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Affinity Chromatography of Rabbit Liver β-Glucuronidase

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An affinity adsorbent was prepared by coupling *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid with CH-Sepharose 4B. β-Glucuronidase from rabbit liver was adsorbed on *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid-CH-Sepharose then eluted with basic buffer or salt solution. The specific enzyme activity increased 46-fold and essentially all of the activity of the enzyme was recovered in elution buffer containing 0.1 M NaCl. N-Acetyl-β-glucosaminidase was adsorbed slightly but β-glucosidase and β-galactosidase were not adsorbed on the affinity adsorbent. The adsorbent retained affinity for the enzyme after being used ten times.

Keywords——affinity chromatography; *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid; rabbit liver; β-glucuronidase; N-acetyl-β-glucosaminidase

Recently, *p*-aminophenyl 1-thio-β-D-glucopyranoside,[2a)] -β-D-galactopyranoside[2b)] and -2-acetamido-2-deoxy-β-D-glucopyranoside[2a,c)] have been used as ligands for affinity chromatography of β-glucosidase, β-galactosidase and N-acetyl-β-glucosaminidase, respectively. Junowicz and Paris[3)] purified β-glucuronidase from rat liver by affinity chromatography using *p*-aminophenyl β-D-glucopyranosiduronic acid as a ligand. It is still not clear whether or not *p*-aminophenyl β-D-glucopyranosiduronic acid bound to the insoluble matrix is hydrolyzed by the enzyme, though this compound is a substrate for β-glucuronidase. Since *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid is a competitive inhibitor of β-glucuronidase, an affinity adsorbent with *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid as a ligand might be more suitable for repeated use than an adsorbent with *p*-aminophenyl β-D-glucopyranosiduronic acid. In this paper we attempted to purify β-glucuronidase from rabbit liver by affinity chromatography with *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid as a ligand.

Materials and Methods

CH-Sepharose 4B and CNBr-activated Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl salt (EDC HCl) was purchased from Sigma Chemical Co. (St. Louis, Mo.), and *p*-nitrophenyl β-D-glucopyranoside was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and β-D-galactopyranoside were prepared according to the method of Snaith *et al.*[4a)] and Heyworth and Walker,[4b)] respectively. *p*-Aminophenyl 1-thio-β-D-glucopyranosiduronic acid (PAPTGA), 1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside, 1-thio-β-D-galactopyranoside, 1-thio-β-D-glucopyranoside were synthesized by the method of Iino.[5)] 5-Bromo-4-chloroindol-3-yl-β-D-glucopyranosiduronic acid was synthesized according to the preceding paper.[6a)]

PAPTGA-succinyl diaminodipropylamino Sepharose was prepared according to the method of Junowicz and Paris.[3)] PAPTGA-CH-Sepharose was usually prepared as follows: 0.5 ml of water (pH 4.5) containing

1) Location: *93, Tamagawa-cho, Minami-ku, Fukuoka-shi, Fukuoka.*

2) *a*) T. Mega and Y. Matsushima, *J. Biochem.* (Tokyo), **79**, 185 (1976); *b*) E. Steers, Jr., P. Cuatrecasas, and H.B. Pollard, *J. Biol. Chem.*, **246**, 196 (1971); *c*) E.E. Grebner and I. Parikh, *Biochim. Biophys. Acta*, **350**, 437 (1974).
3) E. Junowicz and J.E. Paris, *Biochim. Biophys. Acta*, **321**, 234 (1973).
4) *a*) S.M. Snaith, G.A. Levvy, and A.J. Hay, *Biochem. J.*, **117**, 129 (1970); *b*) R. Heyworth and P.G. Walker, *Biochem. J.*, **83**, 331 (1962).
5) N. Iino and K. Yoshida, *Carbohydr. Res.*, **51**, 223 (1976).
6) *a*) K. Yoshida, N. Iino, and I. Koga, *Chem. Pharm. Bull.* (Tokyo), 32, 1759 (1975); *b*) K. Yoshida, N. Iino, I. Koga, and K. Kato, *Anal. Biochem.*, **58**, 77 (1974).

