

$\text{NiL}_2(\text{DMSO})_2$ was isolated, but NiL_2 decomposed in pyridine into non-metal-containing disulfide (I)⁵ instead of NiL_2py_2 . Ligand field stability of NiL_2 is lower than those of PtL_2 and PdL_2 . Therefore, it may likely occur that pyridine molecules coordinate with Ni^{2+} from the axial directions, which dissociate NiL_2 . On the contrary, DMSO which is a weaker base than pyridine does not dissociate NiL_2 and gives $\text{NiL}_2(\text{DMSO})_2$.

PtL_2 and PdL_2 yielded both pyridine- and DMSO-clathrate. On the other hand, CoL_2 and ZnL_2 formed neither clathrate.

These differences are supposed to depend on the stability of the complexes against bases. Therefore, the stability of the metal complexes, ML_2 , seems to be $\text{PtL}_2, \text{PdL}_2 > \text{NiL}_2 > \text{CoL}_2, \text{ZnL}_2$.

Experimental

DMSO clathrates were synthesized by recrystallization of their original complexes from DMSO. Infrared measurements were carried out as potassium bromide disks with a Hitachi Infrared Spectrophotometer Model EPI-S2. Magnetic susceptibility measurements were done by the Gouy method at room temperature ($20 \pm 1^\circ$).

5) S. Kitagawa and H. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **26**, 1629 (1978).

[*Chem. Pharm. Bull.*
26(10)3225-3228(1978)]

UDC 577.152.07 : 543.544.2.061

Bovine Liver β -Acetylhexosaminidase. Purification by Hydrophobic Affinity Chromatography and Heterogeneity

MITSUYA TANAKA, SHIGEHISA KYOSAKA, and SANAE MURATA

Faculty of Pharmaceutical Sciences, Toho University¹⁾

(Received April 26, 1978)

Bovine liver β -acetylhexosaminidase A (Hex A) and B (Hex B) were purified by hydrophobic affinity chromatography. Octyl Sepharose CL-4B was more effective than Phenyl Sepharose CL-4B as an adsorbent. Both the crude and the purified preparations of Hex A and Hex B exhibited extensive heterogeneity when focused on a polyacrylamide gel plate with pH gradient; Hex A gave at least ten bands with pI's ranging from 5.0 to 7.0, and Hex B at least fourteen bands with pI's ranging from 6.5 to 8.5.

Stepwise elution with increasing pH from a CM-cellulose column resulted in rough separation of Hex A and Hex B into overlapping classes.

Keywords— β -acetylhexosaminidase; bovine liver; hydrophobic affinity chromatography; isoelectric focusing; polymorphism of enzyme; isozyme

β -Acetylhexosaminidase from bovine liver consists of two main components, acetylhexosaminidase A and B (Hex A and Hex B),²⁾ like many other mammalian visceral acetylhexosaminidases. Previously, the bovine liver enzymes were purified by affinity chromatography using CH-Sepharose 4B substituted with *p*-aminophenyl β -acetylglucosaminide and the corresponding 1-thio-glycoside as affinity adsorbent, in which multiplicity of Hex B was suggested on the basis of its behaviour in affinity chromatography.^{2b)}

1) Location: 2-2-1, Miyama, Funabashi-shi, Chiba, 274, Japan.

2) a) T.J. Langley and F.R. Jevons, *Arch. Biochem. Biophys.*, **128**, 312 (1968); b) M. Tanaka, S. Kyosaka, and S. Murata, *Chem. Pharm. Bull.* (Tokyo), **26**, 1188 (1978).

To avoid the laborious preparation of the adsorbents hydrophobic affinity chromatography using commercial adsorbents, Phenyl and Octyl Sepharose CL-4B, has been examined in our laboratory. The present paper describes the effectiveness of these adsorbents, especially of Octyl Sepharose CL-4B, and also extensive heterogeneity of the enzymes exhibited by isoelectric focusing on a polyacrylamide gel plate. In addition, an experiment intended to separate the heterogeneous enzyme proteins with a CM-cellulose column is described.

