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Purification and Some Properties of Bovine Liver β-Acetylhexosaminidase

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Three affinity adsorbents, *p*-aminophenyl β-acetylglucosaminide, *p*-aminophenyl β-1-thio-acetylglucosaminide and β-acetylglucosaminylamine, each bound to CH-Sepharose 4B (GO, GS and GN, respectively), were examined for the purification of bovine liver β-acetylhexosaminidases. In preliminary experiments with crude enzyme, columns of GO and GS gave three fractions when eluted successively with 0.05 M citrate buffer, pH 5.0, 0.1 M borate buffer, pH 10, containing 1 M sodium chloride, and 5 M urea.

Purification procedures involve ammonium sulfate precipitation, treatment at pH 3.8 at 37°, Sephadex G-200 gel filtration, DEAE-cellulose column chromatography and affinity chromatography on GS.

A hexosaminidase A was obtained as a electrophoretically pure protein with high specific activity, 137 units per mg. Activity in hexosaminidase B fraction showed multiplicity in its behavior in the affinity chromatography, and the high specific activity (184 units per mg) was obtained only with a GO column.

*K*m values and ratios of acetylglucosaminidase to acetylgalactosaminidase activities were determined for main components. The molecular weights of the hexosaminidase A and B were estimated to be 280000 and 320000, respectively, as determined by gel filtration using the partially purified enzymes.

Keywords——affinity chromatography; CH-Sepharose 4B; β-acetylhexosaminidase; bovine liver; isozyme

β-Acetylhexosaminidase (β-acetylglucosaminidase, 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1. 30) is widely distributed among living organisms and is an exo-enzyme which catalyzes the hydrolysis of β-acetylhexosaminidic linkages. This enzyme activity of mammalian sources has been attributed to several isozymes,[2] among which hexosaminidase A and hexosaminidase B have been extensively studied in relation to the heritable gangliosidoses. The former is an acidic or anodic isozyme moving faster in electrophoresis than the latter. Hexosaminidase A and B from human placenta[2a] and kidney[2b] are the enzyme of homogeneous protein with high specific activity. In the course of our work on the nature of the hydrolysis catalyzed by bovine liver β-acetylhexosaminidase, a pure enzyme preparation was required.

Weissmann[3] had reported the 4400-fold purification of the enzyme from the same source without any aid of column chromatography. Later, Langley[4] separated the enzyme into two fractions by DEAE-cellulose column chromatography following Sephadex G-200 gel filtration, although the specific activity of the separated enzymes was low. Many studies[5]

1) Location: *542, Miyama-cho, Funabashi-shi, Chiba.*
2) *a*) S.K. Srivastava, Y.C. Awasthi, A. Yoshida, and E. Beutler, *J. Biol. Chem.*, **249**, 2043 (1974); S.K. Srivastava, A. Yoshida, Y.C. Awasthi, and E. Beutler, *ibid.*, **249**, 2049 (1974); *b*) S.K. Srivastava, J.E. Wiktorowicz, and Y.C. Awasthi, *Proc. Nat. Acad. Sci. U.S.A.*, **73**, 2833 (1976); *c*) Y. Seyama and T. Yamakawa, *J. Biochem.* (Tokyo), **75**, 495, 947 (1974); *d*) B. Overdijk, W.M.J. van der Kroef, W.A. Veltkamp, and G.J.M. Hooghwinkel, *Biochem. J.*, **151**, 257 (1975); *e*) G.T.N. Besley and D.M. Broadhead, *Biochem. J.*, **155**, 205 (1976).
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have recently been reported on the affinity chromatography of β-acetylhexosaminidase on substrate or its analogues bound to Sepharose 4B, which is suggested to be more effective when introduced in the conventional purification procedures. The present report describes purification of the bovine liver enzyme using affinity column chromatography and some properties of the enzymes separated.

Materials and Methods

Affinity Adsorbents——(a) *p*-Aminophenyl β-Acetylglucosaminide substituted CH-Sepharose 4B (GO) and the Corresponding 1-Thio-glycoside substituted CH-Sepharose 4B (GS): The *p*-aminophenyl glycosides used as affinity ligands were prepared by the procedures essentially the same as described by Kiyohara *et al.*[5f] CH-Sepharose 4B (3 g) washed with 0.5 M sodium chloride (600 ml) was suspended in water (20 ml), to which freshly prepared *p*-aminophenyl glycoside (2 mmol) and N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 600 mg) were added. The mixture was maintained at pH 5.0 at room temperature for 20 hr with continuous shaking. The gel was washed with water, and the unreacted spacer groups were blocked by shaking with 2-aminoethanol (60 μl) in the presence of EDC (200 mg) at pH 4.5 at 4° for 20 hr. The gel was washed successively with water, 2 M sodium chloride in 0.01 M sodium bicarbonate (pH 8.8, 200 ml) and water, and used for affinity chromatography.

