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Purification and Kinetic Studies of β-Acetylhexosaminidase A from Porcine Kidney

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Porcine kidney β-acetylhexosaminidase A was purified to electrophoretic homogeneity by procedures involving ammonium sulfate precipitation, gel filtration and column chromatography on diethylaminoethyl cellulose, octyl Sepharose CL-4B and ε-aminohexanoyl β-acetylglucosaminylamine-Sepharose 4B. Analysis by sodium dodecyl sulfate-gel electrophoresis suggested that the enzyme consists of three subunits with molecular weights of 61000, 31000 and 9000. The molecular weight of the intact enzyme as determined by gel filtration was 129000 at pH 8.0 and 302000 at pH 5.0.

Kinetic analysis with mixed substrates as well as inhibition studies with various substrate analogs, indicated that the enzyme hydrolyzes both β-N-acetylglucosaminide and β-N-acetylgalactosaminide at the same active site.

Among hexoses tested for inhibitory effect, only mannose was an effective competitive inhibitor. A comparison of mannosides with N-acetylglucosaminides with respect to binding ability to the enzyme in terms of K_i or K_m values implied the presence of a site for mannose other than the active site. On the other hand, kinetic studies with mixed inhibitors indicated that mannose competes with N-acetylgalactosamine, N-acetylglucosamine and acetamide.

Keywords——β-acetylhexosaminidase purification; active site; affinity chromatography; enzyme inhibition; enzyme kinetics; porcine kidney; subunit composition; Yagi-Ozawa plot

β-Acetylhexosaminidase (hexosaminidase, 2-acetamido-2-deoxy-β-hexoside acetamidodeoxyhexohydrolase, EC 3.2.1.52) is a common enzyme which hydrolyzes β-glycosides of N-acetylglucosamine and N-acetylgalactosamine. The enzyme is believed to hydrolyze both glycosides at a common active site, but Pope *et al.*[2)] have recently reported that the enzymes from rat colonic tissues and from bovine kidney exhibit kinetic behavior which suggests the presence of different active sites for the two glycosidase activities. The enzyme activity is inhibited by N-acetylglucosamine and N-acetylgalactosamine. Most of the binding affinity of N-acetylglucosaminides as substrates for this enzyme is attributable to their acetamido group.[3)] Recently, Reglero found that mannose, methyl α-mannoside and mannosamine have inhibitory effects on some mammalian hexosaminidases, and he discussed the physiological significance of the inhibitory effect.[4)]

Mammalian hexosaminidases, especially from human sources, have been purified to homogeneity and the subunit structures of some of them have been studied in detail in connection with the significance of the enzyme in heritable gangliosidoses.[5)] Hexosaminidases A and B have been partially purified from porcine kidney and characterized by physicochemical methods.[6)] The present paper describes the purification of the hexosaminidase A from porcine

1) Location: *2-2-1, Miyama, Funabashi, 274, Japan.*
2) A.J. Pope, N. Mian, and D.G. Herries, *FEBS Lett.*, **93**, 174 (1978).
3) T. Mega, T. Ikenaka, H. Arita, K. Fukukawa, and Y. Matsushima, *J. Biochem.* (Tokyo), **73**, 55 (1973).
4) A. Reglero, *Int. J. Biochem.*, **10**, 285 (1979).
5) *a*) B. Geiger and R. Arnon, *Biochemistry*, **15**, 3484 (1976); *b*) D.V. Marinkovic and J.N. Marinkovic, *Biochem. J.*, **163**, 133 (1977).
6) S.J. Wetmore and J.A. Verpoorte, *Can. J. Biochem.*, **50**, 563 (1972).

kidney using affinity chromatography, as well as its enzymatic properties and kinetic studies with emphasis on the active site for the two glycosidase activities and the binding site of mannose.

Experimental Procedures

Chemicals——Carbohydrate compounds were purchased from Nakarai Chemicals Ltd., except for aryl and methyl β-N-acetylglucosaminides and β-acetylglucosaminylamine (2-acetamido-2-deoxy-β-D-glucopyranosylamine) derivatives, which were prepared in the usual way. Other chemicals were as described previously.[7]

Enzyme Assay——The enzyme activity was routinely assayed by incubation with 2 mM *p*-nitrophenyl-β-N-acetylglucosaminide in 0.05 M sodium citrate buffer, pH 4.5, containing 0.01% bovine serum albumin. 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.*[8] using bovine serum albumin as a standard.