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14.4 mg of PAPTGA and 0.5 ml of water (pH 4.5) containing 90 mg of EDC HCl salt were added successively to 10 ml of water containing CH-Sepharose 4B (9 ml, wet volume). After shaking overnight at room temperature, the suspended solution was filtered and the beads were washed thoroughly by the method of Junowicz and Paris.[3] The amount of PAPTGA combined with CH-Sepharose 4B was determined by measurement of unreacted PAPTGA in the filtrate and several washings by the color reaction with sodium 2,4,6-trinitrobenzenesulfonate (TNBS): 2.0 ml of 0.5 M phosphate buffer (pH 8.0) and 2.0 ml of 0.1% TNBS were added to 1.0 ml of the filtrate and several washings. After incubation at 40° for 90 min, 1.0 ml of 6 N HCl was added to the solution and the absorbance at 380 nm was measured. Other affinity adsorbents consisting of CH-Sepharose 4B coupled with *p*-aminophenyl 1-thio-β-D-glucopyranoside, 1-thio-β-D-galactopyranoside and 1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside as ligands were prepared by the same method as PAPTGA-CH-Sepharose.

Rabbit liver β-glucuronidase was prepared as follows: rabbit liver was minced and homogenized in 0.25 M sucrose (1: 3 w/v). The homogenate was centrifuged at 10000 ***g*** for 15 min. The pellet was suspended in 5 mM Tris-acetate buffer, pH 7.8 (liver: buffer=1: 9 w/v). After 1 hr, the suspension was centrifuged at 10000 ***g*** for 15 min and the supernatant was 40% saturated with solid ammonium sulfate. The resulting precipitate was centrifuged off and dissolved in 5 mM Tris-acetate buffer for storage in a cold room.

Activity of β-glucuronidase was usually assayed as follows: a mixture of 0.1 ml of sample, 0.1 ml of 0.6 M acetate buffer (pH 4.5)–0.3% BSA and 0.1 ml of 15 mM *p*-nitrophenyl β-D-glucopyranosiduronic acid was incubated at 37° for 20 or 30 min. After incubation, 2.0 ml of 0.2 M Na_2CO_3 was added. Absorbance of the mixture was measured at 400 nm. Using the same conditions, the activities of N-acetyl-β-glucosaminidase, β-glucosidase and β-galactosidase were assayed with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, β-D-glucopyranoside and β-D-galactopyranoside, respectively. One unit of enzyme is the quantity required to release 1 nmol of *p*-nitrophenol per min under the conditions described above.

Protein was measured according to the method of Lowry *et al.*[7] Specific activity was expressed as units per mg of protein.

Polyacrylamide gel electrophoresis was carried out according to the procedure of Davis.[8] Protein was stained with Amido Black 10B, and enzyme activity was stained according to the preceding papers.[6a,b]

Results and Discussion

Effect of Ligand Concentration on the Enzyme Binding Capacity of the Adsorbent

Affinity adsorbents were prepared by addition of 36 mg of EDC HCl salt and 1.5—47.8 mg of PAPTGA to 3 ml of CH-Sepharose 4B beads (wet volume). The enzyme binding capacity of the adsorbent with 1.4 μmol of ligand per ml of beads was less than those of other adsorbents with more ligand, but the adsorbed enzyme was almost completely eluted with 0.1 M H_3BO_3–NaOH buffer (pH 8.0), as shown in Fig. 1. At 4.7—16 μmol of ligand bound, the capacities of the adsorbents were similar, but the enzyme activities in the eluent decreased with increase of ligand concentration. At 16 μmol of ligand β-glucuronidase, the material left in the column after elution with 0.1 M H_3BO_3–NaOH buffer (pH 8.0) was eluted with 0.1 M $Na_2B_4O_7$–Na_2CO_3 buffer (pH 10.0). At 51 μmol of PAPTGA the amount of adsorbed enzyme was less than in other cases, and the adsorbed enzyme was not eluted with 0.1 M H_3BO_3–NaOH buffer (pH 8.0), though it was eluted with 0.1 M $Na_2B_4O_7$–Na_2CO_3 buffer (pH 10.0). Unless otherwise stated, all operations were carried out with adsorbents containing 4 μmol of ligand, since the adsorbed enzyme could be eluted with less basic buffer. No difference in affinity for β-glucuronidase between affinity adsorbents prepared with CH-Sepharose 4B and succinyl diaminodipropylamino Sepharose 4B as a matrix could be detected. Thus, unless otherwise stated, CH-Sepharose 4B was employed as a matrix. In a control test with CH-Sepharose 4B using 5 mM Tris-acetate buffer (pH 7.8) as a washing buffer and 0.1 M H_3BO_3–NaOH buffer (pH 8.0) as an elution buffer, all the β-glucuronidase activity passed through with the washing buffer.

