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AFFINITY CHROMATOGRAPHY BY ENZYME–SUBSTRATE INTERACTION

PURIFICATION OF SOME RAT LIVER GLYCOSIDASES

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SUMMARY

Derivatives for the affinity chromatography purification of β-glucuronidase and *N*-acetyl-β-glucosaminidase of bovine or murine origin were prepared by coupling modified glycoside substrates to Sepharose 4B by means of suitable extension arms.

Resolution of mixtures of glycosidases was difficult, since these enzymes were found to possess similar affinities towards the binding glycone moieties. The bound glycosidases could be eluted specifically with the corresponding substrates, inhibitors or salt gradients. 40–100-fold purification of the glycosidases with respect to a rat liver autolysate was achieved in a single step, with a recovery of 90% or higher. For β-glucuronidase, the overall purification with respect to the original tissue was about 1250-fold. The described glycoside–Sepharose derivatives are a convenient means of partially purifying β-glycosidases. These supports are easy to prepare and can be reused several times.

INTRODUCTION

Various affinity chromatography procedures are used for the purification of proteins[1,2]. The specific adsorbents employed are prepared by attaching ligands such as competitive inhibitors[3,4], cofactors[5], antibodies[6], natural enzyme inhibitors[7], binding proteins[8] or enzymes[9] to a solid matrix. Usually, selection of a suitable ligand is difficult before some properties of the previously isolated enzymes have been characterized. However, a known property of an enzyme prior to its purification is the specific recognition of a substrate. This substrate can be used as a ligand.

Abbreviation: EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

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The formation of a complex between an enzyme and its insoluble, highly polymerized substrate has been used for the purification of α-amylase[10,11], starch synthetase[12], DNA polymerase[13], lysozyme[14], concanavalin A[15] and collagenase[16]. It appeared of interest to attempt the affinity chromatography purification of enzymes acting on simple, low molecular weight substrates bound to insoluble matrices. Convenient substrates were chemically modified and attached to insoluble supports by means of suitable binding arms for the purification of some rat liver glycosidases.

MATERIALS AND METHODS

Biochemicals

p-Nitrophenyl-β-D-glucuronide, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, phenolphthalein-β-D-glucuronide, bovine liver β-glucuronidase (Type B-10) and *Escherichia coli* β-galactosidase were purchased from Sigma Chemical Co. (St. Louis, Mo.); *o*- and *p*-nitrophenyl-β-D-galactopyranoside were obtained from Calbiochem (La Jolla, Calif.).

CNBr, 3,3'-diaminodipropylamine, sodium 2,4,6-trinitrobenzenesulfonate and succinic anhydride were obtained from Eastman Organic Chemicals (Rochester, N.Y.). Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Aldrich Chemical Co. (Milwaukee, Wisc.). PtO_2 (Adam's catalyst) was obtained from Matheson Coleman and Bell (Norwood, Ohio). Other chemicals were obtained from various suppliers.

Methods

Preparation of p-aminophenyl-β-D-glucuronide. 0.30 mmoles of *p*-nitrophenyl-β-D-glucuronide were dissolved in 2 ml of 95% ethanol. 5 mg of PtO_2 were added, and a reduction was carried out by H_2 under pressure of 40 lbs/inch^2 in a Parr instrument for 90 min. 2 drops of glacial acetic acid were added and the mixture was filtered through a medium pore size glass filter under vacuum, and used immediately for binding to a matrix as indicated below.

Preparation of p-aminophenyl-N-acetyl-β-D-glucosaminide and of p-aminophenyl-β-D-galactopyranoside. 0.293 mmoles of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were dissolved in 0.5 ml of water and 3 ml of 95% ethanol were added. 0.33 mmoles of *p*-nitrophenyl-β-D-galactopyranoside were dissolved in 1 ml water, and 4 ml of 95% ethanol were added.

Both solutions were maintained at 60 °C and transferred to a Parr instrument. 5 mg of PtO_2 were added to each mixture, and H_2 reduction was carried out for 120 min in the manner described above. The reduced compounds were stirred with a few drops of glacial acetic acid, filtered through glass and used immediately.

