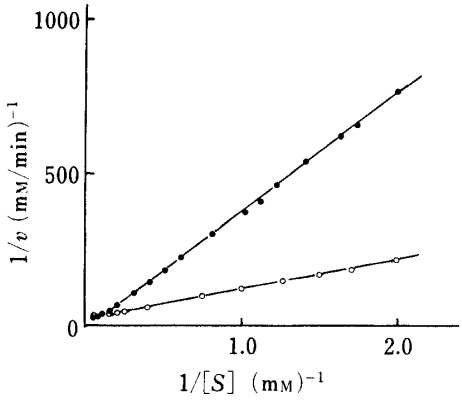

Fig. 9. Lineweaver–Burk Plots for *p*-Nitrophenyl β-Mannoside in the Absence and Presence of *p*-Nitrophenyl α-Mannoside

●, no additive; ○, 10 mM *p*-nitrophenyl α-mannoside. Enzyme concentration, 0.005 units/0.4 ml of incubation mixture.

and kinetic behavior, *i.e.*, deviation from Michaelis–Menten kinetics and activation by various compounds. The molecular weight of the enzyme as determined by gel filtration at pH 5.0 or pH 8.0 was 110000 or 130000, respectively, and that determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was 110000, suggesting that the enzyme is a monomeric enzyme. The enzyme had a pH optimum of 4.0 (Fig. 6) and was stable at 37—45 °C at pH 4.0 for at least 60 min in the presence of bovine serum albumin. Metal ions examined had no significant effect on the enzyme activity except that 10 mM silver nitrate and 10 mM mercury chloride strongly inhibited and 10 mM ferrous sulfate 1.3-fold activated the enzyme.

Figures 7, 8 and 9 show the Lineweaver–Burk plots for three nitrophenyl β-mannosides. The plot for *o*-nitrophenyl β-mannoside exhibited a downward curvature which indicates negative cooperativity in the enzyme reaction.[15] In the case of *p*-nitrophenyl β-mannoside, an upward curvature was observed at high substrate concentrations, and the extrapolation of the linear part of the curve intersects the ordinate at a negative $1/v$ value, which indicates positive cooperativity.[15] *m*-Nitrophenyl β-mannoside apparently obeyed the Michaelis–Menten kinetics.

Addition of *p*-nitrophenyl α-mannoside accelerated the enzymatic hydrolysis of the three nitrophenyl β-mannosides to a greater extent than in the case of barley β-mannosidase,[5] and greatly improved the linearity in the Lineweaver–Burk plots (Figs. 7—9). To examine the specificity of the enzyme for ligand (activator or modifier), the effects of a variety of compounds were examined using *p*-nitrophenyl β-mannoside as a substrate. Methyl α-mannoside was rather inhibitory, tending to rule out the importance of α-mannopyranoside structure in the activation. However, the enzyme was found to be stimulated by various compounds including some chlorinated pesticides and detergents as well as several *p*-nitrophenyl α- and β-glycosides. Activities in the presence of some compounds relative to activity in their absence were as follows (examined with 1.3 mM *p*-nitrophenyl β-mannoside as a substrate): 7.1 mM *p*-nitrophenyl α-xyloside, 3.16; 10 mM *p*-nitrophenyl β-xyloside, 3.15; 5.7 mM *p*-nitrobenzoic acid, 2.50; 11.2 mM *p*-nitrophenyl sulfate, 3.43; 244 μM pentachlorophenol, 3.80; 20 μM aldrin, 1.75; 244 μM dieldrin, 2.03; 0.01% Triton X-100, 3.42.

Discussion

In the present study, β-mannosidase was isolated in a pure state from the 20000 *g* supernatant of guinea pig liver. The β-mannosidase was conveniently obtained from the acetone powder of whole tissue, and the preparation obtained was the same as that from the 20000 *g* supernatant fraction. Gel filtration and sodium dodecyl sulfate-gel disc electrophoresis suggested that the enzyme is a monomeric enzyme.

In this study, the two nitrophenyl β-mannosides exhibited departure from Michaelian kinetics. The reactions were carried out in the presence of bovine serum albumin, because of fluctuations in activity in the absence of bovine serum albumin. If an incubation mixture contains an impurity which combines with the substrate to decrease the substrate concentration, departure from Michaelian behavior can be expected.[16] The possibility that bovine serum albumin combines with the substrate to affect the kinetic behavior was ruled out by the following separate experiments; in the presence of bovine serum albumin, β-mannosidase activity in "glycosidase mixed" from Turbo cornutus (Seikagaku Kogyo Co., Ltd.) did not show such a departure from Michaelian kinetics, though simple substrate inhibition was observed.

Many enzymes, at least *ca.* 13% of known enzymes, exhibit cooperativity.[15] Among glycosidases, several enzymes have been reported to show deviation from Michaelis–Menten kinetics other than substrate inhibition.[17] These deviations were explained in terms of the enzyme having a modifier site or two mutually interacting active sites,[17a-d] or in terms of a

mnemonical enzyme.[17f] On the other hand, positive cooperativity ascribed to transglycosylation (a reaction involving two molecules of substrate) was reported for the β-amylase-catalyzed hydrolysis of β-maltosyl fluoride, an unusual substrate. First, two molecules of the substrate are condensed to maltotetraosyl fluoride by transglycosylation, and in the second step this product is hydrolyzed to β-maltose and β-maltosyl fluoride.[17g]

At first it was tempting to speculate that nitrophenyl derivatives, including the substrates, were good acceptors for glycosyl residues and accelerate the possible rate-determining step of deglycosylation of glycosyl enzyme, which is the intermediate in the enzymatic hydrolysis of glycoside. However, the enzyme was activated by a variety of compounds including aldrin and dieldrin which cannot act as acceptors.

At present, although it is not known whether the activation by a variety of compounds arises from the same mechanism or not, it seems very likely that the activation arises from the binding of a hydrophobic ligand or a hydrophobic moiety of a ligand (including substrate) to a hydrophobic broad-specificity modifier site other than the active site on the enzyme. The departure from Michaelian behavior observed for *o*- and *p*-nitrophenyl β-mannosides can be explained in terms of a mechanism involving two substrate molecules, as discussed by Frieden.[18] The same mechanism could operate in the apparent Michaelian behavior with *m*-nitrophenyl β-mannoside; the mechanism can result in a straight line as well as concave and convex lines in Lineweaver–Burk plots, depending on the values of the kinetic parameters. The presence of an activator (ligand, L) could favor enzyme species LE and LES, and the dominant species would display Michaelian behavior.

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