Chromatography of β-Glucuronidase from Bovine Liver. A Study of the Enzyme Binding Sites of Prepared Adsorbents

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β-Glucuronidase from bovine liver was adsorbed to the adsorbents prepared with CH-Sepharose 4B and either the competitive inhibitor or its analogs such as *p*-aminophenyl 1-thio-β-D-glucuronic aicd, -glucoside, -galactoside, and *N*-acetyl glucosaminide. The adsorbed enzyme was eluted at 0.1 or 0.5 M NaCl by a stepwise gradient. Chromatography of the enzyme was also performed by using the adsorbents prepared with Epoxy-activated Sepharose 6B and amine compounds or other compounds.

In order to see whether the hydroxyl groups of the sugar parts in the ligand are necessary for the adsorption of the enzyme, chromatography was performed by using the adsorbents prepared with sugar derivatives as the ligand. As a result, it was found that β-glucuronidase had an affinity for adsorbents prepared with either acetyl derivatives or methoxy derivatives of glycosides and CH-Sepharose 4B.

From the results of elution of the enzyme with NaCl from adsorbents having amide bonding, it was clarified that the affinity of the enzyme for adsorbents without glycosides in the ligands correlated with acidity of the amide in the adsorbents.

Hydrogen bond chromatography was performed with the prepared adsorbents. The enzyme was adsorbed under a high concentration of ammonium sulfate, and the elution of the adsorbed enzyme from adsorbents was examined by the degradation of salt. The enzyme was most easily eluted from aminoethyl 1-thio-β-D-glucuronic acid–CH Sepharose 4B at 0.9 M ammonium sulfate and at 0.5 M concentration of the salt with *p*-aminophenyl 1-thio-β-D-glucuronic acid–CH Sepharose 4B. Furthermore, the adsorbed enzyme was eluted by the addition of urea as well as ethylene glycol which are known as reagents which weaken hydrogen bonding. The results suggested that the interaction between the enzyme and the adsorbents with an amide bonding may be affected by the electrostatic force in the adsorbents under a high concentration of salt, although the electrostatic force decreases under the high concentration of salt.

We also investigated whether or not the adsorbed enzyme was eluted by sodium cholate, cholic acid and triton X-100 known as hydrophobic reagents.

It was assumed from the results of these chromatographies that the presence of amide bonding in adsorbents with glycosides as the ligand may be essential for the adsorption of the enzyme and that the glycosidic parts of the ligands have an effect on adsorption, however, it may not be essential for adsorption.

Keywords β-glucuronidase; bovine liver; affinity chromatography; amide bonding; imide bonding; hydrogen bond chromatography; hydrophobic chromatography; glycoside–Sepharose

Affinity chromatography has been widely used for the purification of proteins. C. Blanco *et al.* purified β-glucuronidase from *E. coli* with a good yield by the use of phenyl 1-thio-β-D-glucuronic acid as a ligand of an adsorbent at chromatography.[1] However, when E. Junowicz *et al.* attempted to purify β-glucuronidase from bovine or murine by affinity chromatography, both the enzyme and *N*-acetyl β-glucosaminidase were adsorbed to adsorbents in which some modified glycoside enzyme substrates were coupled to Sepharoses, and resolution of mixtures of the glycosidases was found difficult.[2]

We also experimented with the affinity chromatography of β-glucuronidase from rabbits by the use of a competitive inhibitor of the enzyme as a ligand. As a result, the enzyme was adsorbed to the adsorbents with either *p*-aminophenyl 1-thio-β-D-glucuronic acid or other 1-thioglycosides as the ligands.[3]

We were interested in further studying this broad affinity of β-glucuronidase for adsorbents attached to various glycosides as ligands, and thus conducted experiments on the chemical characteristics of the enzyme binding sites of adsorbents. However, it has been speculated in general that the affinity chromatography of glycosidases may be done by utilizing the specificity of enzymes towards the sugar moiety of ligands in the adsorbents.[4] Our experimental results suggested that the affinity of β-glucuronidase for adsorbents in which glycosides were coupled as the ligands was affected first by the mode of binding between the ligands and spacers of the solid matrices, and then by the sugar moiety of ligands.

Materials and Methods

Chemicals CH-Sepharose 4B, Phenyl-Sepharose CL-4B, Octyl-Sepharose CL-4B, Epoxy-activated Sepharose 6B and EAH-Spharose 4B were purchased from Pharmacia LKB Co.; 1-ethyl-3-(3′-dimethylamino-propyl)carbodiimide HCl (EDC HCl), β-D-glucuronidase from the bovine liver were from Sigma Chemical Co.; *p*-nitrophenyl β-D-glucuronide were from Merck Co. The melting points were determined with a Yanagimoto MP-S2 apparatus and were uncorrected. The infrared (IR) spectra were recorded with a Hitachi model EPI-G3 spectrophotometer. The mass spectra (MS) were recorded with a JMS-DX303 spectrometer. Thin-layer chromatography (TLC) was performed on Aluminium sheets coated with Silicagel 60 F_{254} (Merck) with the following solvents (v/v): I, 4:1 methanol–water; II, 3:2:2 ethylacetate–acetic acid–water; III, 1:3:2 methanol–ethylacetate–benzene; IV, 1:1 methanol–acetone. Gas chromatography was done on a Shimadzu gas-chromatography GC-15A, equipped with a hydrogen-flame, by ionization on a capillary column (CBP-1-S-25-050). High performance liquid chromatography (HPLC) was done by using CCPE pumps (Tosoh) with a variable-wavelength detector (Waters 484) with a TSK gel ODS-120T set between them.

