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NON-SPECIFIC PROTEIN BINDING BY ADSORBENTS DESIGNED FOR THE SPECIFIC AFFINITY CHROMATOGRAPHY OF SIALIDASE IN CRUDE BACTERIAL EXTRACTS

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SUMMARY

A number of derivatives of Sepharose 4B gels were prepared containing tyramine, -Gly–Gly–Tyr and the tetrapeptide, Thr (Bu^t)–Phe–Pro–Tyr–. A specific inhibitor of sialidase, *p*-diazo-phenyl oxamic acid, was also coupled to these phenol-substituted Sepharose gels. The effectiveness of these gels to purify sialidase by "affinity" chromatography was examined.

The sialidase from *Vibrio cholerae* was adsorbed by these gels and subsequently re-eluted by a change in pH and temperature. The adsorption and elution of enzyme was independent of the presence or absence of the "specific inhibitor" of the sialidase, phenyl oxamic acid. A mechanism for the adsorption and subsequent elution of the sialidase is proposed.

It is important to re-evaluate the biological data obtained with sialidase prepared by the affinity column recommended by Cuatrecasas and Illiano, Biochem. Biophys. Res. Commun. 44, 178–184 (1971), since glycosidases from *Clostridium perfringens* other than sialidase are also adsorbed and eluted under the conditions specified.

INTRODUCTION

The principle of using an inhibitor of an enzyme to facilitate the purification of that enzyme is now one of the established procedures of affinity chromatography [1–3]. This principle was applied by Cuatrecasas and Illiano to purify sialidase (EC 3.2.1.18) [4, 5] from commercially available crude and partially purified preparations, using a column made of Sepharose to which an inhibitor of this enzyme [6], *N*-(*p*-aminophenyl)-oxamic acid, is attached through an azo linkage to a tripeptide ligand, -Glycyl-Glycyl-Tyrosine.

Abbreviations: Sepharose-Ty, tyramine-substituted Sepharose gel; Sepharose-Tri, Sepharose-Gly–Gly–Tyr; Sepharose-Tet, Sepharose-Tyr–Pro–Phe–Thr(Bu^t); Sepharose-E, ethanolamine-substituted Sepharose. Sepharose-TyO and Sepharose-TriO, the corresponding aminophenyl oxamate-diazotized derivatives of Sepharose-Ty and Sepharose-Tri, respectively.

We were interested in using this technique to achieve two objectives, namely (a) to purify sialidase and (b) to specifically remove contaminating sialidase from bacterial extracts containing other glycosidases, e.g. *Clostridium perfringens*. In the course of these investigations we made a number of observations that question (a) whether the method of Cuatrecasas and Illiano [4] can be designated as true affinity chromatography and (b) the purity of the product thereby obtained.

MATERIALS AND METHODS

Materials

A crude extract of *Vibrio cholerae*, Ogawa strain, was prepared by growing the organisms on soft agar plates. Freezing and thawing released the enzyme into the supernatant, which was then filtered through a bacterial filter to remove organisms and debris and dried from the frozen state.

A crude extract of *Clostridium perfringens* was used as the source of a number of glycosidases [7, 8] and it contained sialidase in high concentration [9]. The organisms were grown as previously described [7]. The supernatant was adjusted to 80% saturation with $(NH_4)_2SO_4$. The resulting precipitate, dissolved in 50 mM sodium phosphate buffer, pH 6.3 (one-tenth of the original volume) was refractionated with $(NH_4)_2SO_4$. The material precipitated at 50–52% saturation was collected, dissolved in a tenth of the original volume and passed through a column of Sephadex G-100, equilibrated and eluted with 50 mM sodium phosphate buffer, pH 6.3, containing 0.1 M NaCl and 0.02% NaN_3. The resulting solution was active with respect to sialidase as well as many other glycosidases.

Each enzyme preparation was dialyzed overnight at 4 °C against 80 vol. of 50 mM sodium acetate buffer, pH 5.5, containing 2 mM $CaCl_2$ and 0.2 mM EDTA [4] and centrifuged at 27 000 × *g* for 30 min prior to application of the supernatant to the affinity columns.