Attachment of extension arms to Sepharose 4B. Sepharose 4B was activated with 300 mg of CNBr per ml of packed gel according to the methods of Cuatrecasas and Anfinsen[1]. The activated Sepharose was reacted with 4 mmoles of 3 3'-diaminodipropylamine per ml of packed Sepharose at pH 9.6 for 16 h at 4 °C. The reaction product: diaminodipropylamino-Sepharose will be referred to as Derivative N hereinafter. An aliquot of Derivative N was succinylated with 3 mmoles of succinic anhydride per ml of Sepharose at pH 6.0 for 5 h at 4 °C. The succinyldiaminodipropylamino-Sepharose thus prepared will be called Derivative S. When a small amount of

Derivative S was mixed with sodium 2,4,6-trinitrobenzenesulfonate, a yellow color developed, indicative of total substitution of the preexisting amino groups[17]. Nitrogen analysis of Derivative S showed a content of 16 μmoles of 3,3'-diaminodipropylaminosuccinyl groups per ml of Sepharose.

Substitution of the terminal carboxyl groups of Derivative S. 10 ml of packed Derivative S were suspended in water, adjusted to pH 4.8 with 0.1 M NaOH and mixed with 2 mmoles of EDC dissolved in 5 ml of water, pH 4.8. To four similar aliquots of the above mixture, 2 mmoles of 2-aminoethanol, ethylamine, propylamine or butylamine were added. These mixtures were maintained at pH 4.8 by careful titration with 0.1 M NaOH for 2 h at room temperature. Afterwards, the mixtures were placed at 4 °C for 14 h, with continuous stirring. In this way, 2-aminoethylsuccinyl-diaminodipropylamino-Sepharose (Derivative A) and ethylamino-, propylamino-, or butylaminosuccinyl-diaminodipropylamino-Sepharose (Derivatives B) were prepared (Fig. 1).

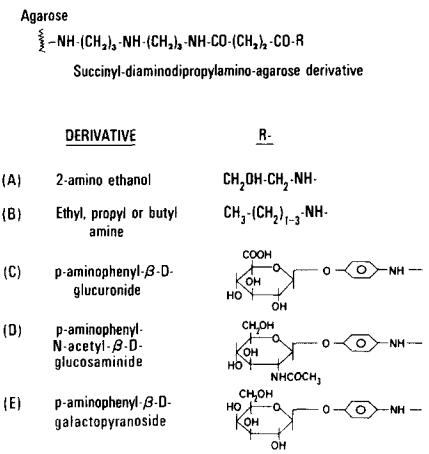

Fig. 1. Affinity chromatography adsorbents. Methods of preparation are given in the text.

Prepararation of glycosidic derivatives. 10 ml of packed Derivative S in water were adjusted to pH 4.8 with 0.1 M NaOH, and 2 mmoles of EDC in 5 ml of water at pH 4.8 were added. After stirring for 1 min, the freshly prepared *p*-aminophenylglycosides described above were added, and the mixtures were maintained at pH 4.8 with 0.1 M NaOH for 2 h at 20 °C and then for 14 h in the cold room. In this manner, Derivatives C, D and E were synthesized (Fig. 1). These derivatives were washed with 1-l batches of: 2 M NaCl in 0.01 M $NaHCO_3$ (pH 8.8), water, 0.05 M sodium acetate (pH 5.0), in that order. The various washed materials were then submitted to nitrogen analysis for the determination of the amount of glycoside attached. The value found for Derivative S, containing no glycosides, was considered as a blank. The following results were obtained: Derivative A, 14 μmoles; Derivative B, 12 μmoles; Derivative C, 13 μmoles; Derivative D, 10 μmoles and Derivative E, 12 μmoles per ml of packed Sepharose.

Unreacted carboxyl groups in the extension arms of Derivatives C, D and E were blocked with 2 mmoles of 2-aminoethanol in the presence of 2 mmoles EDC at

pH 4.8 for 18 h in the cold room. These materials were then washed in the manner described above and used for the affinity chromatography purifications.

