Chem. Pharm. Bull. 30(5)1753—1759(1982)

Adsorption of Some β-Glycosidases on *N*-(ε-Aminohexanoyl)-β-D-glycosylamine-agarose

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(Received August 8, 1981)

Adsorption of β-galactosidase (EC 3.2.1.23), β-glucosidase (EC 3.2.1.21) and β-acetylglucosaminidase (EC 3.2.1.30) on agarose substituted with *N*-(ε-aminohexanoyl)-β-D-galactosylamine, -β-D-glucosylamine, -2-acetamido-2-deoxy-β-D-glucosylamine and -β-D-mannosylamine was studied.

All the glycosidases were adsorbed only at low buffer concentration. Although the adsorptions of Taka-diastase and Sanactase glycosidases were non-biospecific, the adsorption required the presence of the glycone as a ligand and the elution patterns in column chromatography were affected by variation of the glycone moiety of the adsorbent. The adsorptions of the enzymes on galactosylamine-agarose were prevented by low concentrations of hexoses and pentoses.

The adsorption and desorption of emulsin glycosidase were apparently biospecific considering that the enzyme protein has one binding site for glucoside and one for galactoside, although the biospecificity was inexplicable in view of the large K_i values of the spacer-ligand compounds.

Keywords——adsorption; affinity chromatography; inhibition constant; *N*-aminocaproyl-β-glycosylamine-agarose; β-galactosidase; β-glucosidase; β-acetylglucosaminidase; emulsin; *Aspergillus niger*; *Aspergillus oryzae*

Many glycosidases have been effectively purified by affinity chromatography on the substrate or its analog bound to agarose, as shown for human β-D-galactosidase,[2] for example. However, glycosidases and also other enzymes are often adsorbed non-biospecifically on affinity adsorbent, giving a perplexing result inexplicable in terms of affinity between the enzyme and ligand used.[3] Nevertheless, the non-biospecific adsorption is considered to be beneficial when it acts synergistically to produce adsorption with a ligand having only insufficient affinity.[3b] The non-biospecific adsorption is usually ionic strength-dependent and is ascribed to the electrostatic interaction between charged enzyme and adsorbent and also to the hydrophobic interaction due to the hydrophobicity of the spacer arm.[3b] Non-biospecific adsorption was observed more often with glycosidases than with other enzyme proteins, presumably because an effective ligand is difficult to obtain and hence the chromatography was often carried out at low buffer concentrations. On the other hand, some authors have stated that the "non-biospecific" adsorption of glycosidases required the presence of a sugar ligand on the adsorbent.[3a,d,e]

Mega and Matsushima described the non-biospecific nature of the adsorption of Taka-glycosidases and others on agarose substituted with *p*-aminophenyl or *p*-aminothiophenyl β-D-glycosides.[3d,e] This paper deals with a similar comparative study on the adsorption of Taka-glycosidases and others on *N*-(ε-aminohexanoyl)-β-D-glycopyranosylamine-agarose, and describes experiments carried out in order to elucidate the nature of the non-biospecific adsorption.

Experimental

Glycopyranosylamine Derivatives and Related Compounds——*N*-Acetyl-β-D-glycopyranosylamine derivatives of D-galactose (AcGal), D-glucose (AcGlc), and D-mannose (AcMan) were prepared by *N*-acetylation of β-D-glycopyranosylamine with Ac_2O in MeOH in the usual way instead of treatment with Ac_2O–pyridine mixture and subsequent de-*O*-acetylation as described by Isbell and Frush.[4]

