PURIFICATION OF NEURAMINIDASES FROM VIBRIO CHOLERAE, CLOSTRIDIUM PERFRINGENS AND INFLUENZA VIRUS BY AFFINITY CHROMATOGRAPHY

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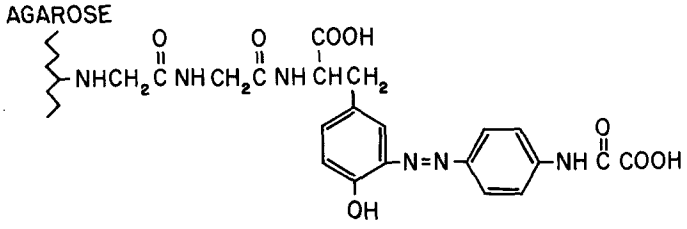
SUMMARY

A selective adsorbent for neuraminidase was prepared by attaching through azo linkage an inhibitor of this enzyme, N-(p-aminophenyl) oxamic acid, to agarose beads containing the tripeptide, glycyl-glycyl-tyrosine. Columns containing this adsorbent can completely extract the enzymatic activity present in extracts of Clostridium perfringens and Vibrio cholerae, and quantitative elution is readily achieved by modifying the pH and ionic strength of the buffer. Intact influenza virus particles are also reversibly adsorbed by these columns, indicating the superficial location of neuraminidase in this virus and emphasizing the feasibility of using functional purification procedures for resolving complex and particulate biological structures.

Sialic acid commonly occurs as a terminal carbohydrate residue in a variety of glycoproteins, mucins, gangliosides, and various oligosaccharides. The function of sialic acid in glycoproteins and cell surfaces has been studied principally by observing the effects of selective removal of this residue by digestion with neuraminidases from influenza virus or Vibrio cholerae ("receptor destroying enzyme"). By such procedures extensive studies have been performed on the contribution of sialic acid to the ability of glycoproteins to participate in enzymatic and hormonal activities, antigen-antibody interactions, clotting mechanisms, metal and hormone transport, and to their role as lubricants (mucins), supportive structures and filtration barriers (basement membranes), and as constituents of cellular membranes of normal and malignant cells (reviewed in Reference 1).

During the course of studies designed to elucidate the possible role of sialic acid in the insulin receptor structures of isolated adipose tissue cells (2), it became apparent that such studies depend critically on the

availability of highly purified neuraminidase preparations which are unfortunately not available commercially. By utilizing the principles of affinity chromatography (3,4) a selective adsorbent was prepared which could be used to rapidly and effectively purify neuraminidases from various sources in essentially a one-step procedure. Based on the report by Edmond, et al. that N-substituted oxamic acids are potent reversible inhibitors of influenza virus neuraminidase (5), N-(p-aminophenyl) oxamic acid was synthesized and attached covalently through azo linkage to agarose beads containing a tripeptide (gly-gly-tyr) (Scheme I). The inhibitor thus extends a considerable distance from the matrix backbone (4).



Scheme I. Adsorbent used in the selective purification of neuraminidases by affinity chromatography. The diazonium derivative of N-(p-aminophenyl) oxamic acid was coupled to agarose-gly-gly-tyr.

Agarose-gly-gly-tyr was prepared by the cyanogen bromide procedure previously described (4). Sepharose 4B was activated with cyanogen bromide (350 mg per ml of packed agarose) and coupled in 0.2 M $NaHCO_3$ buffer, pH 9.5, containing gly-gly-tyr (1 g per 50 ml). By amino acid analysis the derivative contained 8 μmoles of peptide per ml of packed gel. N-(p-Nitrophenyl) oxamic acid (K & K) was converted to the corresponding p-aminophenyl derivative by catalytic hydrogenation (4 hours, 40 mm pressure, in Parr) using dimethylformamide in 10% palladium on charcoal. This compound (80 mg) was dissolved in 30 ml of ice-cold 0.4 N HCl, and 150 mg of sodium nitrite (in 1 ml of cold water) were added over a one-minute period. After 5 minutes an 80 ml slurry of 0.5 M $NaHCO_3$ buffer, pH 8.9, containing 30 ml of packed agarose-gly-gly-tyr was added. The pH of the suspension was adjusted to 8.8, and after stirring

gently for 8 hours at room temperature the coupled agarose derivative was washed with 12 liters of 0.1 N NaCl.

Neuraminidase activity was determined by incubating 0.002-0.02 units of enzyme in 0.2 ml of 0.1 M sodium acetate buffer, 2 mM $CaCl_2$, 0.2 mM EDTA, pH 5.5, containing 0.2 mg of N-acetylneuraminyllactose. After 30 minutes at 37° the sialic acid content was determined by the thiobarbituric acid procedure of Warren (6).

