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BIOSPECIFICITY CHROMATOGRAPHY
The Investigation of the Active Site of *N*-Acetyl-β-D-Hexosaminidase with Affinity Chromatography

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Affinity chromatography of different natural biopolymers, including enzymes, is perhaps the most widely used modern technique for isolation and purification of these molecules. We have synthesized three biospecific sorbents with weak ion exchange properties by coupling ligands to carriers through hydrazide groups. These sorbents have no ion exchange or hydrophobic groups, thereby minimizing the influence of nonspecific binding on the process of affinity chromatography. These biospecific sorbents have been used for purification of *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52), and for study of its active site and of its sorption and elution mechanisms in affinity chromatography. Biospecificity of sorbents was suggested by adsorption of *N*-acetyl-β-D-hexosaminidase at optimum catalytic pH and by elution of the enzyme with minimal variation of pH.

By comparing the known data for mapping the active site of *N*-acetyl-β-D-hexosaminidase and energetic contributions of functional groups of inhibitor molecules with the obtained results, one can infer that sorption of the enzyme on biospecific sorbents is realized only by hydrogen bonds between the ligand used and ionizable groups of *N*-acetyl-β-D-hexosaminidase (p*K* 5.5).

INTRODUCTION

Affinity chromatography of enzymes is based on their ability to interact specifically and reversibly with various active ligands immobilized on insoluble matrices. Competitive inhibitors, being as a rule substrate analogs, are such biospecific ligands. The process of affinity chromatography may be described by the following scheme:

$$E+\bar{S}\text{–}\textcircled{P} \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} [E\bar{S}]\text{–}\textcircled{P} \qquad (1)$$

where E, $\bar{S}$, and Ⓟ are the enzyme, the substrate analog, and the carrier, respectively.

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Since the biospecific ligand in use is the substrate analog, some kinetic principles described by the Michaelis–Menten equation must be realized:

$$E+S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} [ES] \xrightarrow{K_{+2}} [EP] \underset{K_{-3}}{\overset{K_{+3}}{\rightleftharpoons}} E+P \qquad (2)$$

where S is the substrate and P is the product.

If the selective sorbent is prepared using a hydrophilic polymer carrier with long spacer arms containing no hydrophobic or ion exchange groups, it is obvious that the hypothetical equation of affinity chromatography (1) corresponds to the left part of Eq. (2). Therefore, such kinetic principles of enzymatic reactions as the dependence of the Michaelis constant (K_m) or the binding constant upon pH, ionic strength, and temperature must be comparable in this case as in the case of soluble enzyme and substrate.

The theoretical principles just described do not always take place in practice. Apparently this discrepancy may be explained by the presence of different ion exchange and/or hydrophobic regions in the matrices and spacer arms (1–5) and also by diffusion limitation of interaction of enzyme with immobilized ligand (6). The interference by electrostatic and hydrophobic interactions of sorbents used in the process of adsorption and elution of the enzymes markedly influences the character of this process, more or less distorting the expected pattern of chromatography. For this reason much attention has recently been focused on the synthesis of selective affinity sorbents without ion exchange and hydrophobic groups and the study of their properties.

In this paper we have described the biospecific chromatography of *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52) on various selective sorbents at varying pH values and some properties of the process.

EXPERIMENTAL

A mixture of glycosidases containing *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52) and β-galactosidase (EC 3.2.1.23) was isolated from an extract of the marine mollusc *Acmeae pallida* by hydrophobic chromatography (7,8). The activities of the enzymes were determined at 25°C in 0.1 M phosphate buffer (pH 4.2) using *p*-nitrophenyl-β-glycosides of the corresponding monosaccharides as substrates. The reaction was terminated by addition of 1 M Na_2CO_3 (1 ml), and the liberated *p*-nitrophenol was determined spectrophotometrically at 440 nm on a Specol instrument (Carl Zeiss, Jenna, DDR). The protein concentration was determined with Coomassie G-250 (9).

Synthesis of Biospecific Ligands

2-(2′-Acetamido-3′,4′,6′-tri-*o*-acetyl-β-D-glucopyranosyl)-2-pseudothiourea hydrochloride (**1**) was obtained from 2-acetamido-2-deoxy-3,4,6-tri-*o*-acetyl-α-D-glucopyranosyl chloride according to the method of Horton and Wolfrom (10).