Some of the glycosides were prepared as follows: *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid, *p*-aminophenyl 2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside, *p*-aminophenyl 1-thio-β-D-glucopyranoside, *p*-aminophenyl 1-thio-β-D-galactopyranoside were synthesized as described in the preceding paper.[5]

2-Aminoethyl 1-Thio-β-D-glucuronic Acid (A): Compound A was

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TABLE I. Physical Properties and Elemental Analysis of Methylated Glycosides

Compounds	Formula	Elemental analysis (%) Calcd (Found) C	H	N	Rf value[a]	mp (°C) (Recrystn. solvent)	IR ν_{max}^{KBr} cm^{-1}	[M$^+$]
Methyl(*p*-nitrophenyl 2,3,4-tri-*O*-methyl-1-*O*-β-D-glucopyranosid)uronate	$C_{16}H_{21}NO_9$	51.75 (51.96	5.66 5.77	3.77 3.71)	0.65	110—111 (EtOH–H_2O)	1350 (NO_2) 1745 ($COOCH_3$) 2840 (OCH_3)	371
p-Nitrophenyl 2,3,4,6-tetra-*O*-methyl-1-*O*-β-D-glucopyranoside	$C_{16}H_{23}NO_8$	53.78 (53.69	6.44 6.50	3.92 3.99)	0.69	108—109 (EtOH–H_2O)	1350 (NO_2) 2840 (CH_3)	357
p-Nitrophenyl 2,3,4,6-tetra-*O*-methyl-1-*O*-β-D-galactopyranoside	$C_{16}H_{23}NO_8$	53.78 (53.66	6.44 6.52	3.92 3.89)	0.44	99 (EtOH–H_2O)	1750 (CO) 2840 (OCH_3)	357
Methyl(*p*-aminophenyl 2,3,4-tri-*O*-methyl-1-*O*-β-D-glucopyranosid)uronate HCl salt	$C_{16}H_{23}NO_7$ ·HCl	50.86 (49.75	6.36 6.48	3.71 3.49)	0.31 (0.54) (−HCl)	167—169	2840 (OCH_3) 3440 (NH_3)	341 (−HCl)
p-Aminophenyl 2,3,4,6-tetra-*O*-methyl-1-*O*-β-D-glucopyroside HCl salt	$C_{16}H_{25}NO_6$ ·HCl	52.82 (52.24	7.15 7.12	3.85 3.63)	0.21 (0.34) (−HCl)	188—190	2840 (OCH_3) 3440 (NH_2)	327 (−HCl)
p-Aminophenyl 2,3,4,6-tetra-*O*-methyl-1-*O*-β-D-galactopyranoside HCl salt	$C_{16}H_{25}NO_6$ ·HCl	52.82 (52.67	7.15 7.27	3.85 3.53)	0.13 (0.23) (−HCl)	192—194	2840 (OCH_3) 3440 (NH_2)	327 (−HCl)

a) Solvent ratio: benzene/MeOH, 9/1.

synthesized with 2-aminoethanethiol HCl salt and methyl(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate by the same method as mentioned above.[5] The crude product was purified by rechromatography with cellulose packed into a column developed with methanol–water (4:1). The syrupy sample was then made into a powder with ethanol. mp 150—155 °C (dec.). TLC Rf value 0.40 (I), one spot: 0.30 (II), one spot. IR ν_{max}^{KBr} cm^{-1}: 3400—2800 (OH and NH). MS m/z: 253 (M$^+$ + H). *Anal.* Calcd for $C_8H_{15}NO_6S \cdot H_2O$: C, 35.42; H, 6.27; N, 5.17. Found: C, 34.91; H, 6.19, N, 5.01.

Phenyl 1-Thio-β-D-glucuronic Acid (B): Compound B was also synthesized with thiophenol and methyl(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate by the same method as above,[5] and recrystalized from an acetone–petroether. The melting point of B was 159—161 °C and it agreed with the values shown in the reference.[6] TLC Rf value 0.56 (IV), one spot. IR ν_{max}^{KBr} cm^{-1}: 3300—3400 (OH), 1725 (COOH), 753, 693 (phenyl group with mono substituent). MS m/z: 287 (M$^+$ + H). *Anal.* Calcd for $C_{12}H_{14}O_6S$: C, 50.35; H, 4.90. Found: C, 50.14; H, 5.18.

p-Aminophenyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-*O*-β-D-glucopyranoside (D): *p*-Nitrophenyl 2-acetamido-2-deoxy-1-*O*-β-D-glucopyranoside was acetylated with acetic anhydride and pyridine into *p*-nitrophenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-*O*-β-D-glucopyranoside (mp 248—249 °C) (C). The nitro group of C was reduced to amine by the diborane (B_2H_6) system method[7] to become D. The substrate was then recrystalized from ethanol (167—168 °C). TLC Rf value 0.5 (III). IR ν_{max}^{KBr} cm^{-1}: 3420, 3320 (NH_2), 2940, 2870 (CH_3), 1735 (CO), 1655 (NHCO). MS m/z: 438 (M$^+$). *Anal.* Calcd for $C_{20}H_{26}N_2O_9$: C, 54.79; H, 5.94; N, 6.39. Found: C, 54.48; H, 5.91; N, 6.38.

Methyl(*p*-aminophenyl 2,3,4-tri-*O*-acetyl-1-*O*-β-D-glucopyranosid)uronate (E): Methyl(*p*-nitrophenyl 2,3,4-tri-*O*-acetyl-1-*O*-β-D-glucopyranosid)uronate (mp 151—152 °C) was reduced by the same method as D. TLC Rf value 0.66 one spot (III); MS m/z: 425 (M$^+$ + H).

Methyl(*p*-acetamidophenyl 2,3,4-tri-*O*-acetyl-1-*O*-β-D-glucopyranosid)uronate (F): E was acetylated with acetic anhydride and pyridine. Recrystallization from ethanol gave mp 206—207 °C. TLC Rf: 0.7 (III). IR ν_{max}^{KBr} cm^{-1}: 3300 (NH), 2950 (CH_3), 1750 (CO) and 1655 (NHCO). MS m/z: 467 (M$^+$). *Anal.* Calcd for $C_{21}H_{25}NO_{11}$: C, 53.96; H, 5.35; N, 3.00. Found: C, 54.06; H, 5.35; N, 3.02.