Analytical Gel Filtration——The molecular weight was estimated by Sephadex G-200 gel filtration according to Andrews.[9] The enzyme (*ca.* 100 units) was applied to a column (1.5×96 cm) and eluted with 0.05 M sodium citrate buffer, pH 5.0, or 0.05 M Tris 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 Electrophoresis and Determination of Subunit Molecular Weight——The purity of the enzyme was examined by disc electrophoresis in a Tris-glycine buffer system of pH 9.4 as described by Davis[10] and in a potassium acetate buffer system of pH 4.3 as described by Reisfeld *et al.*[11]

For the determination of subunit molecular weight, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the standard procedure of Weber *et al.*;[12] after incubation at 100° 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. In order to obtain complete dissociation of the enzyme, the following denaturation procedures were also examined: (1) treatment at 20° for 1 hr in Tris buffer, pH 8.5, containing 1 mM dithiothreitol and 7 M guanidine hydrochloride, followed by carboxymethylation and further incubation at 100° for 5 min in phosphate buffer, pH 7.1, containing 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol and 8 M urea according to Marinkovic *et al.*,[5b] and (2) treatment at 100° for 5 min with 1 M 2-mercaptoethanol or 50 mM dithiothreitol in 0.01 M phosphate buffer, pH 7.1, containing 1% sodium dodecyl sulfate. These conditions were essentially the same as those described by Geiger and Arnon[5a] except for higher concentrations of reducing agents.

Partial Purification of Hexosaminidases A and B——Porcine kidney (80 g) was twice homogenized in 360 ml each of cold acetone for 2 min at top speed in a homogenizer (Nihon Seiki), filtered, washed with 160 ml of acetone and dried under reduced pressure. The acetone powder from *ca.* 1 kg of wet porcine kidney was stirred for 2 hr in 4.2 l of cold 0.1 M phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, and centrifuged at 10000 rpm for 15 min. The supernatant was adjusted to pH 4.5 with 1 M citric acid and kept at 37° for 1.5 hr. After removal of the resulting precipitate by centrifugation, the protein in the supernatant liquid was fractionated with ammonium sulfate. The precipitate formed between 30 and 60% saturation was dissolved in a small volume of cold water and dialyzed against 0.05 M sodium citrate buffer, pH 4.5, containing 0.1 M sodium chloride. The dialyzed solution was concentrated to *ca.* 10 ml by ultrafiltration and passed through a Sephadex G-200 column (5×90 cm) using the dialysis buffer at a flow rate of 15 ml per hr. The active fractions were pooled, concentrated to *ca.* 30 ml, dialyzed against 0.01 M phosphate buffer, pH 6.0, and applied to a diethylaminoethyl (DEAE)-cellulose column (Whatman DE 52, 2.5×20 cm) previously equilibrated with the dialysis buffer. The column was eluted with 250 ml of the same buffer and the active fractions were pooled (crude hexosaminidase B fraction). The activity adsorbed on the column was eluted with a 500 ml linear gradient of 0 to 0.3 M sodium chloride in 0.01 M phosphate buffer, pH 6.0, at a flow rate of 20 ml per hr. The fractions containing activity, which were eluted between 0.1 and 0.2 M sodium chloride, were pooled (crude hexosaminidase A fraction).

Hydrophobic Chromatography of Crude Hexosaminidase A Fraction——Crude hexosaminidase A fraction (2185 units, 71 mg protein) was concentrated to *ca.* 10 ml, buffered with 0.05 M sodium citrate buffer, pH 5.0, containing 25% saturated ammonium sulfate, and applied to a column (1.8×4 cm) of octyl Sepharose CL-4B (Pharmacia). The column was washed with 200 ml of the same buffer and the enzyme adsorbed on the column

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8) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
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was eluted with 300 ml of the buffer containing increasing ethylene glycol (0—50%) and decreasing ammonium sulfate (25—0%) concentrations at a flow rate of 20 ml per hr at 4°. The eluate was dialyzed immediately against 0.05 M sodium citrate buffer, pH 4.5, using a continuous-flow dialyzer. The activity appeared at 15—50% ethylene glycol concentration.