Ethyl ester of 2-*S*-(2′-acetamido-3′,4′,6′-tri-*o*-acetyl-β-D-glucopyranosyl)-2-thioglycolic acid (**2**): To a solution of compound **1** (4.4 g, 10 mmol) in water (12 ml) and acetone (12 ml), 2 g (12 mmol) of ethyl ester of monobromoacetic acid, 1 g (10 mmol) of $NaHSO_3$, and 1.5 g (11 mmol) of K_2CO_3 were added with stirring. The mixture was stirred for 2 h at room temperature, diluted with water (25 ml), and product **2** was extracted with chloroform (30 ml). The extract was washed with 3% solution of $NaHCO_3$ (10 ml) and water (10 ml) and dried over Na_2SO_4. Chloroform was evaporated and residue was crystallized from 33% aqueous methanol to give compound **2** (3.4 g, 76%): m.p. 158°–158.5°C; $[\alpha]_D^{20}$ −70° (c 1, ethanol); infrared characteristics (ν_{max}^{KBr} cm^{-1}): 3300 (NH), 1739 (OAc), 1656, 1555 (CONH); NMR ($CDCl_3$, δ): 1.29–1.36 (3H; CH_3), 1.95 (3H; NAc), 2.0–2.08 (9H; OAc), 3.21–3.65 (2H; CH_2), 4.06–4.30 (4H; H_2, H_5, $2H_6$), 4.78–4.89 (1H; H_1, J: 11 Hz, β bond), 4.96–5.35 (2H; H_3, H_4), 6.16–6.25 (1H; NH).

Analysis: Calculated for $C_{18}H_{27}NO_{10}S$: C 48.10; H 6.05; N 3.12; S 7.09. Found: C 47.91; H 6.14; N 3.12; S 7.13.

2-*S*-(2′-Acetamido-3′,4′,6′-tri-*o*-acetyl-β-D-glucopyranosyl)-2-thioglycolic acid (**3**): To a solution of **1** (2 g, 4.5 mmol) in water (12 ml) and acetone (12 ml), 0.75 g (5.4 mmol) of monobromoacetic acid, 0.5 g (4.8 mmol) of $NaHSO_3$, and 1.2 g (9 mmol) of K_2CO_3 were added with stirring. The mixture was then stirred for 2 h at room temperature and 2 N HCl (11.2 ml, 22.5 mmol) was added. The solution was saturated with NaCl and product **3** was extracted with chloroform (20 ml). The extract was washed twice with water (10 ml) and dried (Na_2SO_4). Chloroform was evaporated and residue was crystallized from methanol to give compound **3** (total yield 1.3 g, 73%): m.p. 188°–188.5°C; $[\alpha]_D^{22}$–63° (c 0.39, chloroform); infrared characteristics (ν_{max}^{KBr} cm^{-1}): 3325 (NH), 2960, 1400, 920 (dimer, COOH), 1745 (OAc), 1660, 1540 (CONH); NMR (CD_3OD, δ): 1.93 (3H, NAc), 2.03–2.09 (9H, OAc), 3.17–3.39 (2H, CH_2), 3.95–4.22 (4H, H_2, H_5, $2H_6$), 4.73–4.85 (1H, H_1, J: 12 Hz, β bond), 4.94–5.18 (2H, H_3, H_4).

Analysis: Calculated for $C_{16}H_{23}NO_{10}S$: C 45.59; H 5.59; N 3.32; S 7.60. Found: C 45.62; H 5.67; N 3.03; S 7.60.

Methyl ester of 2-*S*-(2′-acetamido-3′,4′,6′-tri-*o*-acetyl-β-D-glucopyranosyl)-2-thioglycolylglycylglycine (**4**): To a solution of methyl ester of

glycylglycine hydrochloride (0.35 g, 2.3 mmol) in 90% aqueous tetrahydrophurane (25 ml), 0.23 g (2.3 mmol) triethylamine, 1 g compound **3**, and 0.56 g (2.7 mmol) dicyclohexylcarbodiimide were added. The mixture was stirred for 2 h at 2°–5°C and 16 h at room temperature. Then water (25 ml) was added and *N,N'*-dicyclohexylurea was collected and washed with water. The combined filtrate and washings were extracted with chloroform (3 × 15 ml). The extract was washed carefully with aqueous $NaHCO_3$ (15 ml), aqueous HCl (15 ml), water (15 ml), and then dried (Na_2SO_4). Chloroform was evaporated *in vacuo* at 40°C and residue was crystallized from ether to give compound **4** (1 g, 77%); m.p. 163°C; $[\alpha]_D^{20}$ −44.5° (c 0.25, ethanol).

Analysis: Calculated for $C_{21}H_{31}N_3O_{12}S$: C 45.91; H 5.69; N 7.65; S 5.83. Found: C 46.23; H 6.02; N 7.19; S 5.56.