Spacer-ligand compounds, *N*-(ε-aminohexanoyl)-β-D-galactopyranosylamine (ACapGal), -β-D-glucopyranosylamine (ACapGlc), and -β-D-mannopyranosylamine (ACapMan) were prepared from β-D-glycopyranosylamines[4] by coupling with *N*-benzyloxycarbonyl-ε-aminohexanoic acid (CbACap) and subsequent removal of the benzyloxycarbonyl group under the conditions described by Gordon *et al.*[5] for β-D-galactopyranosylamine derivative. *N*-Acetyl derivatives of ACapGal, ACapGlc, and ACapMan (AcACapGal, AcACapGlc, and AcACapMan) were obtained by treatment with Ac_2O in MeOH at room temperature; the product separated from the reaction mixture on standing at 4°C. The properties of these compounds were as follows (melting points are uncorrected and specific rotations are those at room temperature). AcACapGal, mp 238—239°C (recrystallized from MeOH–water), yield, 60%. *Anal.* Calcd for $C_{14}H_{26}N_2O_7$: C, 50.29; H, 7.84; N, 8.38. Found: C, 49.82; H, 7.69; N, 8.20. CbACapGlc, mp 169—170°C (from EtOH), $[\alpha]_D$ −9.8° (c=2, dimethylformamide), yield, 75%. *Anal.* Calcd for $C_{20}H_{30}N_2O_8$: C, 56.33; H, 7.09; N, 6.57. Found: C, 56.64; H, 7.24; N, 6.55. ACapGlc, mp 188—189°C (from MeOH–Et_2O) $[\alpha]_D$ −10.5° (c=1, water), yield, 86%. Converted to AcACapGlc without further purification. AcACapGlc, mp 230—231°C (from MeOH–water), yield, 56%. *Anal.* Calcd for $C_{14}H_{26}N_2O_7$: C, 50.29; H, 7.84; N, 8.38. Found: C, 50.00; H, 7.80; N, 8.29. CbACapMan, mp 89—90°C (from MeOH), $[\alpha]_D$ −12.0° (c=1, dimethylformamide), yield, 55%. Converted to ACapMan without further purification. ACapMan, mp 192—193°C (from MeOH–Et_2O), $[\alpha]_D$ −9.8° (c=1, water), yield, 69%. *Anal.* Calcd for $C_{12}H_{24}N_2O_6$: C, 49.30; H, 8.28; N, 9.58. Found: C, 49.26; H, 8.27; N, 9.75. AcACapMan, mp 240—241°C (from MeOH–water), yield, 60%. *Anal.* Calcd for $C_{14}H_{26}N_2O_7$: C, 50.29; H, 7.84; N, 8.38. Found: C, 50.09; H, 7.84; N, 8.30.

Similar derivatives of 2-aminoethanol (CbACapEtOH and ACapEtOH) were prepared. CbACapEtOH, mp 89—91°C (recrystallized from MeOH–water after removal of the faster-migrating CbACap by chromatography on silica gel (Merck, 70—235 mesh) irrigated with $CHCl_3$–MeOH–water, 30: 10: 1 (v/v)), yield, 58%. *Anal.* Calcd for $C_{16}H_{24}N_2O_4$: C, 62.32; H, 7.85; N, 9.08. Found: C, 62.08; H, 8.01; N, 9.02. ACapEtOH, mp 145—147°C (from EtOH–Et_2O), yield, 69%. *Anal.* Calcd for $C_8H_{18}N_2O_2$: C, 55.14; H, 10.41; N, 16.08. Found: C, 55.62; H, 10.40; N, 15.99.

Adsorbents——2-Acetamido-1-*N*-(ε-aminohexanoyl)-2-deoxy-β-D-glucopyranosylamine-Sepharose 4B (GlcNAc-agarose) was prepared according to the procedure of Lotan *et al.*[6] *N*-(ε-Aminohexanoyl)-β-D-galactopyranosylamine, -β-D-glucopyranosylamine, -β-D-mannopyranosylamine, and -2-aminoethanol each bound to Sepharose 4B (Gal-agarose, Glc-agarose, Man-agarose, and EtOH-agarose) were prepared by coupling of ACapGal, ACapGlc, ACapMan, and ACapEtOH to Sepharose 4B by the cyanogen bromide method of March *et al.*[7] Ligand contents were determined by analyzing for ε-aminohexanoic acid (using an amino acid analyzer) after hydrolysis in 6 N HCl at 110°C in an evacuated sealed tube. Ligand contents (μmols per ml of settled gel) were: Gal-, 6.1; Glc-, 3.3; GlcNAc-, 5.0; Man-, 5.0; EtOH-agarose, 3.8.