(b) β-Acetylglucosaminylamine substituted CH-Sepharose 4B (GN): CH-Sepharose 4B (2 g) was washed successively with 0.5 M sodium chloride (400 ml) and dioxane (100 ml), and was suspended in dioxane (8 ml). To the suspension was added 3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosylamine[6] (150 mg). After gentle shaking for 6 hr at room temperature, the gel was filtered off and washed with dioxane (100 ml) and methanol (100 ml), and was resuspended in methanol–dioxane (2: 1, 10 ml). After adding 0.01 M sodium methoxide in methanol (1 ml) to the suspension, deacetylation was carried out with shaking at room temperature for 2 hr. The gel was filtered and washed with methanol (200 ml), dioxane (200 ml) and water (300 ml) successively. Blocking the spacer groups and washing were carried out as in GO adsorbent.

The adsorbents were analyzed for glucosamine by the method of Rondle and Morgan[7] after hydrolysis in 2 N hydrochloric acid at 100° for 10 hr. Glucosamine contents (μmol per ml of gel): GS, 4.6; GO, 7.4; GN, 2.0.

Preparation of Crude Enzyme, HG 200——According to Weissmann's specifications,[3] the extract of acetone powder was treated at pH 3.8 at 37°, and fractionated twice with ammonium sulfate. The "second" ammonium sulfate precipitate which precipitated between 25 and 50% saturation was dissolved in 0.05 M sodium citrate buffer, pH 5.0, and dialyzed against the same buffer at 4° for 24 hr. Such an enzyme solution from 0.5 kg of liver, which contained 1800—1900 units of the enzyme, was concentrated to about 8 ml by ultrafiltration using an Amicon ultrafiltration apparatus and was applied to a column (2.5×95 cm) of Sephadex G-200 previously equilibrated with 0.05 M sodium citrate buffer, pH 5.0, and the column was eluted with the same buffer. The activity was eluted shortly after the void volume, and the active fractions were pooled, designated as HG 200 and stored at 4°.

Enzyme Assay——A mixture (0.4 ml) containing 5 mM *p*-nitrophenyl β-acetylglucosaminide, 0.05 M sodium citrate buffer, pH 4.2, 0.01% bovine serum albumin and enzyme was incubated at 37° for 5—20 min, and analyzed by the method of Levvy and Conchie.[8] The units of enzyme activity refer to micromoles of *p*-nitrophenol liberated per min at 37° from 5 mM *p*-nitrophenyl β-acetylglucosaminide.

Protein——Protein was determined by the method of Lowry *et al.*[9] with bovine serum albumin as a standard.

Polyacrylamide-gel Electrophoresis——Electrophoresis was carried out in 7.6 or 5.3% gel at pH 9.2 by using the discontinuous buffer system of Davis.[10] Gels were stained for protein with Amido Black. The enzyme activity was detected by incubation with 4-methylumbelliferyl-β-acetylglucosaminide,[11] after the gels were bufferized with citrate buffer, pH 4.2, for 10 min.

Results

The "second" ammonium sulfate precipitate prepared from bovine liver acetone powder according to Weissmann *et al.*[3] showed specific activity of 1.6—4.3 units per mg, which was

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Table I. Purification of Bovine Liver β-Acetylhexosaminidase

Procedure[a)]	Fraction or component	Activity[b)] (units)	Yield (%) For each step	Yield (%) Based on acetone powder	Specific[b)] activity (units/mg)
"Second ammonium sulfate precipitation"		3917	—	77.3	3.3
Sephadex G-200 chromatography	HG 200	3740	95.5	73.8	12.7
DEAE-Cellulose chromatography	Fraction A	1805	48.3	35.6	81.2
	Fraction B	2046	54.7	41.4	10.2
GS column chromatography of fraction A (461 units)	Component A_2	284	61.6	24.9	137
	Component A_3	6.6	1.4	0.6	56.5
fraction B (480 units)	Component B_1	100.1	20.9	7.4	5.1
	Component B_2	150.3	31.3	11.1	18.1
	Component B_3	6.3	1.3	0.5	74.1
GO column chromatography of fraction B(240 units)	Component B_1'	14.4	6.0	—	27.5
	Component B_2'	164.6	68.6	—	184

a) In the initial three steps, values are per 1 kg of bovine liver.
b) As assays were carried out at a final concentration of 5 mM *p*-nitrophenyl β-acetylglucosaminide, the activities are expressed slightly higher than those determined at 2 mM substrate concentrations in prevailing assay conditions.