Methyl ester of 2-*S*-(2'-acetamido-3',4',6'-tri-*o*-acetyl-β-D-glucopyranosyl)-2-thioglycolylglycylglycylglycine (**5**) was obtained from methyl ester of triglycine sulfate in a similar manner (total yield 1 g, 72%); m.p. 187°–188°C; $[\alpha]_D^{20}$ −107.5° (c 0.23, ethanol).

Analysis: Calculated for $C_{23}H_{34}N_4O_{13}S$: C 45.77; H 5.18; N 9.28; S 5.31. Found: C 46.01; H 5.28; N 9.16; S 5.12.

2-*S*-(2'-Acetamido-2'-deoxy-β-D-glucopyranosyl-)-2-thioglycolylhydrazide (**6**): To a solution of compound **2** (4.5 g, 10 mmol) in methanol (50 ml), 2.5 g (60 mmol) hydrazine was added with stirring. The mixture was stirred for 12 h at room temperature and compound **6** was collected, washed with methanol, and dried over Na_2SO_4 (total yield 3.2 g, 92%); m.p. 247°–249°C; $[\alpha]_D^{20}$ −58° (c 1, water).

Analysis: Calculated for $C_{10}H_{19}N_3O_6S$: C 38.83; H 6.19; N 13.58; S 10.27. Found: C 38.99; H 6.12; N 13.55; S 10.28.

2-*S*-(2'-Acetamido-2'-deoxy-β-D-glucopyranosyl-)-2-thioglycolylglycylglycylhydrazide (**7**): To a solution of compound **4** (1 g, 1.8 mmol) in methanol (10 ml), 0.35 g hydrazine (10.8 mmol) was added with stirring. The mixture was stirred for 20 h at room temperature and compound **7** was collected, washed with methanol, and dried (total yield 0.68 g, 90%); m.p. 219°–220°C; $[\alpha]_D^{17}$ −13° (c 0.13, methanol–water, 2 : 1).

Analysis: Calculated for $C_{14}H_{25}N_5O_8S$: C 39.70; H 5.96; N 16.54; S 7.57. Found: C 40.02; H 6.04; N 16.32; S 7.38.

2-*S*-(2'-Acetamido-2'-deoxy-β-D-glucopyranosyl-)-2-thioglycolylglycylglycylglycylhydrazide (**8**) was obtained from compound **5** in a similar manner (total yield 0.58 g, 75%); m.p. 225°–226°C; $[\alpha]_D^{22}$ −47° (c 0.18, methanol–water, 2 : 1).

Analysis: Calculated for $C_{16}H_{28}N_6O_9S$: C 39.99; H 5.88; N 17.49; S 6.67. Found: C 40.22; H 6.14; N 17.37; S 6.97.

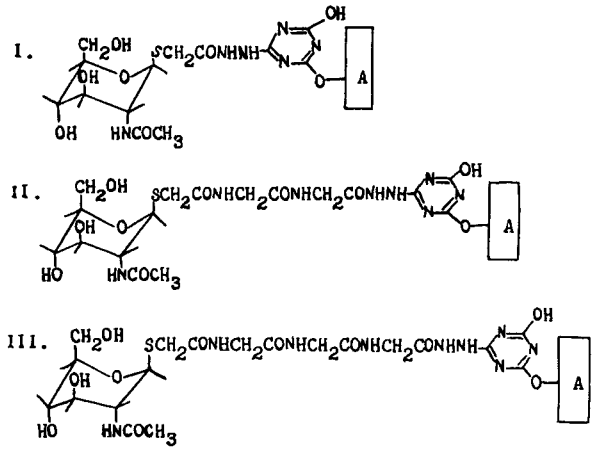


FIG. 1. The structures of the biospecific sorbents.

Preparation of Biospecific Adsorbents

Sepharose 4B (Pharmacia, Sweden) was activated with trichlor-*s*-triazine as described previously (7, 11). The sedimented agarose gel (25 ml) in 10–15 ml of distilled water was combined with 25 ml of acetone containing 1 g of trichlor-*s*-triazine. The pH value of the reaction mixture was adjusted to 8–9 and maintained at this value using a pH stat. The end of the reaction was determined by increasing pH to basic values (above 9) by addition of a small amount of 0.1 M NaOH. Further treatment of activated Sepharose 4B was carried out as described earlier (7, 11). The biospecific ligands containing the terminal hydrazide groups (2.3 mmol) (compounds **6**, **7**, **8**) in 25 ml of 7% $NaHCO_3$ were added to 25 ml of activated agarose gel. The reaction mixture was stirred for 2 h at room temperature. The selective sorbents obtained were washed with 1 liter of distilled water, resulting in complete removal of excess ligand. About 10–20 μmol of the ligand was found to be present per gram of Sepharose 4B. The structures of the biospecific sorbents are shown in Fig. 1.

