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# AFFINITY CHROMATOGRAPHY OF *CLOSTRIDIUM PERFRINGENS* SIALIDASE: NON-SPECIFIC ADSORPTION OF HAEMAGGLUTININ, HAEMOLYSIN AND PHOSPHOLIPASE C TO SEPHAROSYL–GLYCYLTYROSYL-(*N*-(*p*-AMINOPHENYL)OXAMIC ACID)

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## SUMMARY

A Sepharosyl-glycyltyrosyl-(*N*-(*p*-aminophenyl)oxamic acid) (Seph–Gly–Tyr–(NAPOA)) column was constructed in order to purify *Clostridium perfringens* sialidase (mucopolysaccharide *N*-acetyl neuraminylhydrolase, EC 3.2.1.18) by affinity chromatography (Cuatrecasas, P. and Illiano, G. (1971) Biochem. Biophys. Res. Commun. 44, 178–184). The results show that at pH 5.5 haemagglutinin, haemolysin and phospholipase C, as well as sialidase, adsorb to the column. They do not adsorb to Sepharose 4B but will stick to Sepharosyl–glycyltyrosine at the same pH. The latter column appears to be acting as a cation exchange resin because at pH 7.5 this non-specific adsorption is largely overcome. When these proteins are applied to the affinity column at pH 7.5 only sialidase is adsorbed. It is not possible to say whether this is due to specific affinity chromatography or is still due to ion exchange effects. The results show that the method, as used here, is not suitable for the purification of *C. perfringens* sialidase because of this non-specific adsorption of other proteins.

## INTRODUCTION

Sialidase (mucopolysaccharide *N*-acetyl neuraminylhydrolase, EC 3.2.1.18) has been isolated from *Vibrio cholerae* [1], *Clostridium perfringens* [2] and Influenza virus [3].

Edmond et al. [4] showed that *N*-subtituted oxamic acids inhibited influenza virus sialidase. On the basis of this report, Cuatrecasas and Illiano [5] coupled the diazonium derivative of *N*-(*p*-aminophenyl)oxamic acid (NAPOA) to Sepharosyl–glycylglycyltyrosine (Seph–Gly–Gly–Tyr) to produce a selective adsorbant for the purification of sialidase by affinity chromatography. Their results show that siali-

Abbreviations: NANA, *N*-acetylneuraminic acid; NAPOA, *N*-(*p*-aminophenyl)oxamic acid; Seph–Gly–Tyr, Sepharosyl–glycyltyrosine; Seph–Gly–Tyr-(NAPOA), Sepharosyl–glycyltyrosyl-(*N*-(*p*-aminophenyl)oxamic acid); Seph–Gly–Gly–Tyr-(NAPOA), Sepharosyl–glycylglycyltyrosyl-(*N*-(*p*-aminophenyl)oxamic acid).

dases from the three sources above were adsorbed to the Sepharosyl–glycylglycyl-tyrosyl-(*N*-(*p*-aminophenyl)oxamic acid) (Seph–Gly–Gly–Tyr–(NAPOA)) column and, on alteration of the pH and ionic strength, were eluted quantitatively with increased specific activities.

*C. perfringens* sialidase was first purified by Cassidy et al. [2] using conventional protein purification techniques. In the course of our studies on *C. perfringens* sialidase we attempted to use affinity chromatography to purify the enzyme. This paper reports our findings on the non-specific adsorption of other *C. perfringens* proteins to the sialidase "specific" affinity column. These results have been reported in abstract form [6].

## MATERIALS AND METHODS

### *Materials*

*C. perfringens* type A strains CN 3870 (a gift of Dr J. Gardner) and ATCC 10534 were maintained at 4 °C as a spore suspension and cooked meat culture, respectively.