Materials and Methods

Chemicals—Phenyl and Octyl Sepharose CL-4B were obtained from Pharmacia Fine Chemicals, CM-cellulose from Serva, Ampholine PAG plates from LKB Production, and Fast Garnet GBC salt and Coomassie Brilliant Blue R from Sigma. *p*-Nitrophenyl and α -naphthyl β -acetylglucosaminides were prepared by the method of Leaback.³⁾

Enzyme—A 25–50% saturated ammonium sulfate precipitate prepared according to Weissmann's specification⁴⁾ was further purified by the procedures of Langley,^{2a)} as described in the previous paper,^{2b)} using Sephadex G-200 and DEAE-cellulose columns. "Fractions A and B" thus obtained were used, designated as crude Hex A and B. The enzyme was assayed by incubating with *p*-nitrophenyl β -acetylglucosaminide as previously reported.^{2b)} One unit of enzyme activity releases 1 μ mol *p*-nitrophenol per min at 37°.

Protein Determination—Protein was determined by the method of Lowry *et al.*⁵⁾ with bovine serum albumin as a standard.

Isoelectric Focusing—Isoelectric focusing was performed using LKB Ampholine PAG plates (pH 3.5–9.5). Focusing, staining and destaining for protein, and preserving were done under the conditions described in Ampholine PAG Plate Instruction Manual. Enzyme was stained by incubation for 5–15 min at 35–40° in a solution of 0.05 M sodium citrate buffer, pH 4.2 (*ca.* 10 ml) containing 1–2 ml of saturated solution of α -naphthyl β -acetylglucosaminide in water, 1–2 mg of Fast Garnet GBC salt and 0.01% bovine serum albumin, although staining with naphthyl AS-BI- β -acetylglucosaminide has been well established.⁶⁾

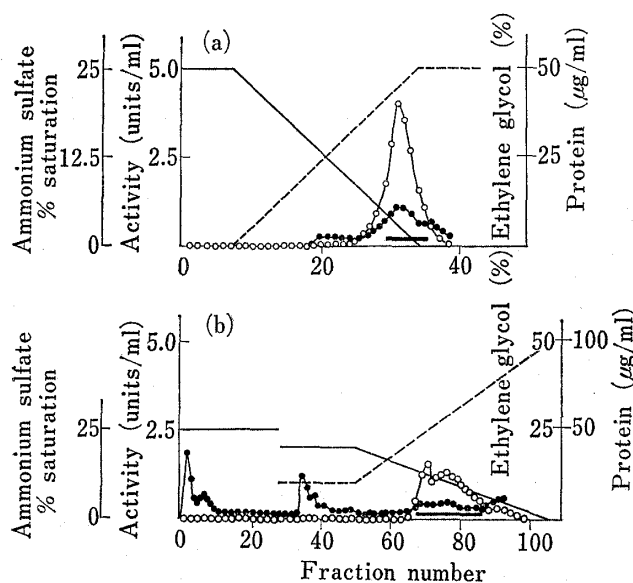


Fig. 1. Elution Patterns of Hex A and Hex B from an Octyl Sepharose CL-4B Column

The crude enzyme in 1 ml of 0.05 M sodium citrate buffer, pH 5.0, containing 25% saturated ammonium sulfate, was applied to a column (1 \times 2.5 cm) of Octyl Sepharose CL-4B. The column was eluted with the same buffer of increasing ethylene glycol and decreasing ammonium sulfate concentration as shown in the figure. The eluates were dialyzed immediately against 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° and fractions under the line were pooled.

(a) Crude Hex A, 112 units, containing 2.24 mg protein and (b) crude Hex B, 114 units, containing 6.2 mg protein, were applied.

—○—: activity; —●—: protein; - - - - -: ethylene glycol; —: Ammonium sulfate.

Results

Crude Hex A and Hex B were prepared by a series of procedures: heat treatment of liver extract at pH 3.8 at 37°, ammonium sulfate precipitation, gel filtration and DEAE-cellulose column chromatography with salt gradient. In polyacrylamide disc electrophoresis (7.5% gel, pH 9.2), crude Hex A moved faster toward anode in a single activity band, whereas crude Hex B almost remained at the top of gel.

3) D.H. Leaback and P.G. Walker, *J. Chem. Soc.*, 1957, 4754.

4) B. Weissmann, S. Hadjiioannou, and J. Tornheim, *J. Biol. Chem.*, 239, 59 (1964).

5) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, 193, 265 (1951).

6) M. Hayashi, *J. Histochem. Cytochem.*, 13, 355 (1965).