Biospecific chromatography was conducted at 4°C and the elution patterns of columns were determined on a Uvicord II instrument (LKB, Sweden).

RESULTS AND DISCUSSION

The synthesis of biospecific sorbents **I**, **II**, and **III** was based on the following precepts. To eliminate any nonspecific interactions of sorbents

with the enzyme, neutral peptides containing no hydrophobic, acidic or basic amino acids were used as spacer arms. Biospecific sorbents with a large number of amido linkages in their composition were used to assure maximal attachment of spacer arms to protein structure. The coupling of ligand to carrier through a hydrazide group made it possible to obtain biospecific sorbents with weak ion exchange properties (pK 3.9). Activation of Sepharose 4B with trichlor-*s*-triazine resulted in a more stable end derivative than is obtained with CNBr (12).

The mixture of 3 mg of *N*-acetyl-β-D-hexosaminidase (specific activity of 21 U/mg) and β-galactosidase (specific activity of 3 U/mg) was chromatographed on specific affinity sorbents **I**, **II**, and **III** at pH 4.0, 5.0, 6.0, and 7.0 under constant conditions of temperature, ionic strength, size of column, and volume of specific sorbents. The amount of the adsorbed enzyme and elution patterns were calculated by determinating total activity of glycosidases and total protein in each fraction of the eluate. The results are shown in Figs. 2–4 and Table 1.

As can be seen in Fig. 2, *N*-acetyl-β-D-hexosaminidase was entirely adsorbed to the specific sorbents **I**, **II**, and **III** at pH 4.0. The β-galactosidase possessing a weak affinity for *N*-acetyl-D-glucosamine was bound rather loosely and eluted at increased ionic strength. When affinity chromatography was carried out at pH 5.0, a decrease in the ability of *N*-acetyl-β-D-hexosaminidase to bind to specific sorbents **II** and **III** was observed, indicated by appearance of this enzyme in the fractions of eluate during application of the sample to the column (Fig. 3, **III**) and washing of column with buffer containing 0.5 M NaCl (Fig. 3, **II**). With respect to sorbent **I**, insignificant increase in ionic strength diminished the adsorption of *N*-acetyl-β-D-hexosaminidase. Only slight retarding of this enzyme at pH 6.0 and no sorption at pH 7.0 were observed (Fig. 4). Comparison of the dependence on pH of adsorption of this enzyme to the specific carrier **II** is given in Table 1. *N*-Acetyl-β-D-hexosaminidase is slightly retarded relative

TABLE 1. **pH Dependence of Biospecific Adsorption of *N*-Acetyl-β-D-hexosaminidase on Sorbent II**

pH	Enzyme unretarding by sorbent (%)	Enzyme bonding by sorbent (%)	Elution of enzyme with 0.5 M NaCl (%)	Elution of enzyme with 50% ethylene glycol (%)
4.0	0	100	0	100
5.0	12	87	87	0
6.0	86	13	13	0
7.0	99	0	0	0