Methyl(*p*-aminophenyl 2,3,4-tri-*O*-methyl-1-*O*-β-D-glucopyranosid)uronate (G): The hydroxyl groups of sugar moiety in *p*-nitrophenyl 1-*O*-β-D-glucuronide were methylated by the Kuhn method, and methyl-esterification of carbonic acid of glucuronide was done with diazomethane. Droplet-counter-current chromatography (DCC) was performed for the purpose of purifying the methylated compounds with two solvent systems. One system with ethylacetate–*n*-butanol–2-propylalcohol–methanol–*n*-hexane–water (50:20:1:1:20:10) was employed in an ascending manner, while the other with benzene–chloroform–methanol–water (15:15:23:12) was done in a descending manner. A catalytic reduction of the methylated nitro compound was made with pt-O_2, and the HCl salt of the amine compound was formed with HCl–dry ether.

Other permethylated sugars were made by the same method as mentioned above and the physical properties and elemental analysis of methylated sugars are listed in Table I.

The Enzyme Assay β-Glucuronidase activity was measured by the method described in our previous paper,[8] except that a buffer containing 0.2% bovine serum albumin (BSA) was used. One unit of the enzyme equals the quantity required to release 1 nmol of *p*-nitrophenol per minute under experimental conditions.

Preparation of Adsorbents Ligand compounds were coupled to CH-Sepharose 4B, EAH-Sepharose 4B or Epoxy-activated Sepharose 6B according to the manufacturer's recommended procedures. CH-Sepharose 4B was condensed with chemical compounds having an amino group in their chemical structures at pH 4.5 under the presence of EDC HCl salt. Phenyl 1-thio-β-D-glucuronide was condensed with EAH-Sepharose 4B. The adsorbents prepared at pH 10 with Epoxy-activated Sepharose 6B and ligand compounds were next washed in an alkali buffer (pH 10) for the purpose of decomposing the remaining epoxy group. The chemical compounds used as ligand compounds as well as the prepared adsorbents are all listed on Table II. The amount of ligand compounds combined with the solid matrices was determined by applying two methods. One was done by the measurement of other unreacted ligand compounds in the filtrates and washing buffers, while the other was done by the measurement of reacted ligand compounds extracted from the hydrolyzed adsorbents.

Estimation of the Concentration of Ammonium Sulfate The concentration of ammonium sulfate was estimated from the conductivities measured with a conductivity meter, model CD-35M II, M&S Instrument Co.

Results

Chromatographies of β-Glucuronidase by the Stepwise Gradient of NaCl Amine compounds are coupled to spacers of CH-Sepharose 4B under the presence of EDC HCl salt over amide (–NHCO–) bonding. Chemical compounds are connected to the spacer of Epoxy-activated Sepharose 6B over imide (–NH–) bonding or ether (–O–) bonding. β-Glucuronidase from the bovine liver was added to the columns of adsorbents which were swollen previously with 5 mM tris–acetate buffer at pH 7.8, and then the adsorbed enzyme was eluted by the stepwise gradient of NaCl. The results of the chromatographies are listed in Fig. 1.

The enzyme was adsorbed to all of the adsorbents with amide bonding except ethylamine–CH Sepharose 4B, and the adsorbed enzyme was eluted by 0.1 or 0.5 M NaCl (Fig.

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TABLE II. The List of Ligand Compounds of Adsorbents

Adsorbents	Ligand compounds	Beads	Ligands (μmol/ml beads)	Determination of ligand compounds
1	Aniline	CH-Sepharose 4B	16.8 (7.7)[b]	TNBS[a] (GLC)[b]
2	Benzylamine	CH-Sepharose 4B	7.1 (4.4)	DNTF[c] (GLC)
3	Phenylethylamine	CH-Sepharose 4B	8.5 (5.2)	DNTF (GLC)
4	Ethanolamine	CH-Sepharose 4B	11.9	DNTF
5	Ethylamine	CH-Sepharose 4B	8.2	DNTF
6	Aminoethyl 1-thio-β-D-glucopyranosiduronic acid	CH-Sepharose 4B	8.5	Naphthoresorcinol reagent
7	*p*-Aminophenyl 1-thio-β-D-glucopyranosiduronic acid	CH-Sepharose 4B	9.9	Naphthoresorcinol reagent, TNBS
8	*p*-Aminophenyl 1-thio-β-D-glucopyranoside	CH-Sepharose 4B	12.2 (7.9)	TNBS (GLC)
9	*p*-Aminophenyl 1-thio-β-D-galactopyranoside	CH-Sepharose 4B	8.0 (3.8)	TNBS (GLC)
10	*p*-Aminophenyl 2-acetamido-2-deoxyl-1-thio-β-D-glucopyranoside	CH-Sepharose 4B	15.9	TNBS
11	2-Amino-2-D-deoxy-glucose	CH-Sepharose 4B	12.8	Elson-Morgan
12	Methyl(*p*-aminophenyl 2,3,4-tri-*O*-acetyl-1-*O*-β-D-glucopyranosid)uronate	CH-Sepharose 4B	13.6	TNBS
13	*p*-Aminophenyl 2-Acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-1-*O*-β-D-glucopyranoside	CH-Sepharose 4B	14.5	TNBS
14	Methyl(*p*-aminophenyl 2,3,4-tri-*O*-methyl-1-*O*-β-D-glucopyranosid)uronate	CH-Sepharose 4B	16.7	TNBS
15	*p*-Aminophenyl 2,3,4,6-tetra-*O*-methyl-1-*O*-β-D-glucopyranoside	CH-Sepharose 4B	14.1 (5.1)	TNBS (GLC)
16	*p*-Aminophenyl 2,3,4,6-tetra-*O*-methyl-1-*O*-β-D-galactopyranoside	CH-Sepharose 4B	16.8 (6.8)	TNBS (GLC)
17	Phenyl 1-thio-β-D-glucopyranosiduronic acid	EAH-Sepharose 4B	5.4	HPLC[d]
18	Aniline	Epoxy-activated Sepharose 6B	13.6	TNBS
19	Benzylamine	Epoxy-activated Sepharose 6B	20.8	DNTF
20	Phenylethylamine	Epoxy-activated Sepharose 6B	16.6	DNTF
21	Ethylamine	Epoxy-activated Sepharose 6B	16.7	DNTF
22	Ethanol	Epoxy-activated Sepharose 6B	15—20	—[e]
23	Ethylene glycol	Epoxy-activated Sepharose 6B	4.7	Iodine method
24	—[f]	Epoxy-activated Sepharose 6B	15—20	—[e]
25	D-Glucuronic acid	Epoxy-activated Sepharose 6B	9.2	Naphthoresolcinol reagent
26	D-Glucose	Epoxy-activated Sepharose 6B	5.6	Phenol–H_2SO_4 method
27	2-Acetamido-2-deoxy-D-glucose	Epoxy-activated Sepharose 6B	18.9	Morgan–Elson method
28	Methyl(*p*-aminophenyl 2,3,4-tri-*O*-acetyl-1-*O*-β-D-glucopyranosid)uronate	Epoxy-activated Sepharose 6B	7.0	TNBS
29	*p*-Aminophenyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-1-*O*-β-D-glucopyranoside	Epoxy-activated Sepharose 6B	16.7	TNBS
30	Methyl(*p*-aminophenyl 2,3,4-tri-*O*-methyl-1-*O*-β-D-glucopyranosid)uronate	Epoxy-activated Sepharose 6B	5.1	TNBS
31	*p*-Aminophenyl 2,3,4,6-tetra-*O*-methyl-1-*O*-β-D-glucopyranoside	Epoxy-activated Sepharose 6B	5.5	TNBS
32	*p*-Aminophenyl 2,3,4,6-tetra-*O*-methyl-1-*O*-β-D-galactopyranoside	Epoxy-activated Sepharose 6B	7.4	TNBS