Preparation of a rat liver autolysate. Male albino Holtzman rats, weighing 180–200 g were used. The animals were fasted 24 h before sacrifice, killed by decapitation and allowed to bleed for 1 min. Minced liver tissue was suspended in ice-cold, unbuffered 0.25 M sucrose and homogenized with six up-and-down strokes of a Potter–Elvehjem device. The crude homogenate was placed in a 37 °C water bath and covered with a thin layer of toluene (0.002 vol.)[18]. Every other day the mixture was stirred and some toluene replaced. Ten days later, the autolysed homogenate was centrifuged at 5000 rev./min for 20 min, the precipitate was discarded and the clear supernatant was kept in the cold room until used as a crude enzyme source.

The autolytic incubation just described was found necessary in order to release glycosidic enzyme activity from the subcellular particles. No bacterial contamination was detected during the long autolysis. This process resulted in the following recovery figures: β-glucuronidase, 70–80%, *N*-acetyl-β-glucosaminidase, 30–50%; β-galactosidase, 5–15%. The liver autolysate retained β-glucuronidase activity for several months in the cold room. However, levels of *N*-acetyl-β-glucosaminidase and β-galactosidase decreased upon storage. It was necessary to add some purified enzyme to the liver autolysate prior to certain experiments.

Binding of glycosidases. Except when otherwise stated, all binding experiments were done at 4 °C. Sepharose derivatives were incubated with enzyme-containing samples in test tubes, with continuous stirring for 10 min. The suspensions were poured into Pasteur pipettes fitted with glass wool plugs. In typical experiments, 0.5–1.0 ml of affinity adsorbent and 1.0–8.0 ml of enzyme-containing solution were used. The non-adsorbed fractions were washed through with 0.05 M acetate buffer, pH 5.0. 1-ml fractions were collected. The elution pattern was followed by monitoring the absorbance at 280 nm and the enzymatic activity of each fraction. After elution, the derivatives were kept in the cold room and washed extensively before re-use.

Enzyme assays and units

β-Glucuronidase (EC 3.2.1.31), *N*-acetyl-β-glucosaminidase (EC 3.2.1.30) and β-galactosidase (EC 3.2.1.23) were assayed as described by Gianetto and De Duve[19], Findlay *et al.*[20], and Conchie and Hay[21], respectively. For the three hydrolases, one enzyme unit is defined as the amount capable of releasing 1 nmole of aglycone per min at 37 °C.

The specific activities of the hydrolases in the fresh liver autolysate were: β-glucuronidase, 10.2 units/mg protein; *N*-acetyl-β-glucosaminidase, 3.1 units/mg protein; β-galactosidase, 0.5 unit/mg protein.

Protein assays

Protein concentration was determined according to Lowry *et al.*[22] with bovine serum albumin as a standard.

Analysis of Sepharose derivatives

Sepharose derivatives were analysed by microdetermination of Kjeldahl nitrogen according to Lang[23]. All determinations were performed on samples previously dried under vacuum and at 70 °C for 12 h.

RESULTS

Effect of pH on affinity binding of glycosidases

A commercial sample of partially purified bovine liver β-glucuronidase was dissolved in 0.01 M buffer solutions of various pH, ranging from 3.0 to 10.0. A suitable number of aliquots of Derivative C were equilibrated at the corresponding pH. Batches of 1 ml of this material were mixed with 1-ml aliquots of β-glucuronidase, incubated as described under Materials and Methods, and the unbound enzyme was removed. The percent enzyme released was determined and plotted against pH (Fig. 2). Binding was more efficient below pH 5.0. The pH optimum of bovine liver β-glucuronidase is 4.8 (ref. 18).

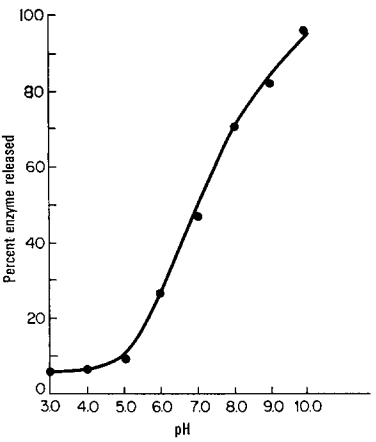

Fig. 2. Effect of pH on desorption of β-glucuronidase from β-glucuronide-Sepharose (Derivative C). 1 ml of Derivative C was equilibrated with 2 ml rat liver autolysate containing 63 units/ml β-glucuronidase at the required pH. The mixtures were equilibrated at room temperature for 20 min and centrifuged at 5000 rev./min for 3 min. The supernatants were tested for enzyme activities. Each point shown represents an average on three separate experiments.