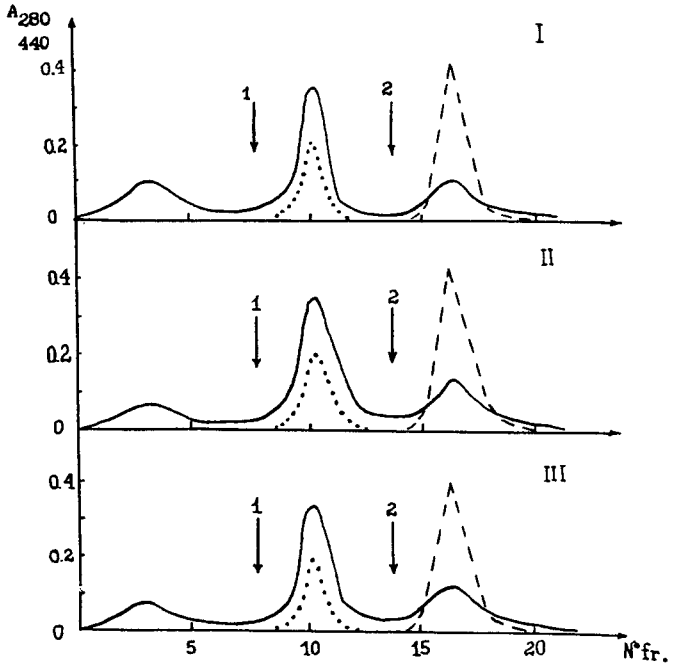


FIG. 2. Chromatography of glycosidases on the biospecific sorbents **I**, **II**, and **III** (pH 4.0). A mixture of enzymes was applied to a column (0.6×5 cm) equilibrated with 0.05 M phosphate buffer adjusted to pH 4.0 with phosphoric acid. Elution was carried out at 4°C with a flow rate of 20 ml/h. Fractions of 2-ml volume were collected. Curves: solid, protein concentration; dotted, activity of β-galactosidase; dashed, activity of *N*-acetyl-β-D-hexosaminidase. (1) 0.05 M phosphate buffer (pH 4.0) containing 0.5 M NaCl; (2) 50% ethylene glycol in buffer 1 (vol/vol).

to the major breakthrough of protein in its downward migration through the column at pH 5.0; binding at pH 6.0 is slight and no sorption is observed at pH 7.0. The data obtained indicate that certain ionizable groups are responsible for the formation of the enzyme–ligand complex in affinity chromatography as in the case of enzyme–substrate interaction. The amount of formed enzyme–ligand complex and its stability decreased with the increase of the degree of dissociation of these groups. Thus, the ionization constant of these active sites may be estimated. Indeed, comparison of pH dependence of the biospecific adsorption of *N*-acetyl-β-D-hexosaminidase with pH dependence of the Michaelis constant for a similar enzyme from *Halosynthia roretzi* (13) shows that point of contrary flexure of both curves

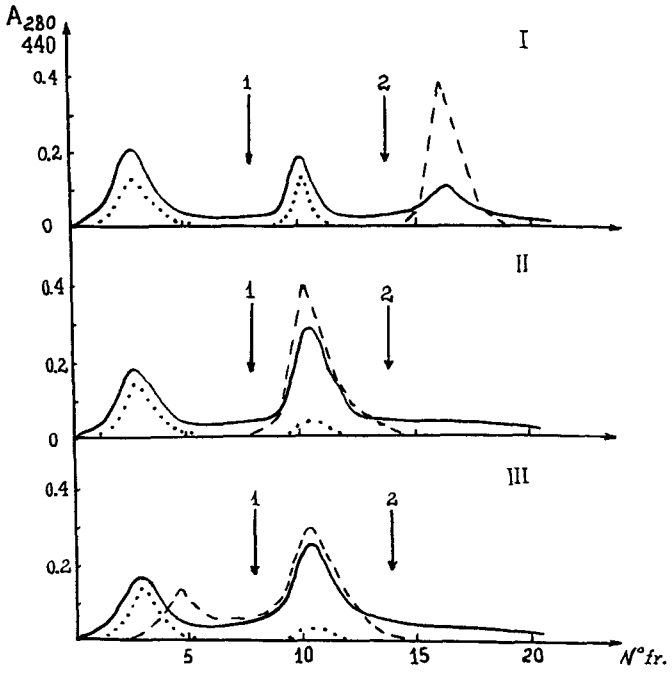


FIG. 3. Chromatography of glycosidases on sorbents **I**, **II**, and **III** (pH 5.0). Conditions similar to those described in Fig. 2 were used except that buffer pH was equal to 5.0. The symbols are the same as those for Fig. 2.

at the same pH value (about 5.5) which is characteristic for pK_a values for imidazole or carboxyl groups (Fig. 5).

Since the sorbents in use contained hydrazide groups with weak ion exchange properties (pK 3.9), we have synthesized a sorbent with a structure corresponding to the nonspecific part of sorbent **II** to confirm biospecificity of these sorbents (Table 2, sorbent 1). If *N*-acetyl-β-D-hexosaminidase interacts with the biospecific sorbents as with ion exchanger sorbents, the behavior of this enzyme on columns with the biospecific sorbents and with its nonspecific analog must be similar. However, *N*-acetyl-β-D-hexosaminidase does not interact with a nonspecific analog at any pH (Table 2). In addition, if the pK is equal to 3.9, then there are about 40% of the hydrazide groups charged at pH 4.0 and only 8–9% charged at pH 5.0. Thus adsorption of *N*-acetyl-β-D-hexosaminidase on the biospecific sorbents **I**, **II**, and **III** cannot be explained by ion exchange interaction. Another proof of biospecificity of the sorbents was obtained when the enzyme was eluted with soluble ligand (0.05 M) **6**.