TABLE I. Hydrophobic Affinity Chromatography of Crude Hex A and Hex B

Adsorbent	Octyl Sepharose CL-4B		Phenyl Sepharose CL-4B	
	Specific activity (units/mg)	Yield (%)	Specific activity (units/mg)	Yield (%)
Hex A	189	82.7	124.4	79.5
Hex B	178	81.1	34.4	63.8

Hydrophobic Affinity Chromatography

Hex A and B were purified to specific activities of 189 and 178 units per mg, respectively, in more than 80% yields by using an Octyl Sepharose CL-4B column which were eluted with increasing ethylene glycol and decreasing ammonium sulfate concentration (Table I and Fig. 1). Purification with Phenyl Sepharose CL-4B led to inferior results (Table I). The above specific activity for the purified Hex A is much higher than the value of 137 units per mg for the Hex A preparation which had been obtained by *p*-aminophenyl β -1-thio-acetylglucosaminide substituted CH-Sepharose 4B chromatography,^{2b)} and the specific activity for Hex B is as high as the value of 184 units per mg for a Hex B preparation obtained by *p*-aminophenyl β -acetylglucosaminide substituted CH-Sepharose 4B chromatography.^{2b)}

Isoelectric Focusing

As depicted in Fig. 2a and 2b, isoelectric focusing on a polyacrylamide gel plate revealed that both Hex A and Hex B, regardless of whether crude or purified, are extensively heterogeneous; Hex A gave at least 10 bands with pI's of 5.0—7.0, and Hex B at least 14 bands with pI's of 6.5—8.5. With Hex A and B purified by using Octyl Sepharose CL-4B, protein bands essentially coincided with activity bands, although, on prolonged enzyme staining, several activity bands appeared at the location where proteins are not detectable.

Treatment of liver extract at pH 3.8 at 37° involved in the purification procedures was first suspected to be a proteolytic step

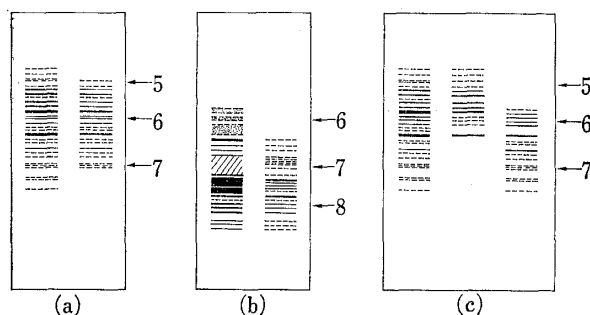


Fig. 2. Diagrammatic Representation of Polyacrylamide Gel Isoelectric Focusing of Bovine Liver β -Acetylhexosaminidase

(a) Purified Hex A, and (b) purified Hex B, each stained for protein (right) and for enzyme activity (left). (c) Crude Hex A before CM-cellulose chromatography (left), eluate from the column at pH 4.0 (middle) and at pH 6.0 (right), all stained for activity. Arrows roughly indicate pH values.

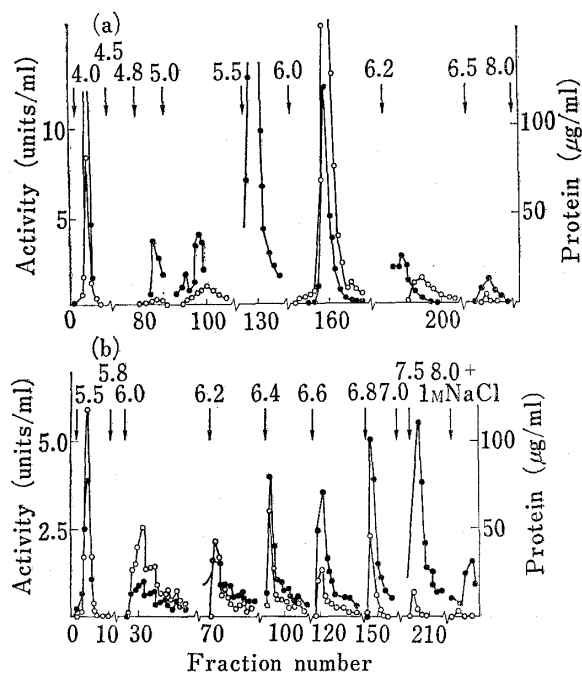


Fig. 3. Elution Patterns of Crude Hex A and Hex B from a CM-Cellulose Column

Stepwise elution was carried out at 4° with 0.01 M buffers of increasing pH as indicated by arrows (pH 4.0—5.0: sodium citrate, pH 5.5—8.0: sodium phosphate). The eluate was dialyzed against 0.05 M citrate buffer, pH 4.2, and 5 ml fractions were collected at a flow rate of 20 ml per hr. (a) Crude Hex A (518 units, 10.5 mg protein) was applied to a column (0.7 × 5 cm) to give 49% recovery, and (b) crude Hex B (228 units, 12.4 mg protein) to a column (0.7 × 2.0 cm) to give 61% recovery.