An identical pH-dependency curve was found for rat liver autolysate β-glucuronidase. It was observed that bovine or murine β-glucuronidase binds to Derivative D (*N*-acetyl-β-D-glucosaminide) and Derivative E (β-D-galactopyranoside). In addition, *N*-acetyl-β-glucosaminidase and β-galactosidase from various sources bind to β-glucuronide-substituted Sepharose (Derivative C) as well as their corresponding derivatives. A study of the degree of binding of the various enzymes to the different adsorbents was then performed. The results of these experiments are given in Table I.

Maximum binding capacity of Sepharose derivatives

The β-glucuronidase binding capacity of Derivatives C, D and E was tested at pH 5.0. 2-ml aliquots of the rat liver autolysate were incubated at room temperature with 1 ml Sepharose Derivatives C, D or E for 10 min. The suspensions were centrifuged at 3000 rev./min for 5 min, and the supernatants were syphoned off and assayed for enzyme activity. Nonspecifically adsorbed proteins were eliminated by stirring the mixtures with 5 ml of 0.05 M sodium acetate buffer, pH 5.0, followed by centrifugation and elimination of the supernatants. This wash was repeated twice,

TABLE I

BINDING OF VARIOUS GLYCOSIDASES TO GLYCOSIDIC SEPHAROSE 4B DERIVATIVES

Reversible indicates that a pH-dependent enzyme binding was found, similar to the one described for β-glucuronidase in Fig. 2. Quantitative enzyme recovery was achieved by salt elution. Irreversible refers to enzymes that could not be eluted by 1 M NaCl solutions from the respective columns. No binding indicates that an enzyme could not be attached to the matrix under the conditions described.

Enzyme and source	*Sepharose derivative (binding type)*		
	β-D-Glucuronide (Derivative C)	*N-Acetyl-β-D-glucosaminide (Derivative D)*	*β-D-Galactopyranoside (Derivative E)*
Rat liver autolysate			
β-Glucuronidase	Reversible	Reversible	Reversible
N-Acetyl-β-glucosaminidase	Reversible	Reversible	Reversible
β-Galactosidase	Irreversible	Irreversible	Reversible
Partially purified β-glucuronidase			
β-Glucuronidase	Reversible	Reversible	Reversible
N-Acetyl-β-glucosaminidase	Reversible	Reversible	Reversible
β-Galactosidase	—	—	—
E.coli β-galactosidase	No binding	No binding	Reversible*

* Although strong binding at low pH was obtained, 90% enzyme activity was released at pH 7.0.

and more rat liver autolysate was added to the mixtures, which were incubated and washed in the same manner. The process was repeated until no further amounts of enzyme could be adsorbed. In this way, approximately 1100 units of β-glucuronidase were adsorbed from an autolysate containing 60 units/ml, with a spec. act. of 9.7 units/mg protein. The same amount of the enzyme could be bound to Derivatives C, D or E. Close to the saturation of the Sepharose derivatives with enzymes, the adsorbents became very viscous.

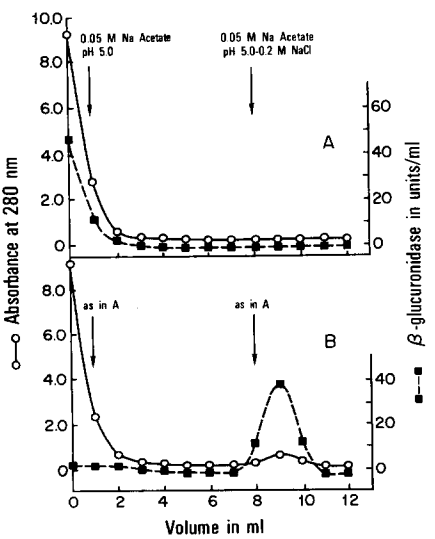

Fig. 3. Affinity chromatography of rat liver β-glucuronidase by salt elution. 1 ml rat liver autolysate containing 63 units/ml was equilibrated with Sepharose Derivatives A, B or C in the presence of 0.05 M acetate buffer, pH 5.0. (A) Unsubstituted Sepharose 4B or Derivatives A or B were used. (B) Derivative C was employed.