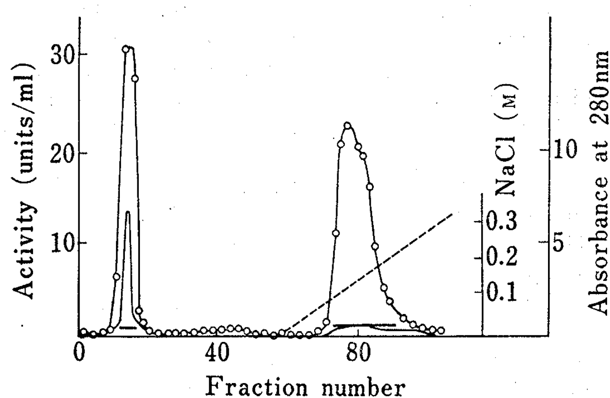

Fig. 1. DEAE-cellulose Column Chromatography of Crude Bovine Liver β-Acetylhexosaminidase

The crude enzyme solution, HG 200, 3740 units, were concentrated to 30 ml, bufferized with 0.01 M phosphate buffer, pH 6.0, and applied to a DEAE-cellulose ("Serva") column (2.5×20 cm) equilibrated with the same buffer. The column was eluted with the same buffer and subsequently with a linear gradient of sodium chloride in the buffer. Ten milliliters fractions were collectde at a flow rate of 20 ml per hr. All the operations were carried out at 4°. Fractions under the line were pooled.

—○—: enzyme activity; ——: absorbance at 280 nm; --------: concentration of sodium chloride.

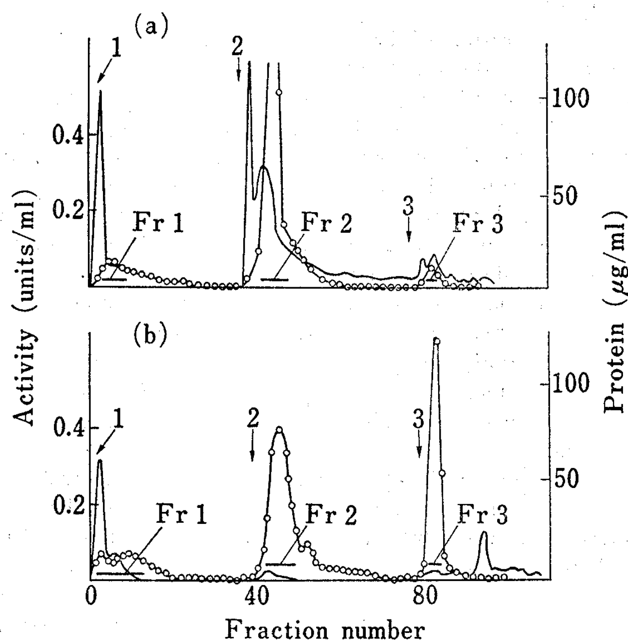

Fig. 2. Affinity Chromatography of Crude Enzyme, HG 200

The crude enzyme solution, HG 200, 0.3 ml, containing 45 units of enzyme (2.3 mg of protein) was applied to each column (5×1.1 cm): (a) GO and (b) GS column. Stepwise elution was performed successively with 0.05 M sodium citrate buffer, pH 5.0 (arrow 1), 0.1 M borate buffer, pH 10.0, containing 1 M sodium chloride (arrow 2) and 5 M urea solution (arrow 3), and the eluates were bufferized immediately with 0.05 M sodium citrate buffer, pH 4.2, using a continuous-flow dialyzer. Five milliliter fractions were collected at a flow rate of 20 ml per hr at 4°.

—○—: enzyme activity; ——: protein.