Enzymes——Partially purified glycosidase mixtures were used. Taka-diastase (Sankyo, *Aspergillus oryzae* enzyme) was used after partial purification by treatment with $Ca(Ac)_2$ and ammonium sulfate precipitation as described by Mega *et al.*[8] Specific activities (units per mg protein) were: β-galactosidase (EC 3.2.1.23), 0.056; β-glucosidase (EC 3.2.1.21), 0.057; β-acetylglucosaminidase (EC 3.2.1.30), 0.105. Sanactase (Meiji Seika, *Aspergillus niger* enzyme) was partially purified by two ammonium sulfate precipitations. Specific activities (units per mg protein) were: β-galactosidase, 0.014; β-glucosidase, 0.023; β-acetylglucosaminidase, 0.027. Almond emulsin (Sigma) used here had specific activities (units per mg protein) of 0.64 (β-galactosidase) and 3.3 (β-glucosidase).

Enzyme Assay——The activities were assayed by incubation at 37°C with 2.5 mM appropriate *p*-nitrophenyl-β-D-glycopyranoside in 0.1 M buffer (sodium acetate, pH 5.0, for β-galactosidase and β-glucosidase, sodium citrate, pH 4.5, for β-acetylglucosaminidase activity).

One unit of activity releases 1 μmol of *p*-nitrophenol per minute under the conditions used. Protein was determined by the method of Lowry *et al.*[9] using bovine serum albumin as a standard.

Column Chromatography——All procedures were carried out at 9°C, a higher temperature than in batch experiments because of equipment limitations. A solution (2—3 ml) of glycosidase mixture previously dialyzed against 20 mM acetate buffer, pH 5.0, was applied to a column (1.5×20.5 cm, 36 ml) packed with adsorbent equilibrated with the same buffer. Elution was performed by stepwise changes of eluent (three column volumes) as shown in Figs. 1—3.

Batch Experiments——Appropriate buffer (2 ml) and enzyme solution (0.1 ml) were added to 2 ml of gel suspension (gel settled overnight–water, 1: 1 (v/v)) in a polyethylene tube, and the mixture was shaken for 1 h and centrifuged at 2000 rpm for 10 min. The adsorption procedures were carried out at 4°C unless otherwise noted. The residual activities in the supernatant were assayed to calculate the amount of adsorbed enzyme.

Results

Figs. 1—3 show the elution patterns of the glycosidase activities and protein from columns of *N*-(ε-aminohexanoyl)-β-D-glycopyranosylamine-agarose (Gal-, Glc-, GlcNAc-, and Man-agarose) at 9°C. All of the β-glycosidase activities passed through the columns packed with

untreated agarose, agarose treated with cyanogen bromide, agarose substituted with ε-aminohexanoic acid, and agarose substituted with ε-aminohexanoyl aminoethanol (EtOH-agarose) even at low ionic strength, as shown for EtOH-agarose, for example.

Taka-glycosidases

As shown in Fig. 1, the adsorptions of the three β-glycosidases were non-biospecific; galactosidase and glucosidase were adsorbed on GlcNAc- and Man-agarose, and acetylglucosaminidase adsorbed on Gal-, GlcNAc- and Man-agarose was eluted with buffer containing 1 M sodium chloride. The use of eluent containing 50 mM counter-ligand, a monosaccharide corresponding to the glycone moiety of the ligand, did not promote the release of glycosidase activity.

The adsorption was also studied by batch experiments. The buffer concentrations at which half the activity was adsorbed were determined from (adsorbed enzyme)–[buffer concentration] plots (not shown). The values (mM) were as follows. Galactosidase; Gal-, 47; Glc-, 11; GlcNAc, 150; Man-agarose, 23. Glucosidase; Gal-, 37; Glc-, 60; GlcNAc-, 430; Man-agarose, 111. Acetylglucosaminidase; Gal-, 180; Glc-, 85; GlcNAc-, 460; Man-agarose, 142. Not all of the values are in accord with the chromatographic behaviors, presumably because of the hysteresis of adsorption[3c] as well as the difference of operational temperature; the batch experiment involves only adsorption while chromatography involves a desorption procedure following adsorption. The adsorptions in 20 mM acetate buffer were markedly weaker at 37°C than at 4°C; for example, Man-agarose adsorbed only 22% of galactosidase activity at 37°C while it adsorbed 96% of the activity at 4°C. The three glycosidases adsorbed at 4°C, however, were not released by raising the temperature to 37°C, but were recovered completely with the buffer containing 1 M sodium chloride in batch experiments.