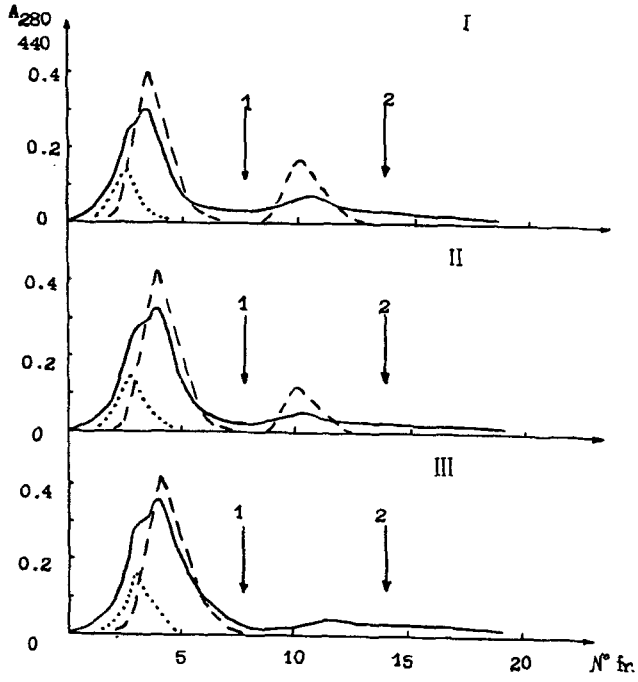


FIG. 4. Chromatography of glycosidases on sorbents, **I**, **II**, and **III** (pH 6.0). Conditions similar to those described in Fig. 2 were used except that buffer pH was equal to 6.0. The symbols are the same as those for Fig. 2.

The capacity of the enzyme–ligand complex, [ES̄]–Ⓟ[Eq. (1)], to dissociation at increasing ionic strength (0.5 M NaCl) or inconsiderable variation of pH (1 or 2 pH units), allows us to assume that the main forces responsible for the organization of this complex are hydrogen bonds or ion exchange interactions.

Lack of any cation or anion exchange groups in the structure of the biospecific ligands allows us to exclude the contribution of ion exchange interaction in the process of biospecific binding. The primary participation of hydrogen bonds in the organization of the enzyme–ligand complex may also be confirmed by calculation of free energy for the affinity process:

$$\Delta F^0_{ads} = \Delta F^0_{ac} + \Delta F^0_{ns} \tag{3}$$

where ΔF^0_{ads}, ΔF^0_{ac}, and ΔF^0_{ns} represent free energy of the chromatography system, specific adsorption, and nonspecific adsorption, respectively (14).

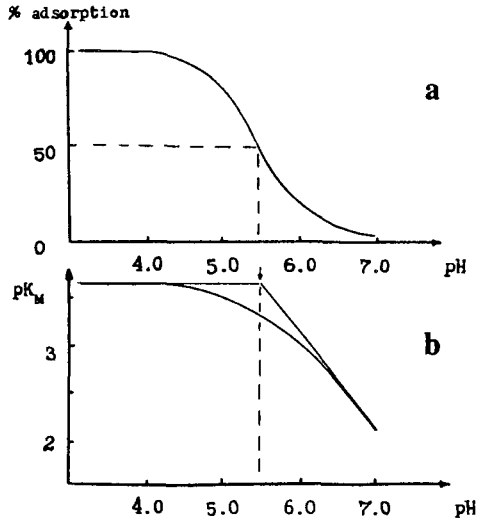
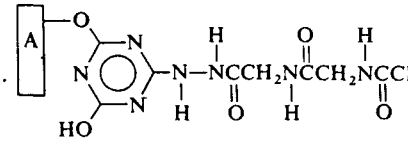
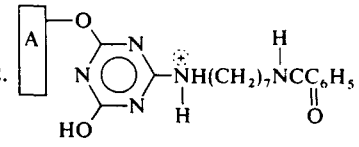
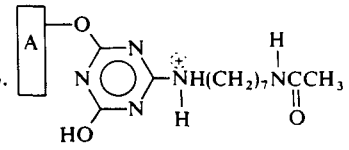


FIG. 5. (a) Dependence of the biospecific adsorption of *N*-acetyl-β-D-hexosaminidase on pH. (b) Dependence of the Michaelis constant for similar enzyme from *Halocynthia roretzi* on pH (13).