Effect of Buffers on Elution of the Adsorbed Enzyme

We examined whether carbohydrates competed with PAPTGA for affinity binding to β-glucuronidase, eluting the adsorbed enzyme. In this experiment PAPTGA-succinyl diamino-

7) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
8) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).

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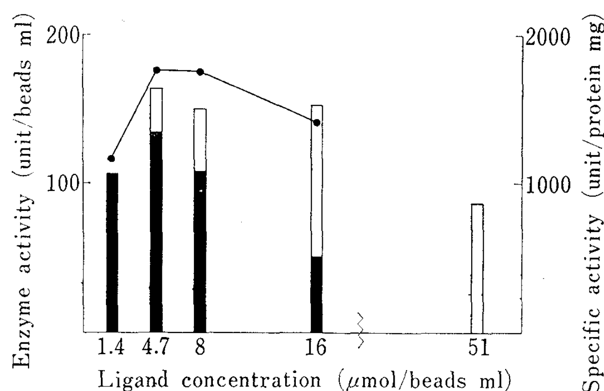

Fig. 1. Effect of Ligand Concentration on Adsorption-Desorption of β-Glucuronidase

PAPTGA-CH-Sepharose 4B (2 ml) was equilibrated with 5 mM Tris-acetate buffer (pH 7.8) and 240 units of enzyme activity were applied to the adsorbent with 1.4 μmol of the ligand and 400 units to the adsorbents with 4.7—51 μmol of the ligand. The enzyme was eluted with 0.1 M H_3BO_3–NaOH buffer (pH 8.0). The data are the means of two experiments.
■ : adsorbed-eluted activity;
□ : adsorbed-uneluted enzyme activity;
●—●: specific activity of adsorbed-eluted enzyme.

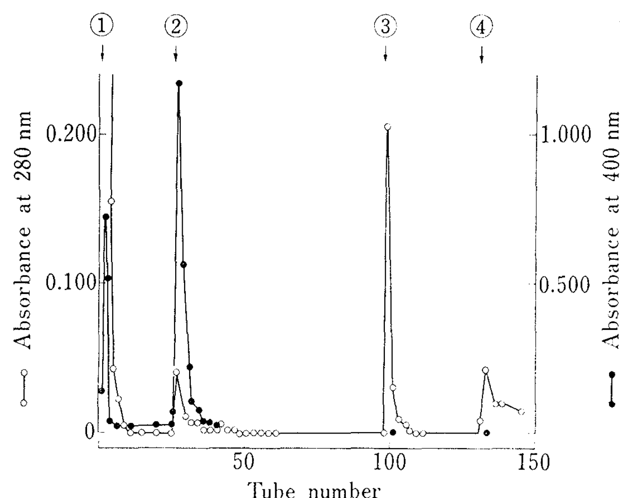

Fig. 2. Elution Profile of β-Glucuronidase from PAPTGA–CH–Sepharose by Stepwise Salt Elution

The ligand concentration was 4.6 μmol per ml of beads. The adsorbent (5 ml, wet beads) was equilibrated with Tris-acetate buffer (pH 7.8) and 1936 units of the enzyme were applied. The enzyme was eluted with ① 0.01 M NaCl–0.05 M acetate buffer (pH 6.0), ② 0.1 M NaCl–0.05 M acetate buffer (pH 6.0), ③ 0.3 M NaCl–0.05 M acetate buffer (pH 6.0), ④ 0.1 M H_3BO_3–0.1 M NaOH buffer (pH 8.0). Fraction volume was 5 ml per tube.

Table I. Purification of β-Glucuronidase from Rabbit Liver by Stepwise Salt Elution from PAPTGA–CH-Sepharose

Enzyme	Before chromatography		After chromatography[a]		
	Total enzyme unit	Spec. act.	Total enzyme unit	Spec. act.	Relative spec. act.
β-Glucuronidase	1936	41.2	1305	1894	46
N-Acetyl-β-glucosaminidase	900	19.2	13	18	1
β-Galactosidase	770	16.4	0	0	—
β-Glucosidase	865	18.4	0	0	—

a) Enzyme activities were measured on the 0.1 M NaCl–0.05 M acetate buffer (pH 6.0) fraction in Fig. 2.