$\alpha_1$-acid glycoprotein, crystalline bovine albumin, and gas-gangrene antitoxin (welchii) were obtained from Commonwealth Serum Laboratories, Melbourne, Australia, the former as a generous gift. *N*-Acetylneuraminic acid (NANA) was obtained from Koch–Light Laboratories, Colnbrook, Great Britain; *N*-(*p*-nitrophenyl)oxamic acid was from K and K Laboratories Inc., Plainview, New York; glycyl-L-tyrosine was from Schuchard, Munich; lecithin (egg) was from British Drug Houses Ltd, Poole, Great Britain; phospholipase C (from *Cl. welchii*) was from Sigma Chemical Co., St. Louis, Mo. and Sepharose 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden.

The main medium used was Fructose Trypticase Soy Broth which contained 17.0 g Trypticase Peptone (Baltimore Biological Laboratories, Baltimore, U.S.A.), 3.0 g Phytone Peptone (Baltimore Biological Laboratories, Baltimore, U.S.A.), 5.0 g NaCl and 2.5 g $K_2HPO_4$ per litre. It was autoclaved for 15 min at 15 lb/inch$^2$ and sterile fructose added to a final concentration of 0.25% (w/v).

### *Methods*

*Preparation of crude concentrate.* 4 ml of a 24-h Fluid Thioglycollate Medium (Difco) culture of the required strain was inoculated into 15 ml of Fructose Trypticase Soy Broth and incubated for 24 h at 37 °C. This culture was used to inoculate 200 ml of the same medium which was then incubated as before and used to inoculate the final 2 l culture.

The culture was harvested after 6 h at 37 °C and the cells removed by centrifugation at 17 000 × *g* for 30 min. The supernatant was cooled to 4 °C and taken to 50% (v/v) acetone with the addition of precooled (−20 °C) acetone. The mixture was allowed to stand overnight at 4 °C so that the flocculant precipitate would settle on the bottom of the container. The bulk of the supernatant was removed by syphoning and the remainder by centrifugation at 5000 × *g* for 10 min. The precipitate was resuspended in sodium acetate buffer (0.05 M, pH 5.5) and dialysed for 24 h at 4 °C against the same buffer. The dialysed material was then centrifuged at 75 000 × *g* for 10 min. The clarified preparation, which shall be referred to as the crude concentrate, was

stored at −20 °C in small aliquots and used as a source of haemagglutinin and sialidase.

*Sialidase Assay.* Sialidase was assayed by determining the amount of NANA released from $\alpha_1$-acid glycoprotein. The free NANA was estimated by the thiobarbituric acid method [7]. The enzyme was incubated in a total volume of 0.5 ml, containing: 100 $\mu$moles potassium acetate buffer (pH 4.5); 1.0 mg $\alpha_1$-acid glycoprotein; and 150 $\mu$g crystalline bovine albumin. Incubation was for 20 min at 37 °C and the reaction was stopped by heating at 100 °C for 2 min. Control tubes lacked either enzyme or substrate. One unit is defined as the amount of enzyme necessary to release 1 $\mu$mole of NANA per min from the substrate under the conditions of the assay.

*Haemagglutinin assay.* Haemagglutinin was estimated by the standard doubling dilution assay using fowl red blood cells as the indicator. The sample was serially diluted 2-fold in 0.25 ml volumes of 1 mM $NiCl_2$ in 0.85% (w/v) NaCl in sodium borate buffer (5 mM, pH 7.2) in a perspex tray; 25 $\mu$l of a 5% (v/v) suspension of fowl red blood cells in saline was added to each well, the tray shaken, and the endpoint read after 60 min at room temperature. $Ni^{2+}$ was included on a routine basis because in some circumstances it was necessary to add $Ni^{2+}$ to obtain haemagglutinin activity (Rood, J. I. and Wilkinson, R. G., unpublished). The endpoint was read by interpolation between the last well showing complete agglutination and the first well with no agglutination. The haemagglutinin titre is the reciprocal of the endpoint dilution and is expressed as the number of haemagglutinin units per ml sample.