The methods of preparation of adsorbents were explained in the Materials and Methods. *a*) TNBS: 2,4,6-trinitrobenzenesulfonate. *b*) A ligand compound extracted from the hydrolyzed adsorbent was analyzed by gas chromatography (GLC). *c*) DNTF: 1-fluoro-2,4-dinitrobenzene. *d*) The unreacted ligand compound in the washing buffer was analyzed by HPLC. *e*) —: the amount of a ligand compound was not determined. *f*) —: Epoxy-activated Sepharose 6B was left under the basic buffer with no ligand compound.

1, 1—16). The adsorbed enzyme was immediately eluted from the adsorbents with imide bonding by the addition of 0.1 M NaCl (Fig. 1, 19—21, 30—32).

After the enzyme was added to the adsorbent 1 in Fig. 1 in which aniline had been coupled over amide bonding to CH Sepharose 4B, the enzyme was then eluted by 0.5 M NaCl. On the other hand, the enzyme could not be adsorbed to the adsorbent with aniline connected to Epoxy-activated Sepharose 6B over imide bonding (Fig. 1, 18). When ethylamine was used as a ligand, the enzyme did not have any affinity for the adsorbent with amide bonding (Fig. 1, 5), but it had an affinity for the adsorbent with imide bonding (Fig. 1, 21).

Whereas the enzyme had an affinity for adsorbents which were prepared by coupling the acetyl derivatives of glycosides to CH-Sepharose 4B (Fig. 1, 12, 13), it passed

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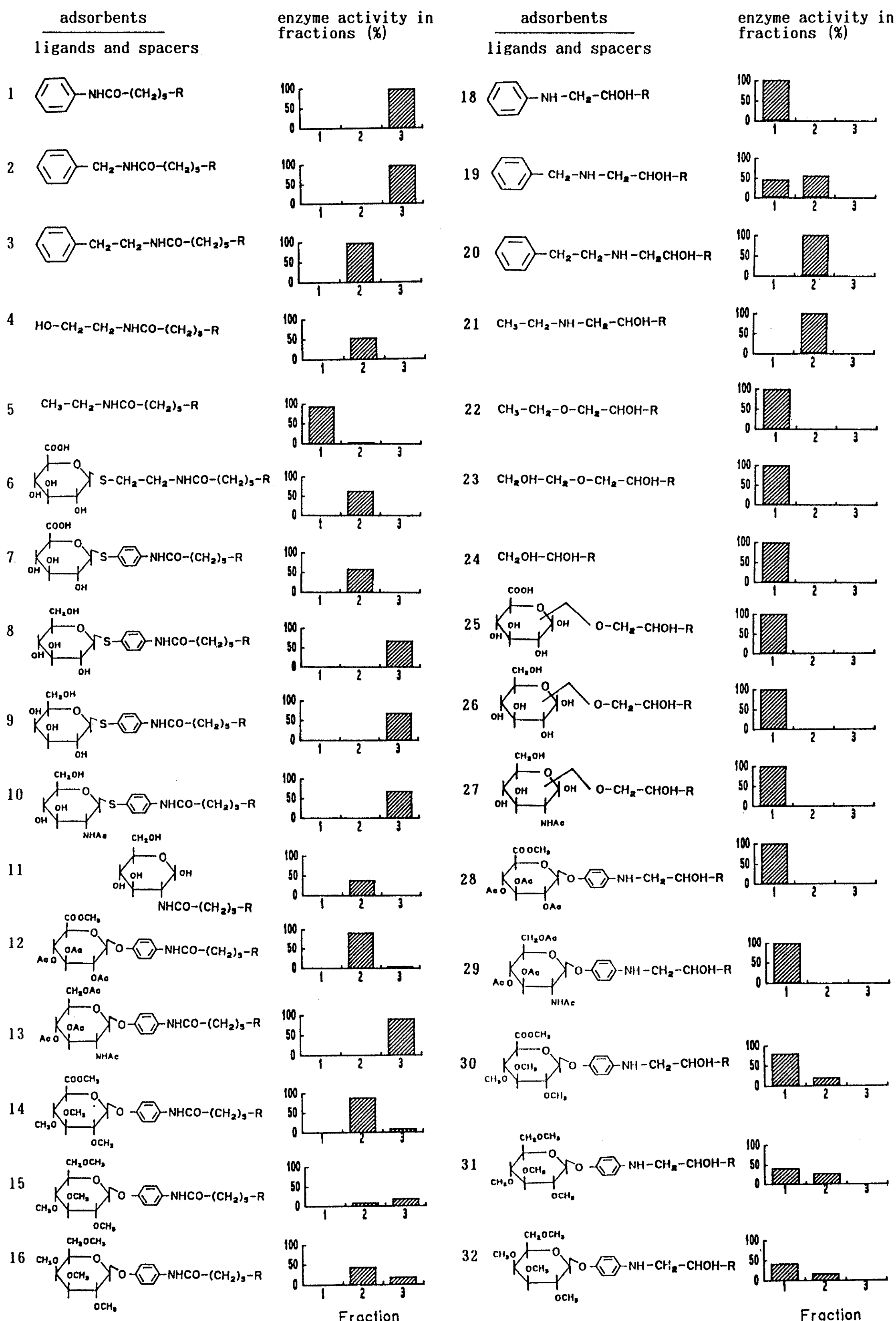