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much lower than the value, 10.6, reported by Weissmann, *et al.* Table I shows a typical result of further purification by using columns. On Sephadex G-200 chromatography, the specific activity rose to 12—18 units per mg (fraction HG 200). This fraction was used for preliminary experiments on affinity chromatography. For the purpose of further purification, fraction HG 200 was applied to a DEAE-cellulose column (Fig. 1). The enzyme fraction "hexosaminidase B" (fraction B) was eluted with 0.01 M phosphate buffer, pH 6.0, and the enzyme fraction "hexosaminidase A" (fraction A) with sodium chloride gradient. Each fraction was then purified separately by affinity chromatography.

Affinity Chromatography

In the preliminary experiments with fraction HG 200, a small amount of enzyme (45 units) in 0.05 M citrate buffer, pH 5.0, was applied to each column (1.1×5 cm). As shown in Fig. 2, a portion of the activity bound strongly to the *p*-aminophenyl glycosides bound to CH-Sepharose 4B (GO and GS) and was recovered to a considerable extent only by elution with 0.1 M borate buffer, pH 10, containing 1 M sodium chloride and with 5 M urea. With the column of the glycosylamine substituted (GN) and the ethanolamine-blocked CH-Sepharose 4B, practically all of the activity was eluted with 0.05 M citrate buffer, pH 5.0 (figure not presented). Stepwise elution of the GO and the GS column resulted in the separation of fractions 1, 2 and 3 in the order of elution (Fr 1, 2 and 3 in Fig. 2). With the GS column, the proportions of the activities in the three fractions were comparable to each other, whereas, with the GO column, the activity in fraction 2 was predominant compensating the decrease of activity in fraction 3. The recovery of activity from the GS and GO columns was 53 and 78%, respectively.

A GS column was chosen for the purpose of purification because of its presumed resistance to the enzymatic attack. As shown in Fig. 3a, substantially all of the activity in fraction A was eluted with the borate buffer, pH 10. The enzyme in the pooled fractions, component A_2, was found to have high specific activity of 137 units per mg.

When fraction B was subjected to GS column chromatography, the activity was separated mainly into two components, B_1 and B_2 (Fig. 3b), where specific activities remained at low values. On the other hand, with a GO column (figure not presented), high specific activity was seen in the fractions eluted with the borate buffer, pH 10 (component $B_{2'}$ in Table I). The different ratios of activity in each component, B_1 to B_2 in GS and $B_{1'}$ to $B_{2'}$ in GO column chromatography, suggest the presence of more than two forms of enzyme in fraction B.

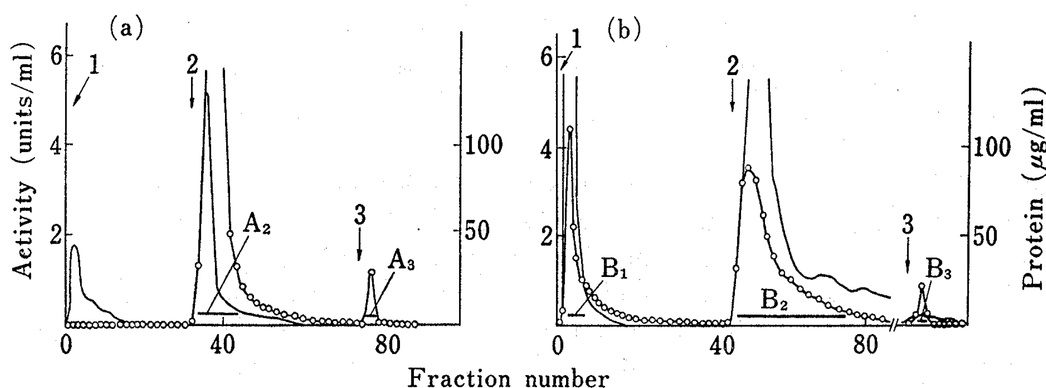

Fig. 3. Affinity Column Chromatography of Fraction A and B on a GS Column

(a) Fraction A, 461 units, and (b) fraction B, 484 units, were applied to GS columns (5×1.1 cm). The operations were carried out as in Fig. 2, and fractions under the line were pooled.
—○—: enzyme activity: ——: protein.