*Phospholipase C assay.* Phospholipase C (phosphatidylcholine: choline phosphohydrolase, EC 3.1.4.3) was assayed by the method of Pastan et al. [8] with egg lecithin as substrate. Each reaction mixture contained 2 mg lecithin, 5 $\mu$M $CaCl_2$ and enzyme in a total volume of 0.29 ml of sodium borate buffer (0.05 M, pH 7.6) containing 0.1 M KCl. Incubations were for 30 min at 37 °C, and the conditions of Pastan et al. [8] were followed for the extraction and assay of total water-soluble phosphorous (phosphoryl choline). One unit of phospholipase C activity is defined as the amount of enzyme required to hydrolyse 1 $\mu$mole of lecithin per min under the conditions of the assay.

*Haemolysin assay.* Haemolysin ($\theta$-toxin) was assayed by lysis of horse red blood cells. Each reaction mixture contained 0.5 ml of a 5% (v/v) suspension of horse red blood cells (prepared from defibrinated horse blood washed four times with isotonic saline), 1 $\mu$mole dithiothreitol, haemolysin sample (up to 0.1 ml) and sodium phosphate buffer (0.15 M, pH 7.6) in a total volume of 1.0 ml. The reaction was initiated by the addition of haemolysin. After 30 min at 37 °C the reaction was stopped by the addition of 2.0 ml of ice-cold isotonic saline containing *C. perfringens* antitoxin (4 units/ml), the mixture agitated on a Vortex mixer and centrifuged at 1500 × *g* for 5 min. The absorbance of the supernatant at 559 nm was converted to percent haemolysis by comparison with standards. Extrapolation to 50% haemolysis was made usually from three levels of activity falling within the linear range of the assay (i.e. between approx. 30 and 60% haemolysis). One unit of haemolysin is defined as that amount of activity causing lysis of 50% of the horse red blood cells under the conditions of assay.

*Protein estimation.* The protein content of column fractions was determined by measuring the absorbance at 280 nm on a Zeiss PMQ II Spectrophotometer.

*Preparation and use of columns. N*-(*p*-Nitrophenyl)oxamic acid was converted to NAPOA by catalytic hydrogenation (6 h 40 mm pressure, in Parr) using dimethyl-

formamide with a Raney nickel catalyst. NAPOA was then coupled to Sepharose 4B as previously described [5], except that Gly–Tyr was used instead of Gly–Gly–Tyr to extend the ligand from the agarose backbone. By acid hydrolysis and amino acid analysis the derivative contained 20 $\mu$moles of Gly–Tyr per ml of packed gel. 4 ml of packed Sepharosyl–glycyltyrosyl-(*N*-(*p*-aminophenyl)oxamic acid) (Seph–Gly–Tyr–(NAPOA)) was used to overlay a 1.5 cm $\times$ 16 cm column of Sephadex G-15. The purpose of the G-15 was to reduce the time the enzyme was exposed to the pH 9.1 elution buffer [9]. A duplicate G-15 column was overlaid with Sepharosyl–glycyltyrosine (Seph–Gly–Tyr) for use as a control column.

Unless stated otherwise the adsorption and elution conditions used for both of these columns were as follows. The sample was applied to the particular column in sodium acetate buffer (0.05 M, pH 5.5) and washed with the same buffer until the absorbance at 280 nm was zero. The column was then washed with sodium bicarbonate buffer (0.1 M, pH 9.1). The buffers were applied at a flow rate of 23 ml/h and fractions were collected using a Toyo Fraction Collector.

## RESULTS

### *Non-specific nature of affinity column*

Cuatrecasas and Illiano [5] used Gly–Gly–Tyr to extend the NAPOA ligand from the agarose backbone whereas we synthesised an affinity column using Gly–Tyr for this purpose. To test the effectiveness of this shorter peptide an aliquot of crude concentrate was applied to the Seph–Gly–Tyr–(NAPOA) column as described in Materials and Methods. As shown in Fig. 1a, the sialidase activity was completely adsorbed and separated from the bulk of the protein in a similar manner to the previous report [5]. This proves that the shorter peptide still produced an effective affinity column.