Affinity Chromatography on N-(ε-Aminohexanoyl)-β-acetylglucosaminylamine-Sepharose 4B——Crude enzyme solutions from different purification steps were subjected to affinity chromatography on a column (1.5×20 cm) of N-(ε-aminohexanoyl)-β-acetylglucosaminylamine-Sepharose 4B (ligand content: 5 μmol per ml) prepared according to the procedure of Lotan *et al.*[13] After application of a sample dialyzed against 0.05 M sodium citrate buffer, pH 4.5, the column was washed with the same buffer and the retained enzyme was eluted with 150 ml of the same buffer but containing 10—40 mM N-acetylglucosamine. The results are summarized in Table I.

Amino Acid and Carbohydrate Contents of Purified Hexosaminidase A——Proteins were hydrolyzed at 110° for 24 hr in 6 N hydrochloric acid in evacuated sealed tubes and subjected to amino acid analysis. Cysteic acid was determined after performic acid oxidation. Carbohydrate contents were determined colorimetrically as described in the previous paper.[7] Amino acid contents (mol per 100 mol) were: aspartic acid, 10.0; threonine, 5.5; serine, 6.8; glutamic acid, 11.4; proline, 7.0; glycine, 7.4; alanine, 5.2; valine, 5.9; isoleucine, 4.2; leucine, 9.1; tyrosine, 4.8; phenylalanine, 7.9; cysteic acid, 1.3; methionine, 1.7; lysine, 4.9; histidine, 2.4; arginine, 4.5; tryptophan not determined. Carbohydrate contents: hexosamine, 3.2% (as glucosamine); hexose, 2.0% (as glucose).

Kinetic Measurements——All the experiments were carried out in the presence of 0.01% bovine serum albumin using the purified enzyme. Initial velocity, v, was determined from the linear time course, and the Michaelis-Menten parameters were calculated from a Hofstee plot by the least-squares method.

Results

Purification and Characterization of Hexosaminidase A

The purification of hexosaminidase A from porcine kidney is summarized in Table I. In this experiment, hexosaminidase B activity was only one-tenth of the hexosaminidase A activity. ε-Aminohexanoyl β-acetylglucosaminylamine-agarose chromatography was effective, even when the 30—60% ammonium sulfate precipitate obtained from the supernatant of kidney homogenate was directly applied to the column (Fig. 1). The purified enzyme (specific activity, *ca.* 160 units per mg protein) was homogeneous in polyacrylamide gel disc electrophoresis at pH 9.4 and 4.3, and also in sodium dodecyl sulfate-gel electrophoresis when applied

Table I. Purification of Porcine Kidney Hexosaminidase A

Procedure[a]	Total activity (unit)	Specific activity (units/mg)
30—60% ammonium sulfate precipitation	6627	3.4
Sephadex G-200 gel filtration	5600	16.5
DEAE-cellulose chromatography		
Crude hexosaminidase A	4086	43.0
Crude hexosaminidase B	396	4.0
Octyl Sepharose CL-4B chromatography of crude hexosaminidase A (2185 units)	1556	102.3
Affinity chromatography of hexosaminidase A (1556 units) from the step of octyl Sepharose chromatography	1221	166.0
hexosaminidases (1508 units) from the step of gel filtration	1020	155.0
30—60% ammonium sulfate precipitate (752 units)[b]	442	162.4

a) In the initial three steps, values are per 1 kg of porcine kidney.
b) Wet porcine kidney (100 g) was homogenized in 1.0 l of 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride. The 30—60% ammonium sulfate precipitate obtained from the supernatant was dialyzed against 0.05 M sodium citrate buffer, pH 4.5, and applied to the column.

13) R. Lotan, A.E.S. Gussin, H. Lis, and N. Sharon, *Biochem. Biophys. Res. Commun.*, **52**, 656 (1973).