Tyramine was a gift from Professor M. Wilchek. The tripeptide glycyl-glycyl-tyrosine was synthesized by Mr Jacobson at the Weizmann Institute of Science. The *t*-butyl ester of threonyl-phenylalanyl-prolinyl-tyrosine was a gift from Dr Rolph Jost.

p-Nitrophenyl oxamic acid (obtained from K & K-ICN) was reduced to the corresponding *N*-(*p*-aminophenyl)-oxamic acid. Some *N*-(*p*-aminophenyl)-oxamic acid was also obtained through the courtesy of Dr G. Ashwell, N.I.H.

Sepharose 4B was substituted with tyramine (Sepharose-Ty), tripeptide, –Gly–Gly–Tyr (Sepharose-Tri) or tetrapeptide, Thr (Bu^t)–Phe–Pro–Tyr– (Tet-Sepharose), according to the published procedure [10, 11]; 5–10 μmoles of the ligand were found to be present per gram of Sepharose 4B. The diazotization and coupling of *N*-(*p*-aminophenyl)-oxamic acid to tyramine or tripeptide-substituted agarose was performed according to the published procedure [4], yielding Sepharose-TyO and Sepharose-TriO, respectively. Ethanolamine-inactivated CNBr-Sepharose 4B (Sepharose-E) was prepared starting from Sepharose or from the commercially available CNBr-Sepharose 4B by the procedure prescribed by the manufacturer (Pharmacia Fine Chemicals).

Some of the other gels were also treated with ethanolamine, after the ligand was attached, in order to block remaining active groups on the Sepharose.

Hog submaxillary glycoproteins used for the assay of the sialidase and α-1,2-

L-fucosidase were prepared by modifications of the procedures described elsewhere [12, 13]. Desialyzed ovine submaxillary glycoprotein used for the assay of the α-*N*-acetylgalactosaminidase was prepared by acid hydrolysis (0.1 M HCl, 80 °C, 45 min) of ovine submaxillary glycoprotein [14].

Sialic acid (NAN), fucose and *p*-nitrophenyl glycosides were obtained commercially as previously described [7].

Methods

Sialidase assay. Sialidase activity was determined by incubating enzyme preparations with hog submaxillary glycoprotein in a total volume of 250 μl and contained 0.23 μmole of bound *N*-glycolyl neuraminic acid, 25 μmoles sodium acetate buffer, pH 5.5, 1 μmole $CaCl_2$ and 0.1 μmole EDTA. After 15–30 min at 37 °C the resulting free sialic acid was determined by the thiobarbituric acid assay [15].

A unit of enzyme is defined as the amount that releases one micromole of *N*-glycolyl neuraminic acid from hog submaxillary glycoprotein per minute and the specific activity as the micromoles of sialic acid released per minute per milligram of protein, determined by the method of Lowry et al. [16].

Preparation of gels for chromatography. The various activated gels, 1 ml, used for affinity chromatography were washed with 10 washing cycles of 20 ml consisting of a wash at pH 4 (0.1 M acetate buffer containing 1 M NaCl) followed by a wash at pH 8 (0.1 M borate buffer containing 1 M NaCl) as recommended by the manufacturer (Pharmacia Fine Chemicals). The gels were then equilibrated at 4 °C by washing with 500 ml of 0.05 M acetate buffer, pH 5.5, containing 2 mM $CaCl_2$ and 0.2 mM EDTA [4]. 1 ml of the gel was then packed into a 1-ml syringe (0.5 cm × 5.5 cm).

1 ml of the *Vibrio* enzyme solution (containing 0.018 unit of sialidase and 7.4 mg of protein) was then applied to 1 ml of gel and washed down with the same buffer until the absorbance of the eluate at 280 nm was negligible. The sialidase was then eluted with 0.1 M $NaHCO_3$ buffer, pH 9.1. The flow rate approximated 9–10 ml/h; 1.2-ml fractions were collected.

Assays for glycosidases. In addition to sialidase the presence of β-galactosidase and β-*N*-acetylglucosaminidase were tested for in the column eluates using the corresponding *p*-nitrophenyl glycosides as substrates. Quantitation was achieved by monitoring the *p*-nitrophenol release at 410 nm [7]. Fucosidase activity was determined as previously described [7], using H-active hog submaxillary glycoprotein, and α-*N*-acetylgalactosaminidase activity using desialized ovine submaxillary glycoprotein as substrate [8].

RESULTS

Adsorption and elution of V. cholerae sialidase from affinity columns

Sepharose-TriO. A typical adsorption/elution profile of *V. cholerae* sialidase from a column of the tripeptide oxamate, Sepharose-TriO, at 4 °C is shown in Fig. 1.