To reproduce the original conditions [5] a similar aliquot of crude concentrate was applied in sodium acetate buffer (0.5 M, pH 5.5) containing 2 mM $CaCl_2$ and 0.2 mM EDTA. The elution profile was identical with that obtained previously (Fig. 1). EDTA and $CaCl_2$ were included in the starting buffer because the *V. cholerae* enzyme is $Ca^{2+}$ dependent [1]. They were omitted from subsequent experiments because *C. perfringens* sialidase does not require $Ca^{2+}$ for activity.

The crude concentrates used as a source of sialidase in these experiments also contained haemagglutinin, a non-enzymic protein that clumps red blood cells [10]. It was expected that haemagglutinin would not be adsorbed to the affinity column and would follow the same profile as the bulk of the protein. However, this was not the case: haemagglutinin was adsorbed and eluted in the same manner as sialidase (Fig. 1a). As before the same profile was obtained in the presence of EDTA and $CaCl_2$.

To see whether the adsorption of haemagglutinin was in some way peculiar to the strain CN 3870 a crude concentrate was made from *C. perfringens* ATCC 10534 and applied to the affinity column. The sialidase and haemagglutinin from this strain behaved identically to those from CN 3870.

Following these observations it was decided to examine the behaviour of two other extracellular enzymes produced by *C. perfringens*, namely phospholipase C and haemolysin. Phospholipase C (Sigma) which was used as a crude source of both enzymes, was added to an aliquot of crude concentrate and dialysed against the starting

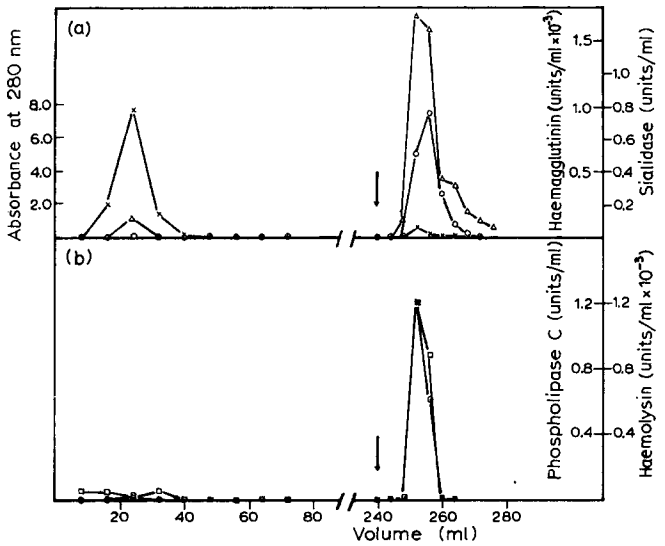


Fig. 1. Elution profiles of sialidase, haemagglutinin, haemolysin and phospholipase C on Seph–Gly–Tyr-(NAPOA) at pH 5.5. (a) An aliquot of crude concentrate was dialysed overnight against sodium acetate buffer (0.05 M, pH 5.5). The dialysed material was applied to a 1.5 cm × 16 cm column of Sephadex G-15 which was covered with 4.0 ml of Seph–Gly–Tyr-(NAPOA) as in Materials and Methods. The column was washed with the same buffer and then eluted with sodium bicarbonate buffer (0.1 M, pH 9.1) as indicated by the arrow. The flow rate was 23 ml/h and fractions were collected and assayed for sialidase (○—○), haemagglutinin (△—△), and the absorbance read at 280 nm (×—×). (b) 4.0 mg of phospholipase C (Sigma) was added to a similar aliquot of crude concentrate. The mixture was dialysed and applied to the Seph–Gly–Tyr-(NAPOA) column as in (a). Fractions were collected and assayed for haemolysin (○—○) and phospholipase C (□—□).

buffer. The dialysed material was then applied to the column and eluted as before. Both phospholipase C and haemolysin behaved in a similar manner to sialidase and were quantitatively adsorbed and eluted (Fig. 1b). Thus we have shown that three other extracellular proteins were also adsorbed to an affinity column supposedly specific for sialidase.