dipropylamino Sepharose was employed as an adsorbent. Neither 0.1 M glucuronic acid nor 0.1 M glucuronolactone eluted β-glucuronidase, while salt or basic buffers such as 0.1 M NaCl–0.05 M acetate buffer (pH 5.0, 6.0), 0.1 M H_3BO_3–NaOH (pH 8.0), 0.1 M $NaHCO_3$–Na_2CO_3 (pH 9.0) and 0.1 M $Na_2B_4O_7$–Na_2CO_3 (pH 10.0) did. Figure 2 shows the elution pattern of β-glucuronidase with stepwise increasing concentrations of NaCl in acetate buffer. On elution with 0.1 M NaCl, the specific activity of the enzyme increased 46-fold with respect to the sample before chromatography, and the recovery of β-glucuronidase activity was 101% of adsorbed enzyme activity (Fig. 2, Table I).

Affinity of Other Glycosidases for PAPTGA-CH-Sepharose

The affinities of other glycosidases for PAPTGA-CH-Sepharose were examined. As shown in Fig. 3 (A), N-acetyl-β-glucosaminidase was adsorbed slightly, and the adsorbed enzyme was desorbed together with β-glucuronidase under the conditions used, but β-galactosidase and β-glucosidase were not adsorbed (Fig. 3 (A), Table I). Junowicz and Paris[3)]

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reported that N-acetyl-β-glucosaminidase as well as β-glucuronidase from rat liver were adsorbed on an affinity adsorbent in which *p*-aminophenyl β-D-glucuronide was used as a ligand. In their report, the recoveries were 98% for β-glucuronidase and 91% for N-acetyl-β-glucosaminidase, and these enzyme were purified 55-fold and 42-fold, respectively. Thus affinity chromatographies with *p*-aminophenyl thio-β-D-glucuronide and *p*-aminophenyl β-D-glucuronide as ligands are similar as regards recovery of activity and the relative specific activity of β-glucuronidase. However, affinity of N-acetyl-β-glucosaminidase for the adsorbent with *p*-aminophenyl thio-β-D-glucuronide as a ligand was different from that in the case of *p*-aminophenyl β-D-glucuronide.

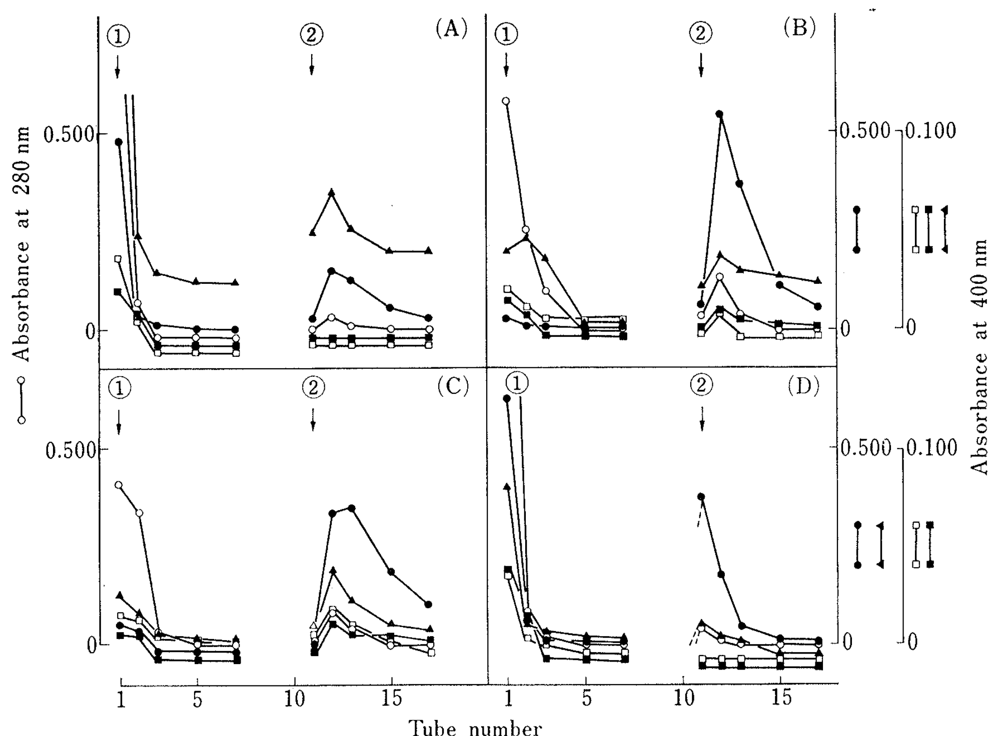

Fig. 3. Elution Profiles of β-Glycosidases from Four Adsorbents Consisting of CH-Sepharose 4B coupled with *p*-Aminophenyl Thioglycosides