Sepharose-TyO. The corresponding tyramine oxamate, Sepharose-TyO, was tested in an attempt to answer two questions: (a) is the length of the peptide ligand crucial? and (b) will an uncharged ligand result in a better affinity column?

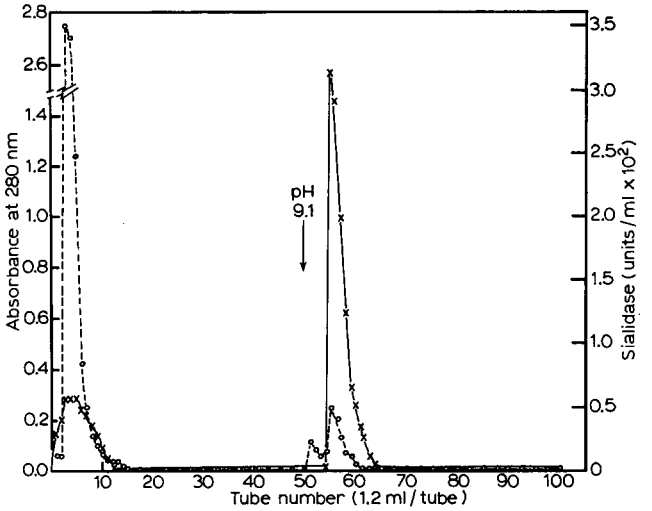

Fig. 1. Elution profile of *Vibrio* sialidase on Sepharose-TriO column at 4 °C. ○---○ absorbance at 280 nm; ×—× sialidase activity.

The results of Sepharose-TyO chromatography at 4 °C are summarized in Table I. Complete adsorption of the sialidase occurs at pH 5.5. Some material absorbing light at 280 nm is eluted at pH 9.1, but the sialidase is still retained on the column. This was readily demonstrated by the release of sialic acid by a suspension of the gel in acetate buffer, pH 5.5, under the usual assay conditions described above.

In contrast, when the Sepharose-TyO chromatography was performed at

TABLE I

CHROMATOGRAPHY OF *VIBRIO CHOLERAE* SIALIDASE ON VARIOUS "AFFINITY" COLUMNS

1 ml column of the different "affinity" gels were equilibrated with pH 5.5-buffered solution (50 mM sodium acetate, 2 mM $CaCl_2$ and 0.2 mM EDTA). The adsorption and elution were carried out at room temperature (RT) or 4 °C as indicated (see text).

Type of "Affinity" Column	Temperature	Effluent at pH 5.5		Eluate at pH 9.1			
		Original activity (%)	Original protein (%)	Original activity (%)	Original protein (%)	Specific activity	Purification factor
Sepharose-ethanolamine	4	107	89.2	0	0	0.02	1.35
Sepharose-tripeptide-oxamate*	4	0	60.0	90.2	13.0	0.14	7.8
Sepharose-tripeptide-oxamate*	4	0	82.2	110.5	6.2	0.35	19.8
Sepharose-tyramine-oxamate	4	0	51.6	0	33.4	0	0
Sepharose-tyramine-oxamate	RT	0	71.6	35.1	11.4	0.06	3.4
Sepharose-tetrapeptide	4	0	44.6	27.8	22.9	0.03	1.5
Sepharose-tetrapeptide	RT	0	28.1	17.1	18.5	0.02	1.0
Sepharose-tyramine	4	0	34.3	0	17.4	0	0
Sepharose-tyramine	RT	0	38.4	0	39.2	0	0
Sepharose-tripeptide	RT	0	40.3	10.6	4.2	0.05	2.9

* Two different preparations.

room temperature, sialidase activity was readily eluted from the column at pH 9.1 (Table I). The sialidase activity, however, was not eluted as quantitatively as it was from the Sepharose-TriO column at 4 °C (Table I).

Sepharose-Tet. In a collaborative experiment with Dr Rolph Jost, a visiting scientist at the Weizmann Institute, from Zurich, Switzerland, we tested his column for the purification of aminopeptidase Thr(Bu^t)–Phe–Pro–Tyr-Sepharose (Jost, R. and Yaron, A. (1974) Eur. J. Biochem., in the press), for its effectiveness to purify sialidase. The effect of temperature on the tenacity of adsorption of sialidase and other proteins is shown in Table I.

These results indicate the non-specific nature of the adsorption and elution of the sialidase from the columns of Sepharose-bound ligands. Table I summarizes the results obtained with columns carrying peptide ligands alone or with phenyl oxamate (the specific inhibitor) attached.