To ascertain which part of the affinity column was responsible for the adsorption of these other proteins a control column consisting only of the Seph–Gly–Tyr backbone was constructed as in Materials and Methods. The results (Fig. 2) show that sialidase, haemagglutinin and haemolysin were adsorbed to the Seph–Gly–Tyr column in the same manner as they were to the Seph–Gly–Tyr-(NAPOA) column. This indicates that the adsorption of all of these proteins, including sialidase, to Seph–Gly–Tyr-(NAPOA) is non-specific. If sialidase was specifically adsorbed to the NAPOA part of the affinity moiety then it would not be expected to be adsorbed to Seph–Gly–Tyr. The adsorption of sialidase could however be a combination of both specific and non-specific interactions. Other experiments showed that phospholipase C also adsorbs to Seph–Gly–Tyr but the behaviour of this enzyme was not followed in the experiments reported here.

To show whether Sepharose 4B or Gly–Tyr was responsible for this non-specific adsorption a mixture of phospholipase C and crude concentrate was applied to Sepharose 4B (Fig. 3). Each of the proteins assayed passed straight through the

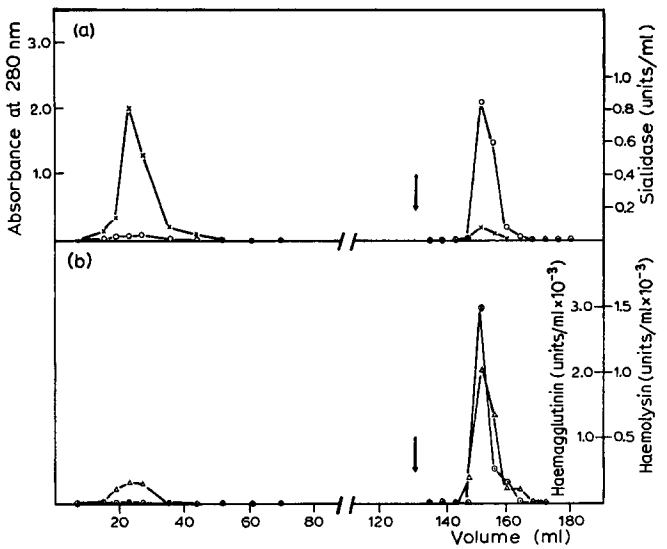


Fig. 2. Elution profiles of sialidase, haemagglutinin and haemolysin on Seph–Gly–Tyr at pH 5.5. 2.0 mg of phospholipase C (Sigma) was added to an aliquot of crude concentrate and dialysed overnight against sodium acetate buffer (0.05 M, pH 5.5). The dialysed material was applied to a 1.5 cm × 16 cm column of Sephadex G-15 which was covered with 4.0 ml of Seph–Gly–Tyr as in Materials and Methods. The column was washed with the same buffer and then eluted with sodium bicarbonate buffer (0.1 M, pH 9.1) as indicated by the arrows. The flow rate was 23 ml/h. Fractions were collected and assayed for sialidase (○—○), haemagglutinin (△—△), and haemolysin (○—⊙). The absorbance was read at 280 nm (×—×).