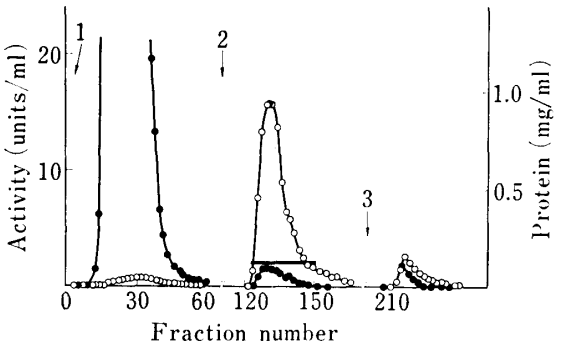

Fig. 1. Affinity Chromatography of Crude Porcine Kidney Hexosaminidase

This elution pattern was obtained in the procedure described in the last line of Table I. Stepwise elution was performed with 0.05 M sodium citrate buffer, pH 4.5 (arrow 1), the same buffer containing 10 mM N-acetylglucosamine (arrow 2) and the same buffer containing 1 M sodium chloride (arrow 3). Fractions of 2 ml each were collected at a flow rate of 30 ml per hr at 4°, and fractions under the line were pooled. ○, enzyme activity; ●, protein.

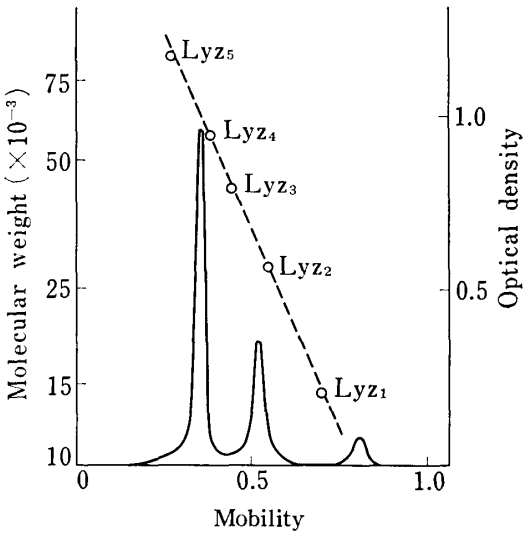

Fig. 2. Densitometric Tracing of a Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoregram

The enzyme was denatured by the standard procedure of Weber *et al.*,[12] and run on 7.5% gel. The molecular weights of subunits were determined by using lysozyme oligomers, Lyz_n, as references. The mobility was expressed relative to the migration of the tracking dye.

without preliminary denaturation (not shown). As described in "Experimental Procedures," the purified enzyme contained carbohydrate, and its amino acid composition was somewhat different from that reported for the partially purified enzyme.[6]

Molecular weight was estimated by Sephadex G-200 gel filtration at three pH's. On elution, the activity appeared in a symmetrical peak corresponding to molecular weights of 302000 at pH 5.0, 170000 at pH 6.0, and 129000 at pH 8.0. For the determination of subunit molecular weight by sodium dodecyl sulfate-gel electrophoresis, the enzyme was denatured by three different procedures involving the use of 7 M guanidine hydrochloride or a high concentration of mercaptoethanol (1 M) or dithiothreitol (50 mM). Essentially the same results were obtained by all three procedures. Three bands of protein appeared with molecular weights

Table II. Effect of Various Inhibitors on the Activity of Porcine Kidney Hexosaminidase A[a]

Inhibitor	Concentration of inhibitor (mM)	Relative[b] activity (%)	Inhibitor	Concentration of inhibitor (mM)	Relative[b] activity (%)
$CaCl_2$	10	100	$HgCl_2$	0.1	95.4
$MgCl_2$	10	97.4		1.0	1.6
$BaCl_2$	10	92.2		10	0.0
$CuCl_2$	10	91.2	$AgNO_3$	0.1	28.8
$NiCl_2$	10	96.9	EDTA	10	95.7
$CoCl_2$	10	98.0		50	92.9
$MnCl_2$	10	100	2-Mercaptoethanol	1	100
$ZnCl_2$	10	98.0		10	100
NaCl	20	99.7	Dithiothreitol	1.6	96.9
$FeSO_4$	10	36.4		10	100
$FeCl_3$	10	63.2			

a) The enzyme was treated with an inhibitor in 0.2 M sodium citrate buffer, pH 4.5, containing 0.01% bovine serum albumin at 37° for 10 min, and assayed for acetylglucosaminidase activity.
b) Relative activities are expressed as percentage of the value obtained for intact enzyme.

of 61000, 31000 and 8800, which on densitometric estimation, appeared to be present in an equimolar ratio (1: 0.98: 0.83) (Fig. 2).