Sepharose-E. In order to elucidate the possible role of unreacted CNBr residues on the activated Sepharose, we examined the effectiveness of such a gel inactivated with ethanolamine, Sepharose-E. As can be seen from Table I most of the protein and enzymatic activity was recovered unchanged in the pH 5.5 effluent. Treatment of some of the other gels with ethanolamine subsequent to coupling with the ligands gave essentially the same pattern of adsorption and elution indicated in Table I for those not treated with ethanolamine.

Effect of pH of eluting buffer on Sepharose-Ty columns

As indicated in Table I, Sepharose-Ty like Sepharose-TyO readily adsorbs the sialidase. The adsorption is much stronger, in that even at room temperature there is no elution of the enzyme at pH 9.1, despite the fact that 39% of the original protein is eluted under these conditions.

The experiment was repeated at 4 °C and the column washed with pH 9.1 buffer until the absorbance at 280 nm dropped to the baseline and then the column eluted with 0.1 M borate buffer, pH 10.0. Some proteins were eluted at pH 10 as determined by the absorption at 280 nm; however, the activity was too weak to be detected. The contents of tubes with some absorption at 280 nm were pooled and concentrated. On analysis this concentrated solution was found to contain 5.7% of the total enzyme activity and 1.3% of the total protein applied to the column (Table I).

Adsorption and elution from Sepharose-TriO of sialidase and other glycosidases in C. perfringens

A typical adsorption and elution experiment was run as with the *V. cholerae* sialidase using Sepharose-TriO gel at 4 °C. The elution profile at pH 5.5 and 9.1 was similar to that obtained from the *Vibrio* enzyme (Fig. 1) in terms of protein and sialidase activity. The contents of the tubes of each peak were pooled and assayed for total protein, by the Lowry procedure [16] and for the various glycosidases. The results are summarized in Table II.

Gel electrophoresis

The effectiveness of the purification achieved was determined by subjecting 25 μg of the original extract and the material eluted at pH 9.1 to electrophoresis on polyacrylamide gels [17]. The electrophoresis was performed in 1% borate buffer,

TABLE II

CHROMATOGRAPHY OF *CLOSTRIDIUM PERFRINGENS* GLYCOSIDASES ON SEPHAROSE-Gly–Gly–Tyr–*N*-*N*-PHENYL OXAMATE

1 ml column was equilibrated with pH 5.5-buffered solution (50 mM sodium acetate, 2 mM $CaCl_2$ and 0.2 mM EDTA). Experiment carried out in the cold room at 4 °C. 4 ml of the enzyme solution was applied (containing 0.53 unit of sialidase and 13.62 mg of protein) and 1.2-ml fractions were collected. The eluate at pH 9.1 was adjusted to pH 6.0 prior to assays for enzymatic activities.

Assay	Effluent at pH 5.5	Eluate at pH 9.1	
	Percent of original	Percent of original	Purification factor
Protein	55.7	3.9	—
Sialidase	12.9	32.7	8.2
β-D-Galactosidase	48.4	14.8	3.7
β-D-*N*-Acetylglucosaminidase	74.6	0	0
α-1,2-L-Fucosidase	55.4	4.4	1.1
α-D-*N*-Acetylgalactosaminidase	66.3	2.1	0.5

pH 9, at 4 °C for 3 h at 5 mA per tube. The protein bands were revealed with Coomassie blue stain [18]. The sialidase activity was determined on a duplicate set of acrylamide gels run in parallel with those stained for protein. Each gel column was then cut into 2.5-mm sections and each slice incubated in 150 μl of solution for 20 h at 37 °C with the substrate under the standard conditions of assay for sialidase. The sialic acid released was detected by the thiobarbituric acid test [15]. Fig. 2 summarizes the results obtained.