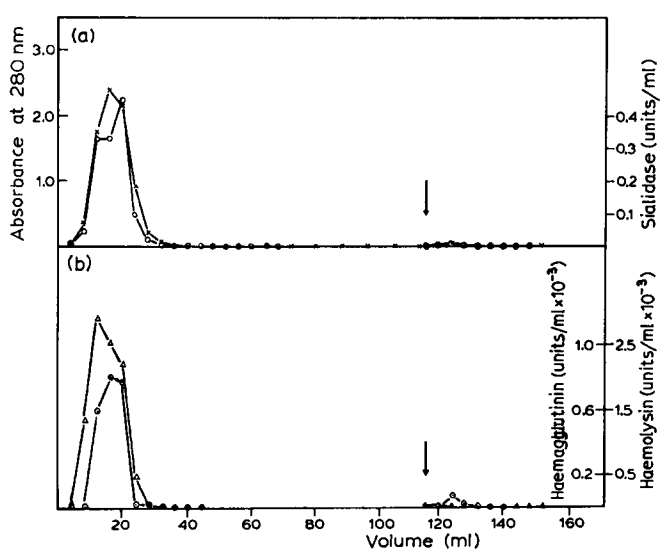


Fig. 3. Elution profiles of sialidase, haemagglutinin and haemolysin on Sepharose 4B at pH 5.5. The sample was prepared as in Fig. 2 and applied to a 1.5 cm × 6.6 cm column of Sepharose 4B. The column was washed with sodium acetate buffer (0.05 M, pH 5.5) and then eluted with sodium bicarbonate buffer (0.1 M, pH 9.1) as indicated by the arrows. Fractions were collected and assayed for sialidase (○—○), haemagglutinin (△—△) and haemolysin (○—○). The absorbance was read at 280 nm (×—×).

column without being significantly retarded. The non-specific binding is therefore due to the Gly–Tyr component of the columns.

*Effect of pH and ionic strength on non-specific adsorption*

To find out whether specific affinity chromatography was operating at all it was decided to look for conditions under which the three proteins, sialidase, haemagglutinin and haemolysin, failed to adsorb to Seph–Gly–Tyr and then to study the effect of these conditions on their adsorption to Seph–Gly–Tyr-(NAPOA).

When a mixture of the three proteins was applied to the Seph–Gly–Tyr column in Tris–HCl buffer (0.05 M, pH 7.5) all of the haemagglutinin and haemolysin activity and about 6% of the sialidase activity, was eluted with the main protein peak (Fig. 4). On further washing of the column with the same buffer most of the remaining sialidase activity was eluted. Thus the change in starting buffer from pH 5.5 to pH 7.5 has meant that haemagglutinin and haemolysin are no longer adsorbed to the column and that sialidase is merely retarded by it.

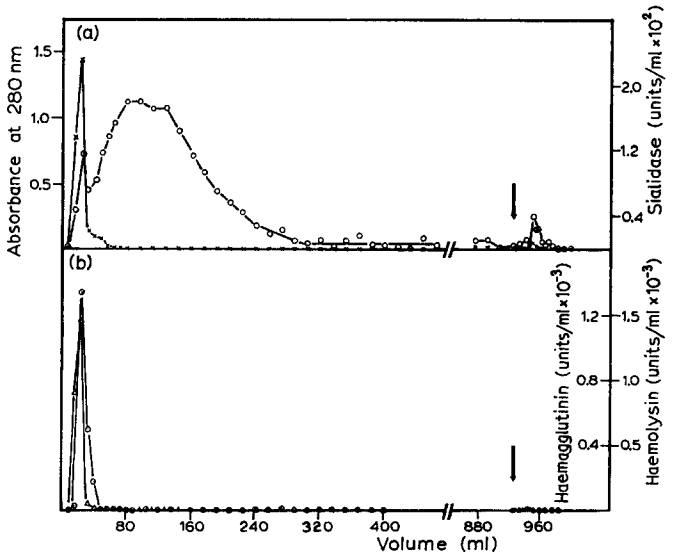


Fig. 4. Elution profiles of sialidase, haemagglutinin and haemolysin on Seph–Gly–Tyr at pH 7.5. 2.0 mg of phospholipase C (Sigma) was added to an aliquot of crude concentrate and dialysed overnight against Tris–HCl buffer (0.05 M, pH 7.5). The dialysed material was applied to the Seph–Gly–Tyr column used in Fig. 2, and the column was washed with Tris–HCl buffer (0.05 M, pH 7.5). The column was then eluted with sodium bicarbonate buffer (arrows) as before (Fig. 2). Fractions were collected and assayed for sialidase (○—○), haemagglutinin (△—△), and haemolysin (⊙—⊙). The absorbance was read at 280 nm (×—×).