Fig. 1. Elution Patterns of β-Glucuronidase from the Bovine Liver from Adsorbents with NaCl

R shows the part of the carriers of immobilized adsorbents. The preparation of adsorbents was described in Materials and Methods and the prepared adsorbents are listed in Table I. One ml of those prepared adsorbents were packed into columns to be followed for 0.5 ml (450—500 units) of the enzyme solution to be added to them. Developing solutions were at first 10 ml of 5 mM Tris–acetate buffer pH 7.8 (fraction 1), next 0.1 M NaCl–5 mM Tris–acetate buffer pH 7.8 (fraction 2), at last 0.5 M NaCl–5 mM Tris–Acetate buffer pH 7.8 (fraction 3), and the enzyme activities in those fractions were assayed by the method mentioned in the Materials and Methods. The enzyme activities were expressed by a percentage of the enzyme activities (units) recovered in fractions to the enzyme activities (units) added on adsorbents in columns.

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through the columns of adsorbents coupled with the same derivatives to Epoxy-activated Sepharose 6B over imide bonding (Fig. 1, 28, 29).

The enzyme was not adsorbed to the adsorbents prepared by coupling ligands to Sepharose over ether bonding, even when the ligand was a monosaccharide (Fig. 1, 25—27).

Neither Epoxy-activated Sepharose 6B, which had been placed in alkali buffer (pH 10) before use (Fig. 1, 24), nor CH-Sepharose 4B (data not shown) had any affinity for the enzyme.

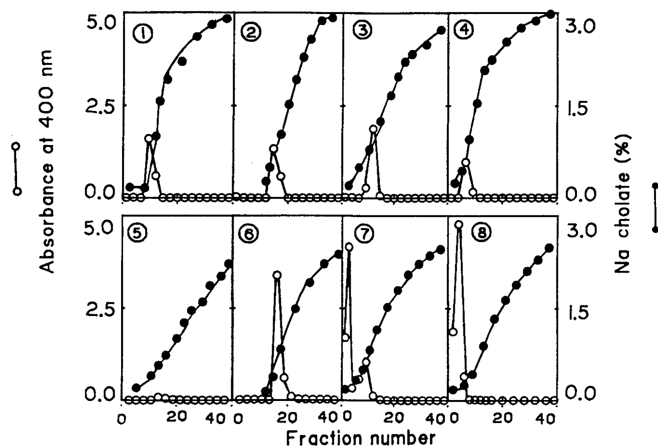

Fig. 2. Elution Patterns of β-Glucuronidase Adsorbed on Adsorbents in Columns by Sodium Cholate

One ml of adsorbents packed into columns were swollen with 5 mM Tris–acetate buffer (pH 7.8) and 0.5 ml (450—500 units) of the enzyme solution were added on those columns, then they were developed by the addition of 5 mM Tris–acetate buffer pH 7.8 containing 0—3% of sodium cholate. 1, adsorbent 7 of adsorbents listed in Table I; 2, adsorbent 8; 3, adsorbent 10; 4, adsorbent 9; 5, adsorbent 1; 6, adsorbent 4; 7, Phenyl-Sepharose CL-4B; 8, Octyl-Sepharose CL-4B.

Hydrophobic Chromatography of β-Glucuronidase We then determined whether or not the hydrophobic parts in prepared adsorbents had any affinity for β-glucuronidase. Figure 2 showed that only a part of the adsorbed enzyme could be eluted by sodium cholate except for the results with adsorbent 4. Both Phenyl Sepharose CL 6B and Octyl Sepharose 4B, which had been purchased as hydrophobic adsorbents, adsorbed little of the enzyme in 5 mM Tris–acetate buffer at pH 7.8 (Fig. 2, 7, 8).

Hydrogen Bond Chromatography An examination was then conducted to find out whether or not the hydroxy groups in a glycoside of ligand interact with enzyme by hydrogen bonding. β-Glucuronidase was added to columns of several adsorbents under the existence of 3 M ammonium sulfate, and the salt concentration was gradually decreased to elute the enzyme (Fig. 3a, b, c). The concentrations of ammonium sulfate at which the enzyme was most eluted are listed in Table III.

Table III. Concentration of Ammonium Sulfate at Which β-Glucuronidase Was Most Eluted

Adsorbents	Ammonium sulfate (M)	Adsorbents	Ammonium sulfate (M)
6	0.9	1	No elution
7	0.5	Phenyl-Sepharose CL-4B	0.05
9	0.5	Octyl-Sepharose CL-4B	0.8
10	0.5	CH-Sepharose 4B	1.2
		4	1.2
		5	1.3

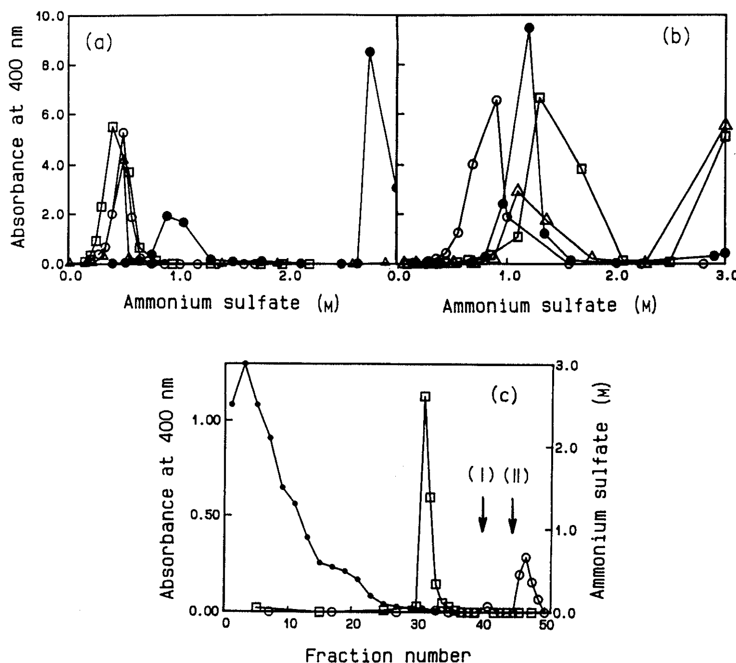

Fig. 3. Elution Patterns of β-Glucuronidase under the Degradation of Concentration of Ammonium Sulfate