TABLE 2. **Adsorption of *N*-Acetyl-β-D-hexosaminidase on Different Nonspecific Sorbents**

N	Structure	Interaction with enzyme			Ref.
		pH 4.0	pH 5.0	pH 6.0	
1.		Not bound	Not bound	Not bound	
2.		Strongly bound	Strongly bound	Strongly bound	27
3.		Strongly bound	Strongly bound	Bound	7

The free energy of specific adsorption in affinity chromatography may be calculated by the method accepted for calculation of free energy for reactions of organization of the enzyme–substrate of enzyme–inhibitor complex (15, 16):

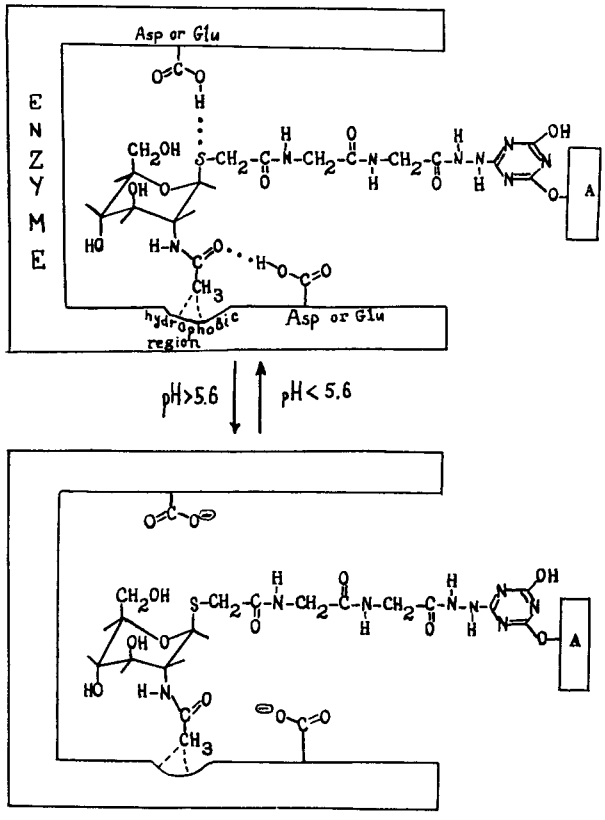
$$-\Delta F^0_{ac} = -(RT \ln K_m + T\,\Delta S) \tag{4}$$

where K_m is the Michaelis constant or inhibition constant.

The Michaelis constant for *N*-acetyl-β-D-hexosaminidase is equal to between 1.1 and 13.9 mM, depending on the β-glycosides of *N*-acetyl-D-glycosamine in use. By substituting these values one obtains ΔF^0_{ac} from −4.83 to −6.43 kcal/mol at 4°C for the free energy of specific adsorption to the ligand. The values obtained for free energy of specific binding of enzymes to the biospecific ligands correspond to the value of the energy of the hydrogen bond in proteins (−6 to −8 kcal/mol) (17, 18). By comparing the known data for mapping the active site of *N*-acetyl-β-D-hexosaminidase and the energetic contribution of each functional group of the inhibitor molecule (15, 16) with the results obtained, one can infer that sorption of enzyme on biospecific sorbents is realized only by hydrogen bonds between the ligand used and the ionizable groups of *N*-acetyl-β-D-hexosaminidase (p*K* 5.5). The proposed models of sorption and elution of *N*-acetyl-β-D-hexosaminidase in the process of biospecific chromatography through sorbent with possible participation of carboxyl groups of the enzyme are represented in Fig. 6.

The results obtained by affinity chromatography of *N*-acetyl-β-D-hexosaminidase give a basis for considering these sorbents to be effective specific sorbents which may be useful for studying the character of enzyme–ligand interaction. Specific sorbents prepared earlier (11,19–27) (Fig. 7) are not the same type because most of them contained ion exchange and hydrophobic groups in their composition.

The accessory effect of these nonspecific interactions on chromatography of *N*-acetyl-β-D-hexosaminidase results in a sharp increase in the importance of factor ΔF^0_{ns} from Eq. (3). If we take into account that free energy of binding of hydrophobic molecules to proteins usually varies from −5 to −12 kcal/mol and decreases about 0.7 kcal/mol for each methylene group and about 2 kcal/mol for each benzene ring (18), then the magnitude of free energy for specific binding (ΔF^0_{ac}) in affinity chromatography of the sorbents shown in Fig. 7 will be equal to or less than the free energy of nonspecific binding (ΔF^0_{ns}). This means that the process of affinity chromatography is determined not only by ΔF^0_{ac} but in general by ΔF^0_{ns}. In practice the influence of nonspecific binding on biospecific chromatography is confirmed by the binding of *N*-acetyl-β-D-hexosaminidase at pH values above 5.5, by the incapacity of the enzyme–ligand complex to dissociation at



FIG, 6. The proposed mechanism of the interaction of *N*-acetyl-β-D-hexosaminidase with the biospecific sorbent. Dotted lines represent hydrogen bonds; dashed, weak hydrophobic interaction by methylene group.

increasing ionic strength, and because elution of the enzyme may be accomplished only by a sharp change in buffer pH to basic values (pH 8.0–10.0) (11, 19–27) or by 50% ethylene glycol (27). These conditions for the elution of the enzyme influence not only hydrogen bonds of the complex but also cause considerable conformational changes in the protein structure, destroying the nonspecific hydrophobic interactions between *N*-acetyl-β-D-hexosaminidase and the ligand used. However, this method of elution is not always desirable because of possible inactivation of the enzyme at basic pH values.