—○—: enzyme activity; —●—: protein.

which causes the heterogeneity of enzyme protein. This was negated as the corresponding enzyme fraction prepared by Langley's method,^{2a)} which is devoid of the procedure of heat treatment at pH 3.8, exhibited an identical zymogram with that of crude Hex A or B.

CM-Cellulose Chromatography

Stepwise elution from a CM-cellulose column with buffers of increasing pH was examined expecting the separation of the heterogeneous enzyme proteins. Fig. 3 shows the results which at first seemed promising. Isoelectric focusing, however, revealed that the separation was unsatisfactory; the activity in each fraction ranged widely over 2—3 pI units and overlapped each other, as shown for two Hex A fractions in Fig. 2c.

Discussion

Octyl Sepharose CL-4B was found to be effective for the purification of crude Hex A and B from bovine liver. Specific activities (units per mg protein) of 189 for Hex A and 178 for Hex B were obtained in this study using *p*-nitrophenyl acetylglucosaminide as substrate. The values are reasonable as compared with the value of 114 reported for the 4400-fold purified bovine liver enzyme,⁴⁾ which was assayed under the same condition. High purification of the enzyme has recently been reported for the enzymes from viscera, such as human kidney⁷⁾ and human placenta,⁸⁾ where the activities were assayed using the methylumbelliferyl glycoside as substrate in different concentrations. When the purified Hex A and B were focused on a polyacrylamide gel plate, all the protein bands exhibited the enzyme activity, suggesting the absence of detectable contaminative protein. This supports the purity of the preparations although association of enzyme with the other charged species should be taken into consideration.

Stepwise elution from CM-cellulose column with increasing pH value was reported to be satisfactory for analyzing proteins, as demonstrated for egg white protein;⁹⁾ proteins can be eluted at pH vicinal to pI and difference of 0.2 pI unit is sufficient for separation. Against expectation, the present experiment employing this method resulted in rough separation into groups.

In the present experiments with isoelectric focusing on a plate, the purified enzyme exhibited heterogeneity more extensive than that reported for ram testis¹⁰⁾ and pig kidney enzyme¹¹⁾ which were focused in a column. Many studies have been reported on hexosaminidase isozymes, among which the isozymes in human liver and kidney, Hex A,B, I₁, I₂, S₁ and S₂, were shown to be characterized by the different combinations of genetically defined α and β subunits.⁷⁾ These isozymes are resolved by DEAE-cellulose column chromatography with salt gradient. The present study deals with further resolution of Hex A and Hex B which are already separated by DEAE-cellulose chromatography, and the heterogeneity is too extensive to arise only from a genetical base. Therefore, it seems likely that the heterogeneity reflects the subcellular stepwise modification of enzyme protein as suggested for rat liver β glucuronidase.¹²⁾

- 7) S. K. Srivastava, J. E. Wiktorowicz, and Y. C. Awasthi, *Proc. Nat. Acad. Sci. U.S.A.*, **73**, 2833 (1976).
- 8) a) S. K. Srivastava, Y. C. Awasthi, A. Yoshida, and E. Beutler, *J. Biol. Chem.*, **249**, 2043 (1974);
b) J. F. Tallman, R. O. Brady, J. M. Quirk, M. Villalba, and A. E. Gal, *J. Biol. Chem.*, **249**, 3489 (1974).
- 9) M.B. Rhodes, P.R. Azari, and R.E. Feeney, *J. Biol. Chem.*, **230**, 399 (1958).
- 10) B.G. Winchester, *Biochem. J.*, **124**, 929 (1971).
- 11) K. Sandhoff, *Z. Physiol. Chem.*, **349**, 1095 (1968).
- 12) J.W. Owens, K.L. Gammon, and P.D. Stahl, *Arch. Biochem. Biophys.*, **166**, 258 (1975).