One ml of adsorbents in the columns was swollen by 3 M ammonium sulfate–5 mM Tris–acetate buffer (pH 7.8), and it was followed by the addition of 0.5 ml of the enzyme solution in 1 M ammonium sulfate–5 mM Tris–acetate buffer (pH 7.8). The elution of the adsorbed enzyme from the columns was developed with the decreased concentration of ammonium sulfate, and at Fig. 3c it was followed with 0.2 M NaCl–5 mM Tris–acetate buffer (pH 7.8) (I→) and 0.05 M acetate buffer (pH 4.5) (II→). The enzyme activities were shown by adsorbance at 400 nm expressed with such symbols as, on Fig. 3a, ●, for adsorbent 6 (the added enzyme activity was 670 units); ○, for adsorbent 7 (790 units); △, for adsorbent 9 (520 units); □, for adsorbent 10 (780 units); on Fig. 3b, ●, for adsorbent 4 (740 units); □, for adsorbent 5 (780 units); ○, for Octyl-Sepharose CL-4B (610 units); △, for CH-Sepharose 4B (680 units); on Fig. 3c, ○, for adsorbent 1 (730 units); □, for Phenyl-Sepharose CL-4B (930 units). The symbol ● represented the concentration (M) of ammonium sulfate in 5 mM Tris–acetate buffer (pH 7.8).

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The results of chromatographies with CH-Sepharose 4B, adsorbents 4 and 5 showed that β-glucuronidase was adsorbed on the insoluble support, Sepharose itself, under high concentrations of salt.

The adsorbed enzyme was eluted at 0.5 M ammonium sulfate from the adsorbent coupled glycosides with an aryl group as aglycone such as adsorbent 7, 9, and 10, and it was eluted at 0.9 M from adsorbent 6, which contained an aliphatic group in the ligand as aglycone. The enzyme that was adsorbed to aniline–CH-Sepharose 4B was not eluted at all from the column under decreased concentrations of ammonium sulfate, while only about 30% of the adsorbed enzyme was eluted with 0.05 M acetate buffer at pH 4.5 (Fig. 3c).

After β-glucuronidase was adsorbed on the columns of hydrophobic adsorbents such as either Octyl-Sepharose CL-4B or Phenyl Sepharose CL-4B under the presence of 3 M ammonium sulfate, all of the adsorbed enzymes were eluted from Octyl-Sepharose CL-4B at 0.8 M (Fig. 3b), while about 50% of the enzyme was eluted at 0.05 M of the salt from Phenyl Sepharose CL-4B but the residue remained in the adsorbent (Fig. 3c). This result suggested that a hydrophobic interaction might have weakly taken place in Octyl-Sepharose, while strongly taking place in Phenyl-Sepharose under the existence of salt.

Discussion

T. Fujita *et al.*[9] noted that some enzymes were purified by hydrogen bond chromatography at which a hydrogen bonding between a protein and a support having many hydroxyl groups was utilized under high concentrations of salt.

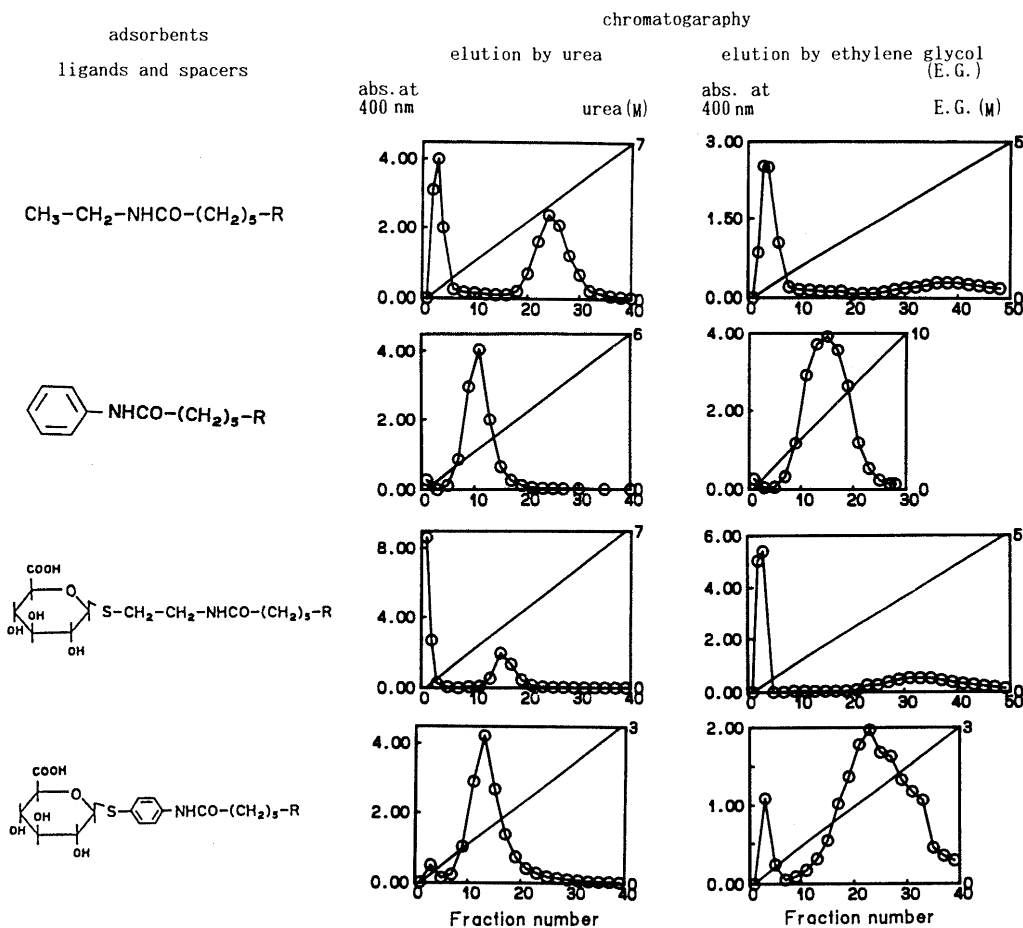

Fig. 4. Elution of β-Glucuronidase from Adsorbents in Columns by the Increased Gradient of Urea and Ethylene Glycol