The heat stability at pH 4.5 at 50°, and the pH optima (4.5 and 4.0 for *p*-nitrophenyl β-N-acetylglucosaminide and β-N-acetylgalactosaminide, respectively) were similar to those reported for the partially purified enzyme.[6] Figure 3 shows the effect of pH on the stability of the enzyme at 37° in the presence of 0.01% bovine serum albumin; the enzyme is fairly stable in the pH range of 3.9—7.7. The effect of various metal ions, ethylenediaminetetraacetic acid (EDTA) and thiols on the enzyme activity are listed in Table II. The effects of mercury and silver ions were not as marked as those reported for the human kidney enzyme, as the experiment was carried out in the presence of bovine serum albumin. Interestingly, ferrous ions were fairly inhibitory. The Michaelis–Menten parameters of the enzyme are listed in Table III for some substrates.

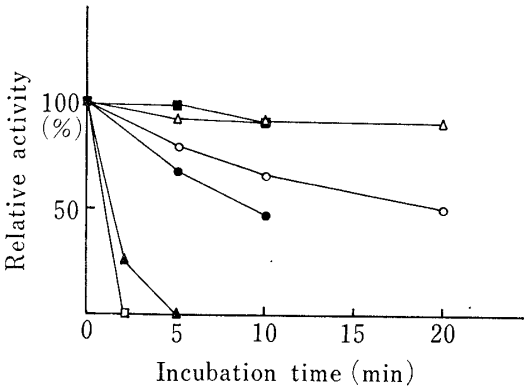

Fig. 3. Effect of pH on the Stability of β-Acetylglucosaminidase Activity at 37°

Enzyme solutions were kept at various pH's (pH 2.1—7.7, 0.05 M phosphate citrate buffer; pH 7.7—9.6, 0.05 M borate buffer). The solution was adjusted to pH 4.2 before estimation of the remaining activity. Relative activities are expressed as percentages of the value obtained for intact enzyme. □, pH 3.0; ○, pH 3.5; △, pH 3.9; ■, pH 7.7; ●, pH 8.9; ▲, pH 9.6.

Table III. Michaelis–Menten Parameters for Some Substrates

Substrate	K_m (mM)	V_{max} (μmol/min/mg protein)
p-Nitrophenyl β-N-acetyl-D-glucosaminide	1.71	342.9
Phenyl β-N-acetyl-D-glucosaminide	4.54	260.6
p-Methoxyphenyl β-N-acetyl-D-glucosaminide	3.62	294.9
p-Nitrophenyl β-N-acetyl-D-galactosaminide	0.20	28.9
Di-N-acetylchitobiose	47.5	6.2

Kinetic Studies

Mixed substrates analysis[14a] was carried out using *p*-nitrophenyl β-N-acetylglucosaminide and *p*-nitrophenyl β-N-acetylgalactosaminide as mixed substrates in four different ratios. The Michaelis–Menten parameters were determined with respect to the sum of the concentrations of the two substrates by following the liberation of the common reaction product, *p*-nitrophenol. As shown in Table IV, the observed values are consistent with the values calculated from the parameters determined with a single substrate, assuming that the two substrates compete for a common active site on the enzyme.