When the sample was applied to the Seph–Gly–Tyr-(NAPOA) column under identical conditions (i.e. at pH 7.5) a different result was obtained (Fig. 5). Most of the haemagglutinin and haemolysin activity was not retarded by the column; a small amount (approx. 3% of the eluted activity) was firmly bound on the column and was only released at pH 9.1. When the Seph–Gly–Tyr column was used (Fig. 4), no haemagglutinin or haemolysin was adsorbed, hence the addition of the NAPOA

ligand has resulted in a slight increase in non-specific adsorption. Sialidase behaved somewhat differently: instead of simply being retarded the enzyme was bound to the column (Fig. 5). However, on continuous washing with the starting buffer it was very slowly leached from the column. After washing with 900 ml of buffer 66% of the activity was removed, the remainder was eluted at pH 9.1 in the normal manner. Presumably further washing at pH 7.5 would have leached all the sialidase activity from the column. These results show that the addition of the NAPOA ligand produced a much greater effect on sialidase than on the other proteins. Accordingly it was of interest to determine whether NAPOA inhibited this enzyme.

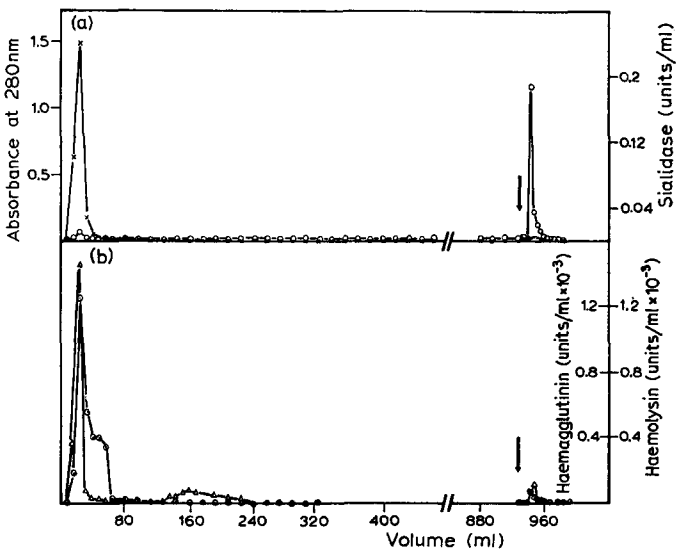


Fig. 5. Elution profiles of sialidase, haemagglutinin and haemolysin on Seph–Gly–Tyr-(NAPOA) at pH 7.5. The sample was prepared as in Fig. 4 and applied to the Seph–Gly–Tyr-(NAPOA) column used in Fig. 1. The column was eluted as in Fig. 4, the sodium bicarbonate wash is indicated by the arrows. Fractions were collected and assayed for sialidase (○—○), haemagglutinin (△—△), and haemolysin (⊙—⊙). The absorbance was read at 280 nm (×—×).

The initial studies on *N*-substituted oxamic acids as inhibitors of influenza virus sialidase [4] did not include NAPOA and no inhibition data has been reported for this particular oxamic acid. Cassidy et al. [11] reported that NANA did not inhibit the *C. perfringens* enzyme, whereas the *V. cholerae* and influenza virus enzymes are inhibited by NANA [12].

Crude concentrate was used as the source of sialidase for inhibition studies with NAPOA. The enzyme was pre-incubated for 65 min at 37 °C at pH 5.5 with various concentrations of NAPOA, the substrate was then added and the enzyme assayed as in Materials and Methods. The results, presented in Table I, show that NAPOA is an effective inhibitor of *C. perfringens* sialidase, 4.3 mM NAPOA causing 57% inhibition.

The main source of the non-specific adsorption has been shown to be Gly–Tyr. Therefore inhibition studies were done for all four proteins by pre-incubating them with 0.02 M Gly–Tyr for 60 min at 37 °C at pH 5.5. No inhibition was detected.

TABLE I

INHIBITION OF SIALIDASE BY *N*-(*p*-AMINOPHENYL)OXAMIC ACID

An aliquot of crude concentrate was pre-incubated for 65 min at 37 °C in sodium acetate buffer (0.05 M, pH 5.5) with various concentrations of NAPOA. The substrate was then added and the enzyme assayed as in Materials and Methods. Control tubes were pre-incubated in the absence of either NAPOA or crude concentrate and the results are presented as the percentage inhibition compared with the control.

| NAPOA (mM) | Sialidase activity Inhibition (%) |
|---|---|
| 0 | 0 |
| 2.1 | 14 |
| 4.3 | 57 |
| 10.8 | 96 |

## DISCUSSION

Previous workers tested the specificity of their affinity chromatography techniques by either of two methods: firstly by using control columns which had the hydrocarbon extending arms but lacked the specific ligands [13]; and secondly, by showing that other enzymes or proteins were not adsorbed to their affinity columns [14, 15].