After one ml of adsorbents in the columns was swollen by 1 or 2 M ammonium sulfate–5 mM Tris–acetate buffer (pH 7.8), 0.5 ml of the enzyme dissolved by the same buffer solution including 1 M ammonium sulfate was added. Urea or ethylene glycol was added with the increased gradient to the columns, and the found enzyme activity was denoted by the symbol ○. A solid line represented the increased gradient of urea or ethylene glycol (they were not determined). The added enzyme activities are summarized on the following table.

Adsorbents	Ammonium sulfate (M)	The added enzyme activity (units)	
		The elution reagents	
		Urea	Ethylene glycol
5	2	420	470
1	1	200	440
6	2	870	270
7	1	310	330

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It was expected that a hydrogen bonding reaction might take place between β-glucuronidase and the sugar of ligand in an adsorbent with a glycoside. So, the hydrogen bonding chromatography was experimented with adsorbents having glycosides. At the chromatography, CH-Sepharose 4B and some other adsorbents with non-glycosidic compounds as ligand were also experimented with, for it was expected that Sepharose itself might adsorb the enzyme under the presence of salt because of its being a polysaccharide. The enzyme was adsorbed to the adsorbents under the presence of ammonium sulfate to which the enzyme had no affinity under the absence of salt, and the adsorbed enzyme was eluted more easily from them than from the adsorbents with glycosides. This result suggested that a sugar part of a ligand might interact with the enzyme under the presence of salt (Fig. 3, Table III).

Although the ligand compounds of both absorbent 6 and 7 were glucuronides, the adsorbed enzyme was eluted from adsorbent 6 at 0.9 M concentration of the salt and at 0.5 M of the salt with adsorbent 7.

We then further investigated whether or not the adsorption of the enzyme under the presence of ammonium sulfate could be attributed to hydrogen bonding. Since both ethylene glycol and urea are well known as reagents for weakening hydrogen bonding, those reagents were added to the adsorbed enzyme (Fig. 4). The enzyme was immediately eluted by the addition of urea, while it was, though slowly, eluted with ethylene glycol. Therefore, it seems that the hydrogen bonding reaction is related to the interaction between the enzyme and the adsorbent in the presence of ammonium sulfate.

The affinity of the enzyme for aniline–CH-Sepharose 4B was stronger than the affinity for ethylamine–CH-Sepharose 4B and the tendency was also similiar to the correlation between adsorbent 7 and 6.

T. Fujita *et al.* elucidated that ion exchange groups coupled to cellulose were more or less functional for the adsorption of the used enzymes even under the presence of 1—3 M ammonium sulfate.[9] It was suggested by their elucidation that the difference of the adsorption of the enzyme between adsorbent 1 and 5 and adsorbent 7 and 6 may be attributed to the difference of the electrostatic forces in the adsorbents with each other. This suggestion was supported by the results in which some of the adsorbed enzymes were eluted from aniline–CH-Sepharose 4B by 0.05 M acetate buffer (pH 4.5) even though it was only about 30%.

About thirty kinds of adsorbents were prepared and the chromatographies by those adsorbents were perfomed. The correlation between the chemical structure of ligand compounds in adsorbents and the affinity of the enzyme for those adsorbents was investigated on the results of chromatographies. 1) However, if an adsorbent with a hydroxy group in the ligand has amide bonding, β-glucuronidase then has an affinity for the adsorbent. However, if the adsorbent does not have such bonding, then it is not able to catch the enzyme even if the ligand is a monosaccharide. 2) Acidic dissociation constants of amines (pK_a) are ethylamine: 10.63,[10a] phenylethylamine: 9.83,[10b] benzylamine: 9.34,[10b] aniline: 4.62.[10c] The acidity of the carbonyl part in the amide in the adsorbents prepared with those amines and CH Sepharose 4B may become stronger in the following order C_6H_5–NHCO– > C_6H_5–CH_2–NHCO– > C_6H_5–CH_2–CH_2–NHCO ≫ CH_3–CH_2–NHCO–, and when chromatography is done by the use of those adsorbents, the tendency of the affinity of β-glucuronidase for those adsorbents correlated with the acidity of the carbonyl group in amide on those adsorbents. 3) As for adsorbents prepared with Epoxy-activated Sepharose 6B and amine, the tendency of basicity on the imide part in those adsorbents was related to the affinity of the enzyme, and all of adsorbed enzymes were eluted by 0.1 M NaCl. This result shows that an adsorbent with imide bonding has a lower affinity for the enzyme than does an adsorbent with amide bonding. 4) In chromatography with an adsorbent in which either acetylated sugars or methylated sugars were coupled over amide bonding to spacer arms of solid matrix, β-glucuronidase was adsorbed to those adsorbents.

As shown in Fig. 1, β-glucuronidase passed through the columns of adsorbents in which acetylated sugars were coupled to spacers over imide bonding, but the enzyme was adsorbed to an adsorbent in which a methylated sugar was coupled. It is interesting to note that various kinds of sugar derivatives of ligands of adsorbents may be concerned in the affinity of the enzyme for those adsorbents.