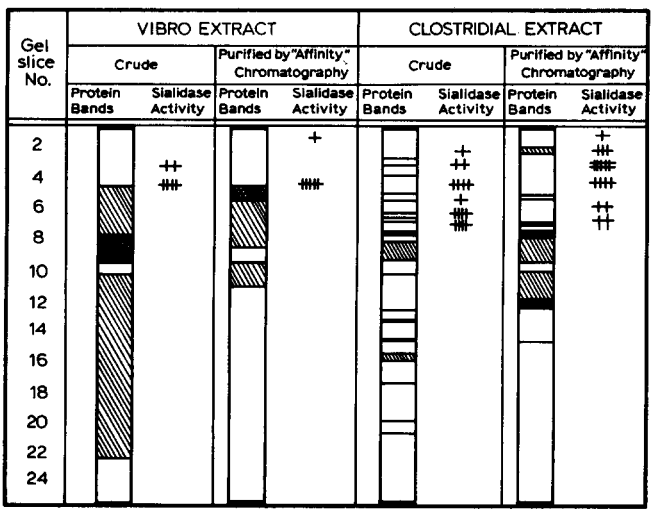

Fig. 2. Identification of proteins and sialidase activity obtained on acrylamide gel electrophoresis.

DISCUSSION

From the data presented it can readily be seen that sialidase from *V. cholerae* is adsorbed on several affinity column gels carrying tyramine or a peptide ligand,

with or without the specific inhibitor phenyl oxamic acid [6]. The tenacity of binding to each gel varies. Nonetheless, manipulation of the conditions in terms of temperature or pH and buffer results in a change in the binding capabilities with subsequent elution of the enzyme.

Using the gel specifically designed to purify sialidase by affinity chromatography [4], viz. Sepharose-TriO, we found that other glycosidases were eluted under the conditions implicated as specific for sialidase alone. Moreover, our results indicate that while there has been effective purification of both the *Vibrio* and *Clostridium* sialidase in the pH 9 eluates, the resulting preparations are still heterogeneous as demonstrated by disc gel electrophoresis (Fig. 2).

While this manuscript was prepared, Rood and Wilkinson [20] reported their observations which indicate that other biologically active factors, such as the hemagglutinin, hemolysin and phospholipase C in the *Clostridium* sialidase preparation used, showed the same adsorption/elution profile as sialidase. Their results could be refuted on the basis that (a) they used a dipeptide, –Gly–Tyr–phenyl oxamate ligand instead of the tripeptide phenyl oxamate recommended by Cuatrecasas and Illiano [4], and that (b) all these activities could be associated with one molecular species as a conjugated protein at pH 5.5. Our results, on the other hand, are not subject to these criticisms. We used the identical type of column used by Cuatrecasas and Illiano [4], Sepharose-TriO, and showed that other glycosidases besides sialidase are adsorbed and eluted. Since the purification factor obtained with the various glycosidases tested is not the same, it would imply that each enzyme behaves independently and argues against a conjugated protein complex for the sialidase with the other glycosidases.

From their results, Rood and Wilkinson [20] conclude that the mechanism of binding of sialidase and other enzymes involves primarily ion-exchange effects. Our results are not in agreement with this hypothesis. At pH 5.5 the uncharged tyramine-conjugated gel readily adsorbed the sialidase and other proteins. This it did more strongly than the other charged peptide–ligand gels, such that even at 4 °C the sialidase was not eluted at pH 9.1 and required at least borate buffer (pH 10) for partial elution. On the other hand, ethanolamine-bound Sepharose did not differ from the un-derivatized Sepharose, having no affinity for sialidase or other proteins (Table I). The implication, that still needs verification, would be that the aromatic ring might be involved; the adsorption of sialidase and other proteins on the tyramine gel is due to non-ionic forces, e.g. hydrophobic interactions between ligand and protein.

In general it would appear that the adsorption and elution of enzymes (or antigen and antibody) from an affinity column could involve a number of different mechanisms: (a) true affinity chromatography, due to the interaction of the enzyme with its specific substrate or inhibitor (or antigen with its antibody), (b) ion-exchange chromatography involving the charged residues on the ligands (in the case discussed here the tri- or tetrapeptide), and/or (c) non-ionic interactions.

We would propose the term amphipathic to such gels which consist of a hydrophilic matrix (Sepharose) with a hydrophobic ligand (tyramine). At any given pH, the type and number of hydrophobic sites available would vary in the different proteins and thereby offer a variety of conditions for the adsorption and elution of these proteins. Judicious exploitation of these conditions will result in an effective chromatographic method for separation of proteins and enzymes [21–24].

In conclusion we can state that Cuatrecasas and Illiano [4] have described a very useful method for concentrating and purifying sialidase. However, the rationale and mechanism proposed for its action is no longer tenable. Moreover, with the heterogeneity of the product obtained, as has been emphasized previously [20], greater caution is necessary in the interpretation of the biological effects observed using sialidase prepared by this method [25–28].

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