Purification of glycosidases by ionic strength elution

β-Glucuronidase. Rat liver β-glucuronidase was bound to Derivative C in the manner described. The enzyme was eluted by washing with stepwise increasing concentrations of NaCl in 0.05 M acetate buffer, pH 5.0. Enzyme desorption began at 0.1 M NaCl, and was complete with 0.2 M NaCl (Fig. 3B). Unsubstituted Sepharose 4B or Derivatives A or B, lacking the glycosides, did not retain enzymes or protein (Fig. 3A). By salt elution, β-glucuronidase recovery was 95%, with a purification of 58-fold with respect to the original liver autolysate. This enzyme could be purified equally well on Derivatives E and D, with recovery figures of 90% in both cases. Specific activities increased 47-fold and 80-fold, respectively.

Salt desorption from Sepharose derivatives was strongly dependent on the presence of unsubstituted carboxyl groups in the extension arms. If unblocked Sepharose derivatives were used, complete enzyme release required concentrations of 0.3 M NaCl or higher.

N-Acetyl-β-glucosaminidase. N-Acetyl-β-glucosaminidase was bound to Derivatives C, D or E in the manner described, and eluted with a continuous NaCl gradient of increasing molarity in 0.05 M sodium acetate buffer, pH 5.0. The results of an experiment using Derivative D are shown in Fig. 4. Desorption of β-glucuronidase was monitored simultaneously for comparison. It was found that elution of *N*-acetyl-β-glucosaminidase requires slightly higher NaCl concentrations than those used for β-glucuronidase.

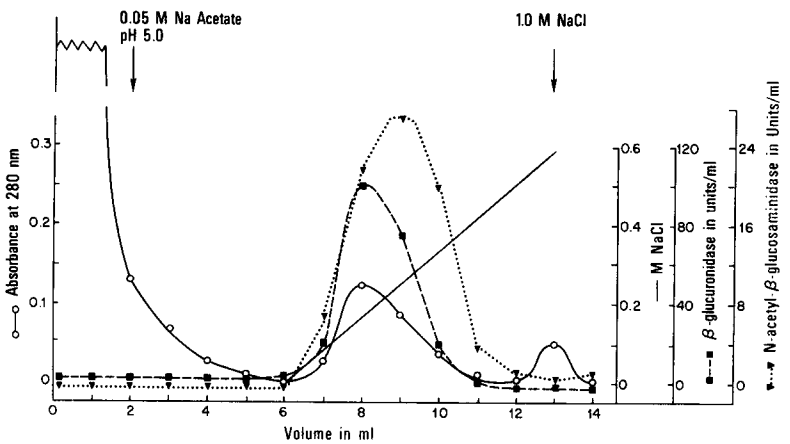

Fig. 4. Affinity chromatography of rat liver β-glucuronidase and *N*-acetyl-β-glucosaminidase by salt elution from Derivative D. 3 ml rat liver autolysate containing 63 units/ml β-glucuronidase and 36 units/ml *N*-acetyl-β-glucosaminidase were equilibrated with 1 ml Derivative D in the presence of 0.05 M sodium acetate buffer, pH 5.0. The enzymes were eluted with a linear gradient solution of NaCl in the same buffer. 101% β-glucuronidase and 87% *N*-acetyl-β-glucosaminidase were recovered after 1.0 M NaCl elution.

β-Galactosidase. 60% of β-galactosidase activity was recovered from the corresponding adsorbent (Derivative E) by elution with 0.1 M NaCl in buffer. Specific enzyme activity increased 36-fold in one step.

Purification of rat liver glycosidases by pH-gradient elution

β-Glucuronidase adsorbed to Derivative C was eluted with 0.1 M sodium borate, pH 10.0. 85% of the enzyme was recovered, and specific activity increased 15–20-fold.

Resolution of mixtures of rat liver glycosidases by affinity chromatography

A rat liver autolysate was adsorbed to Derivatives C, D or E. Gradient elution from Derivatives C or E with continuously increasing molarities of NaCl failed to separate β-glucuronidase from *N*-acetyl-β-glucosaminidase. However, elution from Derivative D resulted in a partial separation of enzymes. Data corresponding to these experiments are shown in Table II and Fig. 5.