It has been reported [16, 17] that commercial sialidase preparations are contaminated with other enzymes or proteins. Cuatrecasas and Illiano [5] obtained a 45-fold purification of commercial *C. perfringens* sialidase using affinity chromatography. However, no data were presented as to the state of purity of the resultant enzyme although it was stated that the specific activity of their enzyme was comparable to that of the purified enzyme [2]. They did not show that other enzymes which may have been present in their crude preparation were not adsorbed nor did they add other enzymes to their preparation to show that these were not bound to the column. Finally they did not test for non-specific adsorption of sialidase to the Seph–Gly–Gly–Tyr backbone of the column, i.e. it was not shown that the sialidase adsorption was dependent only upon the NAPOA ligand. This does not necessarily mean that their resultant enzyme preparation was impure since their starting material may have been free of proteins which could non-specifically adsorb to the column.

The results presented in this paper showed that *C. perfringens* haemagglutinin, haemolysin and phospholipase C, as well as sialidase, were adsorbed to and quantitatively eluted from an affinity column similar to that used by Cuatrecasas and Illiano [5]. There were two differences between the method employed here and their method: the use of Gly–Tyr instead of Gly–Gly–Tyr and the source of sialidase. They used a commercial preparation of sialidase whereas we used a crude acetone precipitate.

Non-specific effects previously reported [13, 18–20] with affinity chromatography systems were generally attributed to ionic interactions with charged groups on the affinity columns. The non-specific adsorption observed by us was largely overcome at pH 7.5. This is consistent with the explanation that the Seph–Gly–Tyr column acts as a cation exchange resin by virtue of the free carboxyl group of tyrosine.

At pH 7.5 sialidase was bound more firmly to the affinity column than the other proteins. Since inhibition studies showed that *C. perfringens* sialidase was inhibited by NAPOA it is possible that specific affinity chromatography was indeed operating. However, this cannot be said for certain. If the Gly–Tyr moiety is acting as a cation exchange ligand then the addition of NAPOA will increase the number of free carboxyl groups on the column. Since sialidase is still retarded by the Seph–Gly–Tyr column at pH 7.5 it appears to be more subject to non-specific interactions than the other proteins and hence the addition of the NAPOA ligand could be expected to have a greater effect on sialidase.

The observation that sialidase was firmly bound to the affinity column at pH 7.5 is at variance with Cuatrecasas and Illiano [5] who stated that at pH 6.5 sialidase was not adsorbed to, but merely retarded by, the affinity column. However, this could simply reflect differences in the number of peptide molecules coupled to the Sepharose 4B backbone. They obtained 8 $\mu$moles peptide/ml packed gel whereas the Seph–Gly–Tyr column used here contained 20 $\mu$moles peptide/ml packed gel.

Several workers [16, 21, 22] prepared *C. perfringens* sialidase by methods based on that of Cuatrecasas and Illiano [5] and used the resultant enzyme in studies on mammalian cell membranes. None of them established the purity of the sialidase although Bach and Brashler [16] showed that it was free of phospholipase C. It is quite possible that enzymes or proteins other than sialidase were present in these preparations.

If the Seph–Gly–Tyr-(NAPOA) column used here was acting as a cation exchange resin then it is probable that Cuatrecasas and Illiano's [5] column would act in a similar manner. Modifications of the affinity procedure may be required for the purification of *C. perfringens* sialidase. This could be done by removing the charge from the spacer (e.g. by using the amide or an ester) or by using a different spacer which contains no charged groups. However, there is still the free carboxyl group on the NAPOA ligand itself. This carboxyl group cannot be modified as it is required for *N*-substituted oxamic acids to inhibit sialidase [4].

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