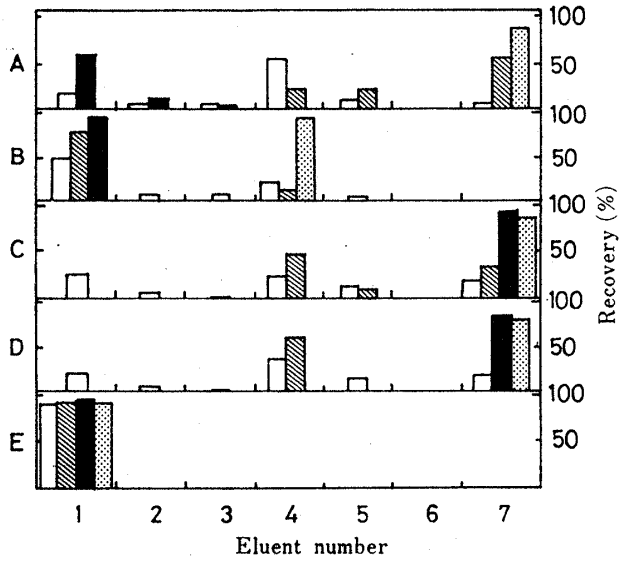

Fig. 1. Elution Patterns of Taka-glycosidases

A solution of enzyme (106 mg as protein) was applied to each column (1.5×20.5 cm). Elution was performed at 9°C by stepwise changes of eluent (sodium acetate buffer, pH 5.0): 1, 20 mM buffer; 2, 20 mM buffer containing 50 mM counter-ligand; 3, 20 mM buffer; 4, 50 mM buffer; 5, 50 mM buffer containing 50 mM counter-ligand; 6, 50 mM buffer; 7, 50 mM buffer containing 1M NaCl. Eluents 3 and 6 were used to remove counter-ligand from the column. The activity or activities appeared almost simultaneously with protein in each step.

Adsorbent: A, Gal-; B, Glc-; C, GlcNAc-; D, Man-; E, EtOH-agarose. □, protein; ▨, β-galactosidase; ■, β-glucosidase; ▩, β-acetylglucosaminidase activity, each expressed in percentage recovery.

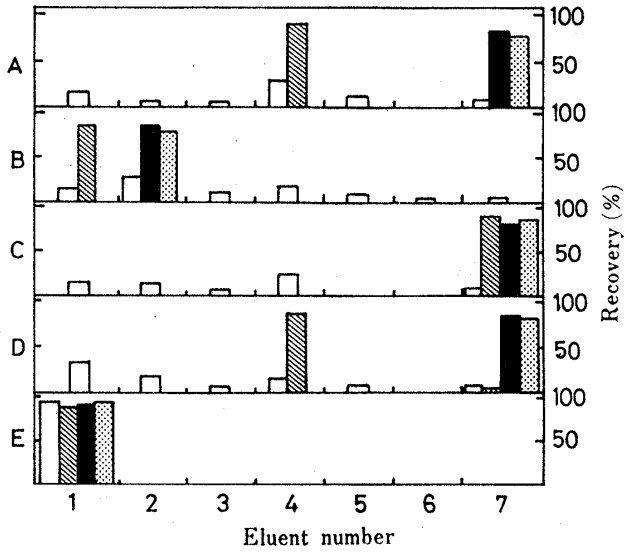

Fig. 2. Elution Patterns of Sanactase-glycosidases

An enzyme solution (106 mg as protein) was applied. Procedures and symbols are the same as in Fig. 1.