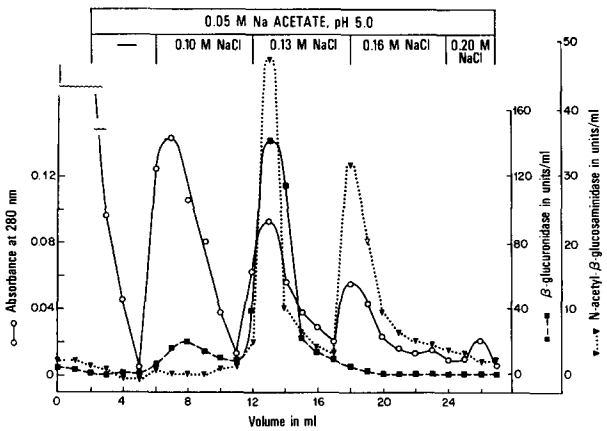

Fig. 5. Affinity chromatography of rat liver β-glucuronidase and *N*-acetyl-β-glucosaminidase on Derivative D by stepwise salt elution. 8 ml rat liver autolysate were equilibrated with 1 ml Derivative D in the presence of 0.05 M acetate buffer, pH 5.0. The enzymes were eluted with a discontinuous NaCl gradient. A data sheet for this experiment is shown in Table II. 92% of the initial activity of β-glucuronidase and 99% of the *N*-acetyl-β-glucosaminidase were recovered.

As shown in Table II, β-glucuronidase and *N*-acetyl-β-glucosaminidase can be purified about 100-fold by salt elution from Derivative D. As indicated in Fig. 5, 0.13 M NaCl elutes 83% of the β-glucuronidase, mixed with 45% of the *N*-acetyl-

TABLE II

PURIFICATION OF β-GLUCURONIDASE AND *N*-ACETYL-β-GLUCOSAMINIDASE ON *N*-ACETYL-β-GLUCOSAMINIDASE SEPHAROSE

All NaCl solutions used contained 0.05 M sodium acetate buffer, pH 5.0. Experimental details are given in the text.

Fraction	*β-Glucuronidase*				*N-Acetyl-β-glucosaminidase*			
	Total activity (units)	*Spec. act. (units/mg protein)*	*Percent enzyme recovery*	*Spec. act. increase (times)*	*Total activity (units)*	*Spec. act. (units/mg protein)*	*Percent enzyme recovery*	*Spec. act. increase (times)*
Autolysate	440	8.9	—	—	139	2.8	—	—
0.10 M NaCl	72	132.6	17	15	9	11.4	7	4
0.13 M NaCl	356	872.4	83	98	61	151.2	45	54
0.16 M NaCl	0	—	—	—	51	262.4	37	94
0.20 M NaCl	0	—	—	—	15	72.0	11	26

β-glucosaminidase. 0.16 M NaCl elutes a second protein peak, containing 37% of the *N*-acetyl-β-glucosaminidase, and negligible amounts of β-glucuronidase.

Elution characteristics of bovine liver glycosidases

A sample of partially purified, commercial β-glucuronidase (6400 units/mg protein) was found to be contaminated with *N*-acetyl-β-glucosaminidase (10 700 units/mg protein) and β-galactosidase (800 units/mg protein). This sample was incubated with Derivatives C, D or E. Stepwise elution with NaCl at pH 5.0 revealed that the ionic strength desorption curve of bovine β-glucuronidase from Derivative E is identical to that of the rat liver enzyme. This procedure purified the commercial β-glucuronidase about 3-fold. The resulting sample still contains a mixture of the three hydrolases.

Effect of carbohydrates on patterns of glycosidase elution

Glycosidase binding is related to the affinity of the enzyme for the glycosidic moiety of the substrates attached to the inert support. Based on the assumption that affinity binding could be specifically reversed in the presence of suitable concentrations of structurally related sugars, a series of mono- and disaccharides was tested as eluents for the enzymes. The sugars used were: glucose, fructose, galactose, rhamnose, mannose, xylose, glucosamine, lactose and sucrose. A column packed with Derivative C was loaded with rat liver autolysate and washed with 0.05 M sodium acetate, pH 5.0, until 94% of the initial protein amount was eluted. Further elution with 0.1 to 1.0 M carbohydrate solution eliminated an additional 2.5% of the initial protein. Up to this point, no glycosidase activity was detectable in the eluates. These enzymes were desorbed with 0.2 M NaCl in acetate buffer (Fig. 6).