Properties of the Enzymes

All the components hydrolyzed both *p*-nitrophenyl β-acetylglucosaminide and β-acetylgalactosaminide. As shown in Table II, the ratios of β-acetylglucosaminidase to β-acetyl-

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galactosaminidase activities were *ca.* 6 for components B_1 and B_2 in agreement with the values for the enzymes from beef spleen, 5.6,[12] and bull epididymis, 6.5,[5e], while a smaller ratio, 2.4, was obtained for component A_3. The main components, A_2, B_1 and B_2 showed similar pH-activity curves with pH optima at pH 3.9—4.2 for the glucosaminide and at pH 3.5—3.7 for the galactosaminide (Fig. 4).

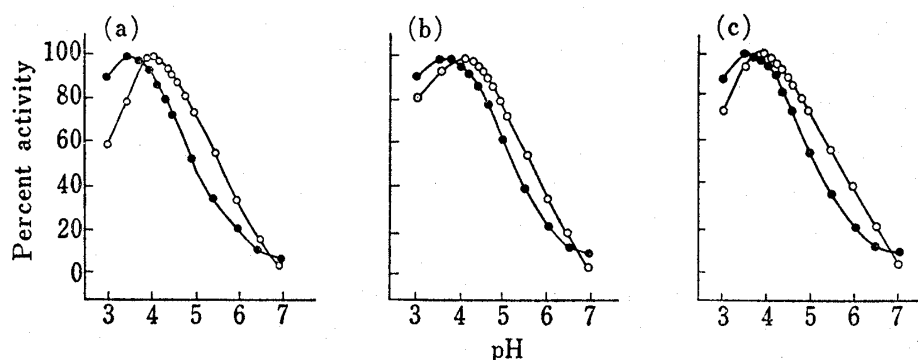

Fig. 4. The Effect of pH on the Activity of Bovine Liver β-Acetylhexosaminidases

Enzymes were incubated with 5 mM *p*-nitrophenyl β-acetylglucosaminide (final concentration) or 2 mM *p*-nitrophenyl β-acetylgalactosaminide in 0.1 M sodium phosphate-citric acid buffers at 37° in the presence of 0.01% bovine serum albumin.
(a) component A_2, (b) component B_1 and (c) component B_2
—○—: β-acetylglucosaminidase activity; —●—: β-acetylgalactosaminidase activity.

Table II. Some Properties of Bovine Liver β-Acetylhexosaminidases

Component	Ratio of β-acetylglucosaminidase to β-acetylgalactosaminidase activity[a]	*Km* (mM)[a] mean ±S.D.	Residual activity[b] after heat treatment for 2 hr at 50° (%)	56° (%)
A_2	4.9	1.4 ±0.09[c]	92	89.6
A_3	2.4	—	—	—
B_1	5.9	1.0 ±0.10[d]	94.3	62.5
B_2	5.7	0.96±0.089[d]	88.1	69.6
B_3	6.0	—	—	—

a) Determined at pH 4.2.
b) At pH 4.5 in the presence of 0.01% bovine serum albumin.
c) Based on six runs.
d) Based on three runs.

Heat stability of the main components at pH 4.5 was studied at 50° and 56° in the presence of bovine serum albumin: in the absence of albumin, the dilute enzyme solutions showed serious fluctuation in the inactivation process without any reproducible results. Table II shows that component A_2 is more stable than components B_1 and B_2 at 56°, contrary to the general recognition[13] that the anodic or acidic form is more labile than component B.

The crude enzymes were stored at 4° without any appreciable loss of activity at least for a month and were not affected by freeze and thawing. Components A_2, B_1 and B_2 were also stable at pH 4.2 at 4° but lost their activity by about 15% on one process of freeze and thawing. Addition of 3.2 M ammonium sulfate protected the enzymes from inactivation caused by freeze and thawing.

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Polyacrylamide gel disc electrophoresis of component A_2 revealed a single protein band moving faster than B_1 and B_2 and containing the enzyme activity when stained with 4-methylumbelliferyl β-acetylglucosaminide. Component B_1 and B_2 moved slowly and could not be distinguished.

The molecular weight of the enzymes in fraction A and B was estimated to be approximately 280000 and 320000, respectively, by Sephadex G-200 gel filtration using 0.05 M citrate buffer, pH 5.0 (Fig. 5). Component B showed a slight shoulder in front of the activity peak, indicating the inclusion of enzyme having a larger molecular weight. Similar values, 280000 and 340000 were obtained for the corresponding enzyme fractions prepared according to Langley's method, which is devoid of procedures of acetone powder preparation and heat treatment at pH 3.8 at 37°.