Table V shows the inhibitory effects of substrate analogs on the hydrolysis rates of the *p*-nitrophenyl glycosides. K_i values for acetylglucosaminidase activity were very similar to those for acetylgalactosaminidase activity, supporting a common active site responsible for the two activities. Acetamido compounds were effective competitive inhibitors with the exception of N-acetylmannosamine, though its parent sugar, mannosamine, was more inhibitory. The K_i values for mannose, methyl α-mannoside and mannosamine were found to be similar to those reported by Reglero for the porcine placenta and bovine epididymis enzymes.[4]

14) *a*) I.H. Segel, "Enzyme Kinetics," A Wiley-Interscience Publication, 1975, p. 113; *b*) *Ibid.*, p. 481.

Mannose was the only hexose that had a K_i smaller than 100 mM. The porcine kidney enzyme did not show any hydrolytic activity for methyl α-mannoside and *p*-nitrophenyl α- and β-mannosides. Interestingly, the K_i values of the mannosides given in Table V were quite different from the values expected on the basis of the variation of K_i (or K_m) found upon C-1 sustitution of N-acetylglucosamine. *p*-Nitrophenyl β-mannoside has a K_i greater than that of mannose while *p*-nitrophenyl β-N-acetylglucosaminide has a K_m smaller than the K_i of the parent sugar. Methyl α-mannoside has a K_i smaller than that of mannose, while the K_i for methyl α-N-acetylglucosaminide is far larger. These findings suggested the possibility that the enzyme has two different binding sites, one specific for acetylhexosamine and the other for mannose. This is implicitly supported by the observation by Mega *et al.* that most of the binding affinity of N-acetylglucosaminide as a substrate is attributable to its acetamido group.[3]

Table IV. Kinetic Studies with Mixed Substrates

Substrate ratio[a]	Calculated[c]		Observed	
	V_{max}(μmol/min)[b]	K_m (mM)[b]	V_{max}(μmol/min)[b]	K_m (mM)[b]
0 : 1	—	—	0.0149	0.21
1 : 5	0.0173	0.25	0.0181	0.25
1 : 2	0.0209	0.30	0.0211	0.28
1 : 1	0.0264	0.38	0.0270	0.37
2 : 1	0.0359	0.52	0.0335	0.44
1 : 0	—	—	0.1349	1.98

a) [*p*-nitrophenyl β-N-acetylglucosaminide]/[*p*-nitrophenyl β-N-acetylgalactosaminide].
b) Parameters with respect to the sum of the concentration of the two substrates.
c) Calculated from the equation, $v=([A]V_a/K_a+[B]V_b/K_b)/(1+[A]/K_a+[B]/K_b)$, where K_a and K_b are K_m values and V_a and V_b are maximum velocities for the single substrates A and B, respectively (refs. 2 and 14*a*).

Table V. Inhibition of Porcine Kidney Hexosaminidase A by Substrate Analogs

Compound	K_a[a] (mM)	K_b[a] (mM)
N-Acetyl-D-glucosamine	6.2	6.8
N-Acetyl-D-glucosaminide		
Methyl α-	124	(170)
Methyl β-	8.7	8.3
β-Acetylglucosaminylamine		
N-Acetyl	8.3	—
N-Acetamidohexanoyl	15.0	—
N-Acetyl-D-glucosaminol	55.5	—
N-Acetyl-D-galactosamine	0.50	0.50
N-Acetyl-D-mannosamine	32.5	—
D-Mannosamine	2.0	—
D-Mannose	21.0	20.7
D-Mannopyranoside		
Methyl α-	16.9	17.6
p-Nitrophenyl α-	(73)	—
p-Nitrophenyl β-	(101)	—
D-Glucose	287	(426)
L-Glucose	(1282)	(1279)
D-Galactose	157	(189)
Sucrose	408	401
Acetamide	7.2	6.8

a) K_a and K_b are inhibition constants for β-acetylglucosaminidase and β-acetylgalactosaminidase activity, respectively. The values in parentheses were roughly calculated from the relative velocity assuming competitive inhibition. The other values were obtained from Hofstee plots, which indicated completely competitive inhibition.