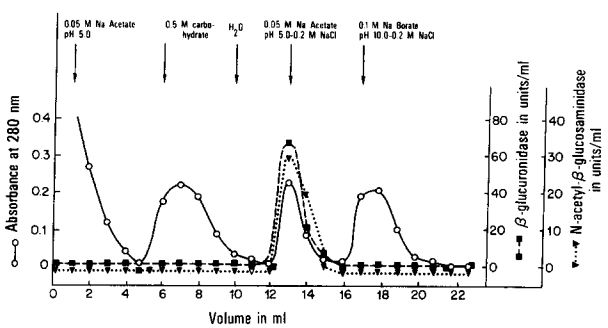

Fig. 6. Effect of various carbohydrates on rat liver glycosidase elution from Derivative C. 2 ml rat liver autolysate containing 105 units β-glucuronidase and 112 units *N*-acetyl-β-glucosaminidase were equilibrated at room temperature with 1 ml freshly prepared derivative C in the presence of 0.05 M sodium acetate buffer, pH 5.0. After elution with 0.01 M sodium borate, pH 10.0 in 0.2 M NaCl, recovery figures of the pooled eluates were 98% for β-glucuronidase and 91% for *N*-acetyl-β-glucosaminidase. These enzymes were purified 55-fold and 42-fold, respectively. The carbohydrate used in the experiment shown was 0.05 M glucose. Further details are given in the text.

Effect of enzyme substrates or inhibitors on glycosidase elution

p-Nitrophenyl-β-D-glucuronide, *N*-acetylglucosaminide or galactosaminide were dissolved in 0.05 M sodium acetate buffer, pH 5.0, in concentrations of $1 \cdot 10^{-5}$

to $1 \cdot 10^{-1}$ M and used for the elution of glycosidases from a rat liver autolysate bound to a column of Derivative C. 0.05 M solutions of any of the *p*-nitrophenyl glycosides elute a protein peak containing the three glycosidases studied. It was not possible to resolve these protein peaks into individual enzymes by specific substrate elution. Overall purification figures were not better than those obtained by salt elution.

Finally, phenolphthalein-β-glucuronic acid (sodium salt), a routine substrate for the spectrophotometric assay of β-glucuronidase, was tested in the same manner. 0.01 M solutions of this substrate in water eluted a sharp peak of β-glucuronidase (Fig. 7). After elution, however, separation of the enzyme from its substrate by dialysis, gel chromatography or ultrafiltration through Diaflo was very difficult.

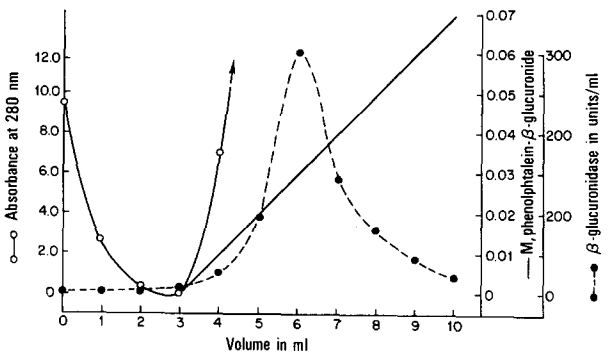

Fig. 7. Effect of phenolphthalein-β-glucuronic acid (sodium salt) on elution of rat liver β-glucuronidase from Derivative C. 4 ml rat liver autolysate containing 262 units β-glucuronidase were equilibrated with 1 ml Derivative C in the presence of 0.05 M sodium acetate buffer, pH 5.0. The column was washed with 3 ml water and eluted with successive 1-ml aliquots of increasing molarities of phenolphthalein-β-glucuronic acid (sodium salt) adjusted to pH 5.0.