It is assumed from these results that the sugar part of the ligand of an adsorbent may not be essential to the adsorption of the enzyme. However, whereas 1 ml of ethylamine–CH-Sepharose 4B adsorbed only 6 units of the enzyme, ethyl 1-thio-β-D-glucuronide–CH-Sepharose 4B adsorbed 800 units or more in 5 mM Tris–acetate buffer (pH 7.8). So it was suggested that the sugar part of the ligand may be involved in an interaction with the enzyme.

About 20—30% of the adsorbed enzymes left in the columns at elution by 0.5 M NaCl from the adsorbents coupling glycosides over an amide bonding. It seems that a hydrogen bond reaction may have taken place between the enzyme and the adsorbents under a high concentration of NaCl. Therefore, in the chromatography with adsorbents coupling glycosides care must be taken in the use of high concentrations of NaCl on the elution of enzymes from adsorbents with glycosides as ligands.

The adsorbed enzyme was eluted from glucuronide–Sepharose such as adsorbent 6 and 7 by 0.1 M NaCl, but could not be eluted from other glycosides–Sepharose such as adsorbent 8, 9, 10 by 0.1 M NaCl. This result explained that the affinity of the enzyme for glucuronide–Sepharose may be lower than that for other glycosides–Sepharose. The tendency of the affinity could not be changed by modifying the free carbonic acid in the glucuronides in the adsorbents with either methyl ester or ethylamide (data not shown).

C. Blanco *et al.* purified *E. coli* β-glucuronidase with phenyl 1-thio-β-D-glucuronic acid–AH-Sepharose 4B (TPU-Sepharose). We examined the adsorption of the enzyme to TPU-Sepharose under the presence of ammonium sulfate (Fig. 5). Some of the enzyme passed through a column of the adsorbent under a 3 M concentration of salt and a part of the adsorbed enzyme was eluted at 1.7 M.

The affinities of the enzyme for some adsorbents under the presence of salt were compared (Table IV). The affinity

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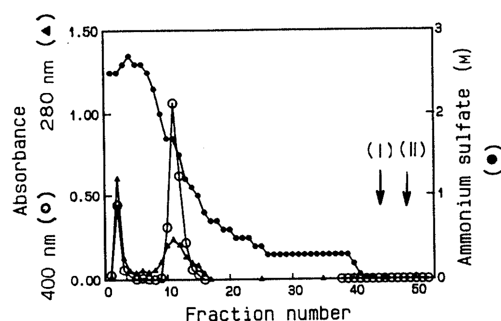

Fig. 5. Elution Patterns of β-Glucuronidase under the Degradation of Concentration of Ammonium Sulfate with TPU-Sepharose

The experimental method was the same as described in Fig. 3c. The added enzyme activity was 240 units. A symbol ○ showed the enzyme activity in the fraction tube; ●, expressed the concentration (M) of ammonium sulfate. The elution by 0.2 M NaCl was shown by (I→) and the elution by 0.05 M acetate buffer (pH 4.5) was shown by (II→).

TABLE IV. Effect of Concentration of Ammonium Sulfate on Adsorption of β-Glucuronidase to the Adsorbents

Adsorbent[a]	Concentration of ammonium sulfate (M)			
	0	1	2	3
Adsorbent 1	Adsorbed	Adsorbed	Adsorbed	Adsorbed
Adsorbent 5	Not[b]	Not[b]	A little[c]	Partly[c]
Adsorbent 7	Adsorbed	Adsorbed	Adsorbed	Adsorbed
Adsorbent 6	Adsorbed	Not[b]	A little[c]	A little[c]
TPU-Sepharose[d]	Adsorbed	Not[b]	A little[c]	A little[c]

a) Adsorbent 1, 5, 6, 7 are listed in Table II. *b*) Not indicates that the added enzyme was not adsorbed. *c*) A little or partly were concluded by the comparison of the extent of adsorption with the result of the chromatography with adsorbent 7. *d*) TPU-Sepharose presents phenyl 1-thio-β-D-glucuronic acid–EAH-Sepharose 4B.

of the enzyme for TPU-Sepharose was similiar to the affinity for adsorbent 6. This result showed that the enzyme had a lower affinity for TPU-Sepharose than for adsorbent 7 under the presence of salt although the phenyl group was included in TPU-Sepharose and it may be explained by a weaker acidity of the amide part in TPU-Sepharose than in adsorbent 7.

Hydrophobic interaction chromatography based on the affinities between the hydrophobic groups in adsorbents and the hydrophobic part of proteins has been utilized in the purification of proteins.[11] We investigated whether or not β-glucuronidase was adsorbed by hydrophobic interaction. Figure 2 shows that β-glucuronidase had no affinity for either Octyl Sepharose CL-4B or Phenyl-Sepharose CL-4B under 5 mM Tris–acetate buffer at pH 7.8, while the adsorbed enzyme was only slightly eluted from adsorbents 1, 6, 7, 9 by sodium cholate. It was then determined that the enzyme adsorbed to adsorbent 7 was not eluted either by cholic acid (0.1%), Triton X-100 (0 to 3%) or Tween-80 (0 to 3%) (data not shown). Thus, it appears that the affinity of β-glucuronidase for adsorbents with amide bonding was not attributable to a hydrophobic interaction under 5 mM Tris–acetate buffer at pH 7.8.

The adsorbed enzyme under the existence of 3 M ammonium sulfate left about 70% on aniline–CH-Sepharose 4B although 0.05 M acetate buffer (pH 4.5) was added after the degradation of salt. On Phenyl-Sepharose CL-4B the adsorbed enzyme left about 50% and it left about 60% on TPU-Sepharose under the degradation of salt. This result suggests that the enzyme may have interacted with a free phenyl group in the adsorbent under the existence of ammonium sulfate.

We thus concluded from the experimental results that an interaction between β-glucuronidase and an adsorbent having a glycoside as ligand may be done at first on the amide part in the adsorbent, and secondly on the sugar part of the ligand. We are now planning further studies on the binding between the enzyme and the amide parts in adsorbents and on the effect of the sugar part in the ligand on the adsorption. It has been previously described[8] and confirmed by preliminary tests in this study that the adsorption of β-glucuronidase to adsorbent 7, 9, 10, Phenyl-Sepharose CL-4B and others did not occur at its enzyme active sites but somewhere else, yet its binding sites still unclear.

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