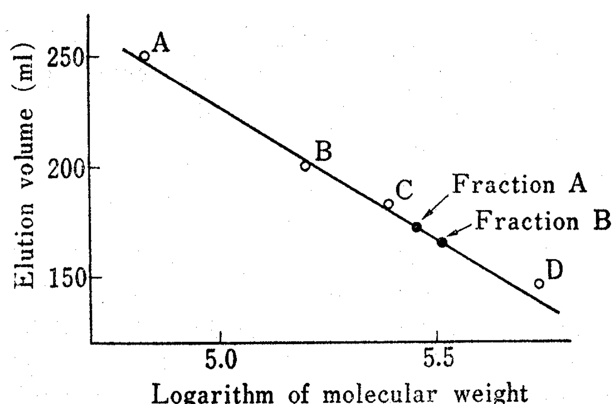

Fig. 5. Estimation of the Molecular Weights of Bovine Liver β-Acetylhexosaminidases by Gel Filtration on Sephadex G-200

The estimation was carried out according to Andrews.[14] A column (2.5×78 cm) was eluted with 0.05 M sodium citrate buffer, pH 5.0, at 4° with a flow rate of 10 ml per hr. Elution of the enzymes in fraction A and B were followed by estimating the enzyme activity. Standard proteins were, A, bovine serum albumin (67000), B, aldolase (158000) and C, catalase (240000). The point of ferritin (540000), D, was omitted from the calibration (refer to ref. 14).

Discussion

Experiments showed that the GS and the GO column chromatography serve as a means of purifying the enzymes. β-Acetylglucosaminylamine linked to Sepharose has been reported to be effective for the purification of human placenta hexosaminidase A and B.[5c] In the present study, however, a similar adsorbent, GN, seemed not to possess the affinity for the enzyme under the condition used, although the low ligand content in GN should be taken into consideration. Component A_2 which has a greater *K*m value (1.4 mM) bound more strongly to the adsorbent GS than the component B_1 (*K*m: 1.0 mM) against expectation. This may be relevant to the following indication by Mega, *et al.*[5d] concerning the affinity chromatography of Taka β-acetylglucosaminidase: "Interaction between the gels and the glycosidases does not involve the active site of the enzyme." It was inexplicable that a considerable amount of activity appeared in 5 M urea eluate in GS column chromatography of crude enzyme (HG 200) but after DEAE–cellulose chromatography this activity almost disappeared (compare Fig. 3 with Fig. 2).

Component A_2 was obtained as an electrophoretically pure protein. This enzyme, as well as the other hexosaminidases A, moves faster in electrophoresis, but differs from the enzymes of human sources[13] in stability at 50° and rather resembles to the enzyme A in porcine kidney,[15] which retained 85% of its original activity at 50° for 1 hr. On the other hand, the enzyme in fraction B seemed to comprize several isozymes, as suggested by the different elution profiles shown when the fraction was applied to a GS and a GO column. The multiplicity of component B was reported for porcine kidney.[15]

In the present study, the *K*m value for component A_2 was found to be 1.4 mM using *p*-nitrophenyl β-acetylglucosaminide as a substrate. This value differs significantly from

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the value, 0.9 mM, described in our previous report[16] dealing with a partially purified acidic component prepared by Langley's method. Examination of the preparation by Langley's method from the same material as used here gave the value of 1.4 mM. A *K*m value of 1.02 mM for a purified bovine liver enzyme was reported by Weissmann,[3] and 1.4 mM both for the acidic and basic form by Langley.[4] Thus, the discrepancy in *K*m values may arise from the individual differences.

The molecular weights of the enzymes in fraction A and B were found to be *ca.* 280000 and 320000, respectively, where the latter value is an average molecular weight as the enzyme in fraction B was shown to be heterogeneous. These values are larger than the values reported for visceral enzymes, 140000 for both enzyme A and B in beef spleen,[12] 125000 for enzyme A and B, and 250000 for enzyme A' in equine kidney.[2c] However, the present values are smaller when compared with those calculated for the isozymes in human kidney and liver according to the "common and unique subunit theory"[2b] that the isozymes are hetero or homohexomers comprizing the α subunit of 47000 daltons and/or β subunit of 120000 daltons, thereby hexosaminidase A and B being expressed as $(\alpha\beta)_3$ and β_6, respectively.

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