DISCUSSION

Covalent binding of *p*-aminophenyl enzyme inhibitors to insoluble polymers has been shown by others to be of value for the preparation of affinity adsorbents[3,4,24]. In this paper, the coupling of modified glycoside enzyme substrates to Sepharose extension arms has been studied. These Sepharose derivatives are efficient for the partial purification of β-glucuronidase and *N*-acetyl-β-glucosaminidase from bovine or murine origin. Resolution of mixtures of the glycosidases, however, was found difficult. The similar behavior of the glycosidases studied on various glycoside derivatives is thought to be the result of similar affinity towards the binding glycone moieties.

It has been reported that β-glucuronidase[25,26] and *N*-acetyl-β-glucosaminidase[27] show little specificity towards the aglycone moiety of their respective substrates. However, glycone requirements are rather strict. The β configuration of the glycosidic bonds in the substrates appears to be essential for enzymatic cleavage[25–27].

It has been shown that β-glucuronidase will cleave glucosiduronic acids as well as β-galactopyranosiduronic acids[25]. This lack of specificity for the C-4 hydroxyl groups may explain why this enzyme binds to β-galactoside substituted derivatives. It was also shown in this paper that β-glucuronidase binds to *N*-acetyl-β-glucosaminide derivatives. It appears that there is unspecific recognition of C-2 as well as of C-4 substituted glycosides. During synthesis of β-glucuronide Sepharose deriva-

tives, free –COOH groups in the extension arms had to be blocked in order to avoid non-specific adsorption. In the process, –COOH groups at C-6 in the glucuronide moiety were also blocked, a change that did no interfere with β-glycosidase attachment. Apparently, enzyme binding occurs whether free carboxylic or amidic groups at C-6 are employed. However, only substrates with a free C-6 carboxyl group can be cleaved by β-glucuronidase[25,26]. *N*-Acetyl-β-glucosaminidase requires *N*-acyl substitutions in the glycone for cleavage. This enzyme is active towards β-*N*-acetylglucosaminyl or β-*N*-acetylgalactosaminyl linkages as well[28].

It was found that *E. coli* galactosidase binds reversibly only to galactoside derivatives. These results are in good agreement with the reported strict requirements of the enzyme for the C-2, C-4 and C-6 positions in the glycopyranoside[29,30]. *E. coli* β-galactosidase was purified by Steers *et al.*[31] in a similar manner. These authors bound β-D-thiogalactoside, a competitive inhibitor of β-galactosidase, to a Sepharose matrix. A 7-fold enzyme purification was reported.

In general, our results by salt elution were equal or better than those of experiments with substrates or inhibitors as eluents. These are more expensive, require higher concentrations and are difficult to separate from the eluted enzymes. Preliminary experiments with the corresponding (1–5) aldonolactones, which are powerful glycosidase inhibitors[32], indicate that further enzyme purification may be achieved.

Two peaks of activity of *N*-acetyl-β-glucosaminidase were detected by stepwise salt elution of a rat liver autolysate. These peaks may correspond to the two forms of this enzyme described by Beck *et al.*[33]. The present results show that affinity chromatography yields 40–100-fold purification of some rat liver glycosidases from a partially purified autolysate, in a single step. The overall purification of β-glucuronidase with respect to the original tissue was 1250-fold, and recovery figures are in the range of 90% or higher. These results can be compared to those of Bonnichsen[34], who reported about 1800-fold purification of bovine β-glucuronidase and Plapp and Cole[18], who described a 4400-fold increase of specific activity for the same enzyme.

The difficulty in achieving complete resolution of the glycosidases studied may be attributed to their marked similarity in substrate recognition requirements, as well as in their physical properties[25–28]. As shown in this paper, most of the protein contents of a rat liver autolysate will not bind specifically to a glycoside-substituted Sepharose. The protein remainder, which can be displaced by specific or non-specific eluents, may represent an array of molecules exhibiting various degrees of affinity towards the glycoside moiety. Several other β-glycosidases known to exist in rat liver may be present in this fraction[35].

The present data are insufficient to determine whether the bound substrates act as substrate-analog inhibitors or are still prone to enzymatic cleavage at a reduced rate. The described glycoside derivatives are convenient for the partial purification of β-glycosidases. Coupling of other substrates to inert matrices in a similar manner may be useful as a general tool for enzyme purification.

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