The influence of ΔF^0_{ns} on binding of *N*-acetyl-β-D-hexosaminidase to affinity sorbents shown in Fig. 7 is confirmed by strong interaction of this

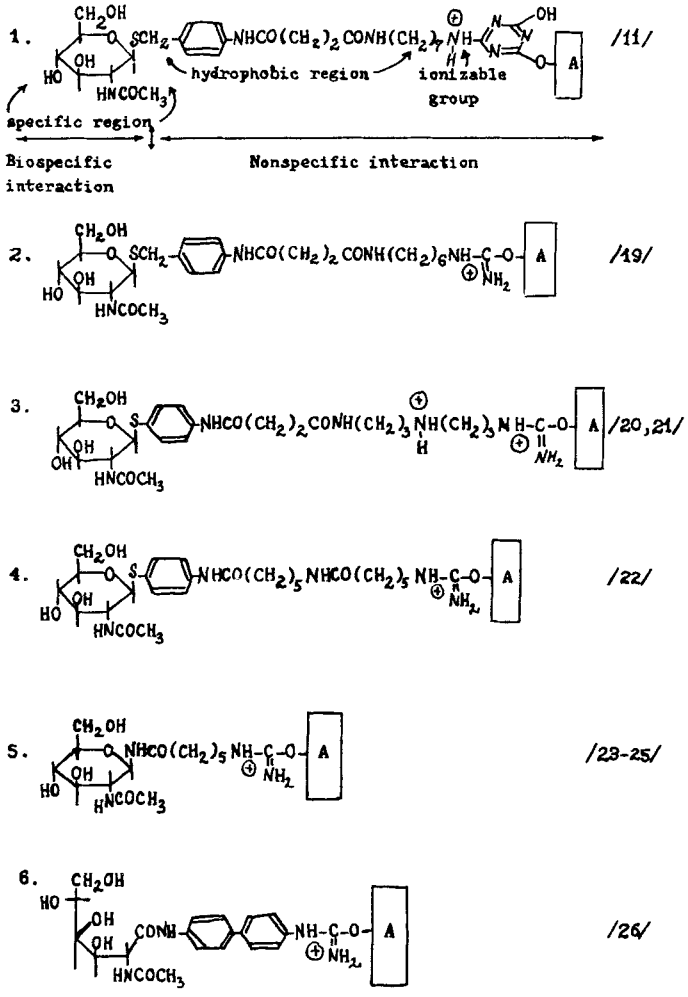


FIG. 7. The structures of sorbents used in affinity chromatography of *N*-acetyl-β-D-hexosaminidase described previously.

enzyme with hydrophobic matrices corresponding to "spacer arms" of these affinity sorbents (27) (Table 2). Similar nonspecific affinity chromatography was reported in early studies on other enzymes and is discussed in reviews by O'Carra et al. (28) and Guilford (29). As seen in Table 2, adsorption of *N*-acetyl-β-D-hexosaminidase on nonpolar matrices is realized in general by hydrophobic interactions (7,27). Stronger sorption of N-acetyl-β-D-hexosaminidase on affinity sorbents containing hydrophobic radicals located near

ligands permits us to assume that the active site of this enzyme or its close environment is formed by nonpolar amino acid residues. This results in the appearance of nonspecific hydrophobic interactions, strengthening adsorption values calculated from ΔF^0_{ac}. Apparently the aromatic triazine ring located near the ligand of sorbent **I** weakly interacts with the hydrophobic region of the enzyme, and therefore sorbent **I** adsorbs *N*-acetyl-β-D-hexosaminidase more strongly as compared with sorbents **II** and **III**. The use of diglycine and triglycine as spacer arms of sorbents **II** and **III** increases the distance between the enzyme active site with its hydrophobic environment and the triazine molecule. This abolishes the nonspecific hydrophobic effect in these cases.

Thus biospecific chromatography may be a procedure which will facilitate determination of various functional enzyme groups involved in the formation of active sites.

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