Ligands and their concentrations were (A) PAPTGA: 4.7 μmoles, (B) *p*-aminophenyl 1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside: 4.1 μmol, (C) *p*-aminophenyl 1-thio-β-D-galactopyranoside: 3.6 μmol, (D) *p*-aminophenyl 1-thio-β-D-glucopyranoside: 4.6 μmol. Each adsorbent (2 ml, wet beads) was equilibrated with 1 mM acetate buffer (pH 6.0) and 0.5 ml of enzyme solution which contained 97 units of β-glucuronidase, 71.3 units of N-acetyl-β-glucosaminidase, 12.2 units of β-galactosidase and 3.3 units of β-glucosidase was applied. The adsorbents were washed with ① 1 mM acetate buffer (pH 6.0) and then ② 0.1 M NaCl–acetate buffer (pH 6.0). Fraction volume was 5 ml per tube. The assay procedures for the glycosidases are described in the text.
●—●: β-glucuronidase; ▲—▲: N-acetyl-β-glucosaminidase;
□—□: β-galactosidase; ■—■: β-glucosidase.

Affinity of β-Glucuronidase for CH-Sepharose 4B coupled with Thio-glycosides Other than PAPTGA

Affinity adsorbents were prepared with *p*-aminophenyl thio-β-D-glucoside, -β-D-galactoside and -N-acetyl-β-D-glucosaminide as ligands. The affinities of β-glucuronidase and other glycosidases for these affinity adsorbents were examined. As shown in Fig. 3 (B), (C) and (D), β-glucuronidase was adsorbed on all of the adsorbents prepared, although the affinities were different. The adsorbents coupled with *p*-aminophenyl thio-β-D-galactoside and -N-acetyl-β-D-glucosaminide adsorbed all four glycosidases tested. The adsorbent coupled with *p*-aminophenyl thio-β-D-glucoside adsorbed β-glycosidases poorly except for β-glucuronidase.

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Disc electrophoresis of the crude enzyme and purified enzyme eluted from the affinity column was carried out in 7.5% (W/V) polyacrylamide gel. As shown in Fig. 4, the purified sample was still heterogeneous, with several contaminating proteins.

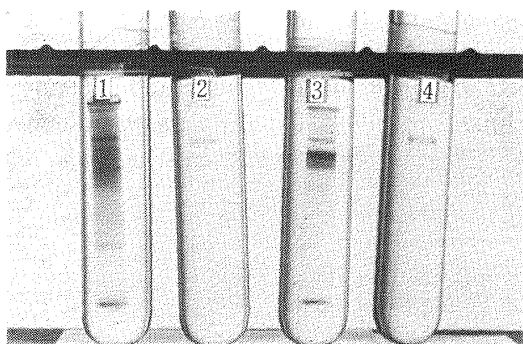

Fig. 4. Polyacrylamide Gel Electrophoresis Pattern of β-Glucuronidase from Rabbit Liver

(1) Polyacrylamide gel electrophoresis pattern of 200 μg of crude enzyme stained for protein.
(2) Same as (1), stained for activity.
(3) Polyacrylamide gel electrophoresis pattern of 40 μg of enzyme partially purified by salt elution from PAPTGA-CH-Sepharose, stained for protein.
(4) Same as (3), stained for activity.

After using the adsorbent with PAPTGA as a ligand ten times, it still adsorbed β-glucuronidase. Although Junowicz and Paris[3] reported that the adsorbent with *p*-aminophenyl β-D-glucuronide could be reused several times, the bound substrate may still be cleaved by the enzyme. Mega and Matsushima[9] reported that *p*-aminophenyl β-D-N-acetylglucosaminide as an affinity adsorbent ligand was hydrolyzed by N-acetyl-β-glucosaminidase. Since *p*-aminophenyl thio-β-D-glucuronide is a competitive inhibitor of β-glucuronidase, the adsorbent with *p*-aminophenyl thio-β-D-glucuronide as a ligand should be reusable repeatedly without cleavage. *p*-Aminophenyl thio-β-D-glucuronide-CH-Sepharose is a useful adsorbent because it can be prepared conveniently and is stable under repeated use.

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