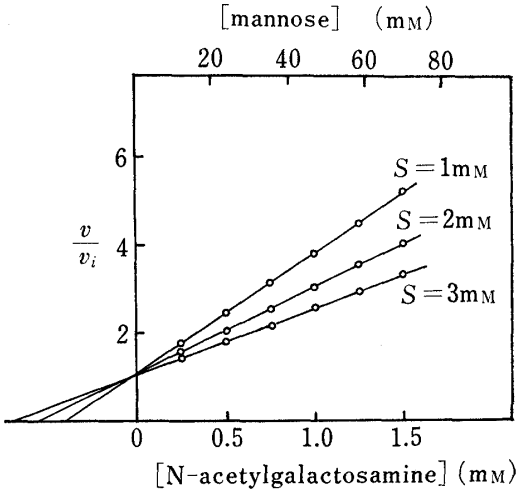

Fig. 4. Yagi–Ozawa Plots for the Inhibition of β-Acetylglucosaminidase Activity

The inhibitory effect of mixed inhibitors, mannose and N-acetylgalactosamine, was determined in 0.05 M citrate buffer, pH 4.5, using *p*-nitrophenyl β-N-acetylglucosaminide as a substrate. v_i, ": initial velocity in the presence or absence of inhibitor.

The above-mentioned possibility was examined by using the Yagi–Ozawa plot,[14b] in which the reciprocal of relative velocity, v/v_i is plotted against the simultaneously increasing concentrations of two inhibitors, I and X. The plot is based on the following equation,

$$v/v_i = (1-v/V_{max})([I]/K_i+[X]/K_x+[I][X]/\alpha K_i K_x) + 1$$

where K_i and K_x are the inhibitory constant of I and X, respectively, and α is a factor representing the effect of one binding inhibitor on the binding ability of the other inhibitor, α being unity in the absence of any effect. If the enzyme has different binding sites specific for each inhibitor and α is not much larger than unity, a parabolic curve is obtained. Figure 4 shows the Yagi–Ozawa plot for the inhibition by N-acetylgalactosamine and mannose as mixed inhibitors. The plot is linear, indicating that the term, $[I][X]/K_iK_x$, is insignificant as compared with the other terms. This means that no XEI complex is formed. Similar results were obtained in experiments using a mixture of N-acetylglucosamine and mannose and a mixture of acetamide and mannose.

Discussion

In the present study, affinity chromatography on ε-aminohexanoyl β-acetylglucosaminylamine-Sepharose 4B was successfully used for the purification of porcine kidney hexosaminidase A, although the K_i values of the ligand and its derivatives and the ligand content of the adsorbent alone cannot account theoretically for the effective retention of the enzyme on the column.

It has previously been reported that, of the hexosaminidases A and B from bovine liver, only hexosaminidase A exhibited pH-dependent dissociation-association.[7] In the present study, porcine kidney hexosaminidase A also showed a similar phenomenon. At present, consideration of the molecular weight of the intact enzyme determined by gel filtration and those of the three subunit chains determined by sodium dodecyl sulfate-gel electrophoresis does not lead to any clear-cut conclusion concerning subunit structure.

Studies with mixed substrates and comparison of the K_i values of various inhibitors for acetylglucosaminidase activity with those for acetylgalactosaminidase activity indicated that a single site is responsible for the two activities. Pope *et al.*[2] have presented kinetic data supporting the presence of two different active sites using a commercial hexosaminidase preparation from bovine kidney. In a separate experiment, the kinetics with mixed substrates were studied here, using the bovine kidney enzyme partially purified by ammonium sulfate precipitation and gel filtration; the results supported the common active site mechanism. Further experimental work on the purification procedures, enzymatic properties and kinetic behaviour of this enzyme is necessary, however.

Consideration of the relation between chemical structure and binding ability of mannosides and N-acetylglucosaminides suggested that the enzyme might possess separate binding sites for N-acetylhexosamine (active site) and for mannose, even though mannose is a competitive inhibitor. It is possible that mannose binds at an aglycone site, excluding substrates having a bulky aglycone, such as *p*-nitrophenyl, from the active site by steric hindrance. However, kinetic analysis with mixed inhibitors did not favor this possibility; mannose was competitive even with the small molecule of acetamide, which probably binds to the active site,[3] as mentioned above. This suggests that mannose binds to the active site itself, since it seems unlikely that mannose could induce a marked conformational change of the active site sufficient to exclude acetamide by binding to a separate site.