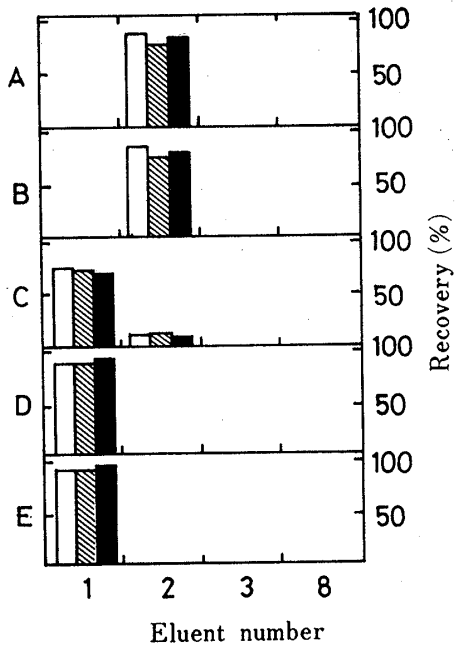

Fig. 3. Elution Patterns of Emulsin

An enzyme solution (2.1 mg as protein) was applied. Procedures and symbols are the same as in Fig. 1 but step 3 was followed by step 8, elution was 20 mM buffer containing 1 M NaCl.
At step 1 with c, both activities appeared concomitantly in two peaks.

The following experiments suggested that the adsorbed glycosidases were active in the state bound to the adsorbent. A solution of Taka-glycosidases was shaken with the adsorbents (Gal-, GlcNAc-, and Man-agarose) in 20 mM acetate buffer at pH 5.0 and the adsorbents were filtered off and washed with the same buffer to remove the free glycosidases. The glycosidases adsorbed on the adsorbents exhibited 57—80% of the activities adsorbed in the above procedure, when assayed by incubation with substrate in the same buffer at 37°C. After incubation, the adsorbent was removed by filtration at 37°C while the reaction was continuing, and the filtrate was dialyzed to removed the substrate and products. The filtrate showed no activity, while in control experiments with an incubation mixture containing enzyme, substrate and unsubstituted gel, 86—91% of the activities were recovered after dialysis.

Table I shows the effect of hydroxy compounds on the adsorption of galactosidase and acetylglucosaminidase on Gal-agarose. Interestingly, the adsorption of the glycosidases was suppressed considerably by pre-equilibration at

Table I. Effects of Various Sugars and Related Compounds on the Adsorption of Some β-Glycosidases on Gal-agarose

Compound / Concn. of compound	Activity not adsorbed[a] (%)					
	Taka-diastase				Emulsin	
	Galact-osidase		Acetylgluc-osaminidase		Galact-osidase	Gluc-osidase
	1 mM	10 mM	1 mM	10 mM	10 mM	10 mM
Ethanol	0	0	0	0	0	0
Ethylene glycol	0	0	0	0	0	0
Glycerin	0	0	0	0	0	0
D-Arabinose	70	94	80	92	0	0
L-Arabinose	78	95	76	88	0	0
D-Xylose	94	95	90	89	0	0
L-xylose	97	92	88	96	0	0
D-Galactose	80	93	77	96	98	96
D-Glucose	92	97	91	90	5	4
L-Glucose	93	91	98	97	0	0
D-*N*-Acetylglucosamine	99	97	94	96	0	0
D-Mannose	90	93	92	96	0	0
D-Glucitol	5	40	9	38	0	0
D-Mannitol	10	49	14	45	0	0
D-*N*-Acetylglucosaminol	8	50	10	53	0	0
AcGal	95	95	93	94	90	90
AcGlc	94	95	92	96	0	0
AcMan	94	98	94	97	0	0

a) A mixture for adsorption experiments contained enzyme solution (0.1 ml) and dilute gel suspension (1: 1, 4 ml), each pre-equilibrated with an appropriate sugar (1 or 10 mM) in 20 mM acetate buffer, pH 5.0, at 4°C for 1 h.

Table II. Inhibition Constants[a] of Spacer-ligand and Related Compounds for Taka-glycosidase Activities

Compound	K_i values (mM)[a] for β-Galactosidase			β-Glucosidase			β-Acetylglucosaminidase		
Buffer concn.	20 mM	50 mM	20 mM 1 M NaCl	20 mM	50 mM	20 mM 1 M NaCl	20 mM	50 mM	20 mM 1 M NaCl
D-Galactose	5.2	5.6	—	(180)	(260)	—	(790)	(640)	—
D-Glucose	N.I.[b]	N.I.	—	1.6	1.6	2.0	(350)	(370)	—
D-*N*-Acetylglucosamine	N.I.	N.I.	—	N.I.	N.I.	—	7.3	7.4	7.0
D-Mannose	N.I.	N.I.	—	40.2	35.5	39.0	(280)	(300)	—
N-Acetyl-β-D-glycopyranosylamines									
AcGal	88.5	90.4	80.4	N.I.	(450)	—	(350)	(370)	—
AcGlc	N.I.	N.I.	—	5.0	5.0	3.8	(100)	(100)	(90)
AcGlcNAc[c]	N.I.	N.I.	—	(700)	(450)	—	17.8	20.3	18.2
AcMan	N.I.	N.I.	—	(550)	(690)	—	10.5	10.4	11.2
N-(ε-Acetamidohexanoyl)-β-D-glycopyranosylamines									
AcACapGal	245	245	208	N.I.	N.I.	—	N.I.	N.I.	N.I.
AcACapGlc	N.I.	N.I.	N.I.	5.1	5.5	5.4	(350)	(410)	N.I.
AcACapGlcNAc[d]	N.I.	N.I.	N.I.	(460)	(540)	—	27.9	28.2	22.5
AcACapMan	N.I.	N.I.	N.I.	(700)	(1400)	—	N.I.	N.I.	N.I.

a) Experiments were carried out at 37°C. K_i values were obtained from Hofstee plots which indicated competitive inhibition, except for the values in parentheses, which were roughly calculated from the percentage inhibition by 50 mM inhibitors at substrate concentrations of 0.15 mM assuming competitive inhibition. K_m values used were 0.75, 0.21 and 0.65 mM for *p*-nitrophenyl β-galactoside, β-glucoside and β-acetylglucosaminide, respectively.
b) N.I. indicates that no detectable inhibition was observed under the conditions described in *a*).
c) 2-Acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine.
d) 2-Acetamido-1-*N*-(ε-acetamidohexanoyl)-2-deoxy-β-D-glucopyranosylamine.

4°C with 1 mM each of galactose and acetylglucosamine. Any activity once adsorbed in 20 mM buffer in the absence of sugar could not be released by addition of 50 mM sugar. Glycerine, ethylene glycol, and ethanol exhibited no sxppression even at 50 mM concentrations.

To confirm the above indications that the binding of glycosidase to immobilized ligand does not involve the active site, the inhibition constants, K_i's, of the spacer-ligand and related compounds were determined. Table II shows the K_i values measured at 37°C, where *N*-acetyl derivatives were examined instead of amino compounds capable of interacting electrostatically. The K_i values of spacer-ligand compounds were not sufficiently small for adsorption. The K_i values (and K_m values, not shown) were essentially unaffected by increase in buffer concentration, in contrast to the adsorption, which was dependent on buffer concentration. Substitution with ε-acetamidohexanoyl rather decreased the affinity of glycopyranosylamine derivatives.

Other β-Glycosidases

Elution patterns of Sanactase were again indicative of non-biospecific adsorption (Fig. 2); all three glycosidases were adsorbed on Gal-, Glc-, GlcNAc- and Man-agarose, except for galactosidase on Glc-agarose, and addition of 50 mM glucose to eluent promoted the release of acetylglucosaminidase as well as glucosidase from a Glc-agarose column.

As shown in Fig. 3, emulsin glycosidase behaved in a biospecific manner in chromatography provided that the glycosidase is a single protein having two different binding sites, one for galactose and the other for glucose structure.[10] Both activities retained on a Gal- or Glc-agarose column were eluted with 50 mM counter-ligand.

Batch experiments showed that the adsorption of the glycosidase activities was dependent on ionic strength and temperature; 80—95% of the activities that were adsorbed in 20 mM buffer was recovered by elution with 50 mM buffer or by raising the temperature to 37°C. The buffer concentrations at which half the activity was adsorbed were the same for both activities:

Gal-, 36; Glc-agarose, 39 mM. Both activities adsorbed on a Glc-agarose column were completely released by treatment with 50 mM glucose but not with galactose or other hexoses. Similarly, the activities adsorbed on Gal-agarose were released only with 50 mM galactose. As shown in Table I, galactose and *N*-acetylgalactosylamine (AcGal) specifically prevent the adsorption on Gal-agarose. On the other hand, the K_i values of the spacer-ligand compounds and the counter-ligands were large; K_i values (mM) for galactosidase and glucosidase in 20 mM acetate buffer at 37°C were roughly as follows: AcACapGal, 900, 2300; AcACapGlc, 1000, 200 (120 at 4°C); galactose, 720, 4700; glucose, 4900, 140, respectively. These values were substantially unaffected by raising the buffer concentration to 50 mM, whereas adsorption hardly occurred in the 50 mM buffer. These findings stand against the biospecificity of the adsorption.

Discussion

Non-biospecific or anomalous adsorption of glycosidases on affinity adsorbent has been reported.[3] However, the anomaly at low ionic strength was discussed on the basis of the supposed affinity of the ligand for the enzyme deduced from the structural similarity of the ligand to the substrate, without estimating the K_i values of ligand or spacer-ligand. In the present study, the K_i values of ligand, spacer-ligand and counter-ligand compounds were evaluated.

The adsorption experiments were carried out under conditions such that the amount of applied enzyme was much smaller than the adsorption capacity of the adsorbent, that is, the amount of adsorbed enzyme was proportional to the amount of added enzyme. In this case, the biospecific retardation, R, expressed in column volume units, is given by $R=[L]/K_{EL}$, where $[L]$ is the effective concentration of the immobilized ligand and K_{EL} is the dissociation constant of enzyme-immobilized ligand complex.[3b] In the present study, effective biospecific adsorption was not expected in view of the K_i values of the spacer-ligand compounds used here. These K_i values at 37°C represent a measure of effectiveness as a ligand, even though the adsorption procedures were performed at lower temperatures.

The modes of adsorption of Taka-glycosidases and Sanactase glycosidases on the adsorbents used were non-biospecific in view of the ionic strength dependence of the adsorption and the lack of ability of the enzymes to discriminate the glycone moiety of the ligand according to the enzymatic specificity. Elution patterns of glycosidase activities varied with change of the adsorbent and with the source of the enzyme. The variations of elution pattern of Taka-glycosidases could not be explained in terms of the K_i values of spacer-ligand compounds. The adsorbents used were effective to some extent for purification, however. As shown in Figs. 1 and 2, partial resolution and enrichment of specific activity of the glycosidases were observed.

Taka-glycosidases adsorbed on adsorbents were active without being released into the aqueous phase, indicating binding through a site other than the active site, as proposed by Mega and Matsushima[3d,e] using a different approach. Their proposal is based on the observation that the enzymes did not distinguish the difference in ligand structure and on the finding that a substrate bound to an adsorbent as a ligand was hydrolyzed and released by the enzyme faster when the adsorption of the enzyme was inhibited by 1 M sodium chloride than when all the enzyme was adsorbed. In the present study, it was demonstrated that the non-biospecific adsorption (examined only with Gal-agarose) was markedly suppressed by hydroxy compounds of pyranose structure. The suppression of adsorption by sugar, together with the requirement for a sugar ligand on the adsorbent for adsorption and the variation of elution pattern with change of the sugar moiety of the ligand, indicate that the non-biospecific adsorption does not simply arise from the hydrophobic effect of the spacer arm or electrostatic interaction between enzyme and adsorbent.

Recently, Kiss *et al.*[10] proposed that the active site of sweet almond emulsin β-glucosidase is composed of a single catalytic site and two distinct substrate binding sites, one for glucoside and the other for galactoside. The adsorption and desorption of emulsin enzyme were in accord with the above view. However, the large K_i values of the spacer-ligand and related compounds, and the occurrence of adsorption only at low ionic strength at low temperature suggest the non-biospecific character of the adsorption of this glycosidase.

It is very likely that glycosidases including the emulsin glycosidase have a high or low specificity sugar-binding site other than the active site, operative only at low ionic strength at low temperature.

Acknowledgement The authors are grateful to Dr. T. Morikawa for elemental analyses.

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