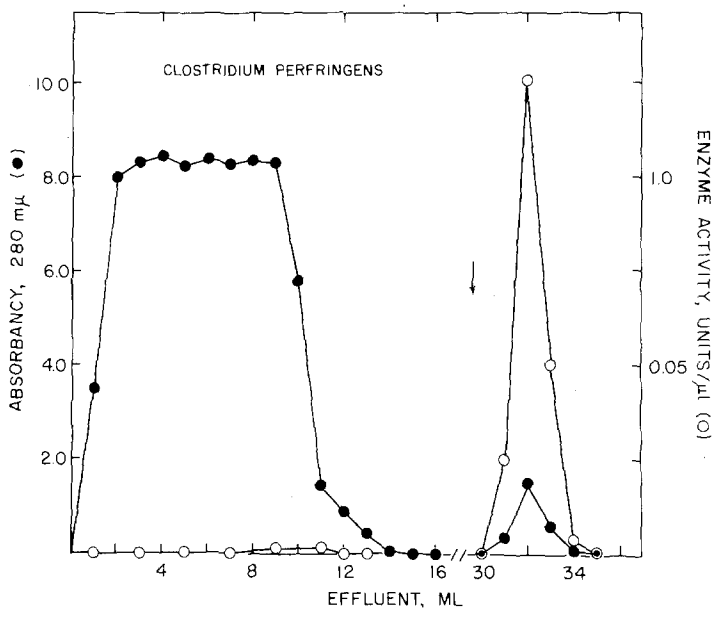


Figure 1. Affinity chromatography of neuraminidase from Clostridium perfringens on the agarose derivative shown in Scheme I. Seven ml of a commercially (Worthington) "purified" enzyme containing 73 mg of protein were dialyzed for 16 hours at 4° against four liters of 0.05 M sodium acetate buffer, pH 5.5, containing 2 mM $CaCl_2$ and 0.2 mM EDTA. This material was applied to a 0.4 x 4 cm column containing the adsorbent which had been equilibrated with the same buffer. Elution was achieved with 0.1 M $NaHCO_3$ buffer, pH 9.1 (arrow); the pH of the eluted sample was immediately lowered to 6.0 with 1 N NaOH. The progress of the eluting buffer through the column can be followed conveniently by the intensification of the color of the adsorbent which results upon ionization of the azo moiety by the higher pH of the buffer. The purification data of this experiment is summarized in Table I.

Virtually all of the neuraminidase from a "purified" preparation of Clostridium perfringens (Figure 1) and from a crude Vibrio cholerae filtrate (Figure 2) adsorbs quite strongly to columns containing the specific adsorbent. Influenza virus particles adsorb similarly well to these columns

TABLE I

AFFINITY CHROMATOGRAPHY OF NEURAMINIDASES FROM VARIOUS SOURCES

These data summarize the chromatographic experiments for purification of the Clostridium perfringens and Vibrio cholerae enzymes, illustrated in Figures 1 and 2, respectively. Influenza virus was purified under identical conditions.

Purification Step	Source of Enzyme: Clostridium perfringens	Vibrio cholerae	Influenza virus[1]
Sample applied on column			
Protein (mg)[2]	73	92	1.2[3]
Volume (ml)	7	12	5
Specific activity[4]	3.6	0.071	0.86
Fraction eluted from column			
Protein (mg)[2]	1.1	0.2	0.5[3]
Specific activity	164	30	1.9
Yield of activity (%)	105	97	91
Purification	45	420	2.2

[1] Purified monovalent inactivated influenza virus vaccine strain A2/Aichi/2/68, containing 400 CCA units per ml, was a kind gift of Dr. Allen F. Woodhour, Merck Institute for Therapeutic Research.
[2] Determined by absorbancy at 210 mμ (7).
[3] For convenience, this was determined by ultraviolet spectroscopy, using $E_{280}^{0.1\%} = 3.0$.
[4] Micromoles of N-acetylneuraminic acid formed per minute per mg of protein.

(Table I). The columns can be washed with large volumes of buffer without loss of enzyme in the effluent. The enzyme is quantitatively eluted by simply altering the pH and ionic strength of the buffer, and the specific activities (Table I) of the purified enzymes are at least as high as those previously reported for the most highly purified enzymes (8). Despite the very small size of the columns, the volume of sample applied to the column can be quite large and the enzyme is eluted in a small volume. These columns

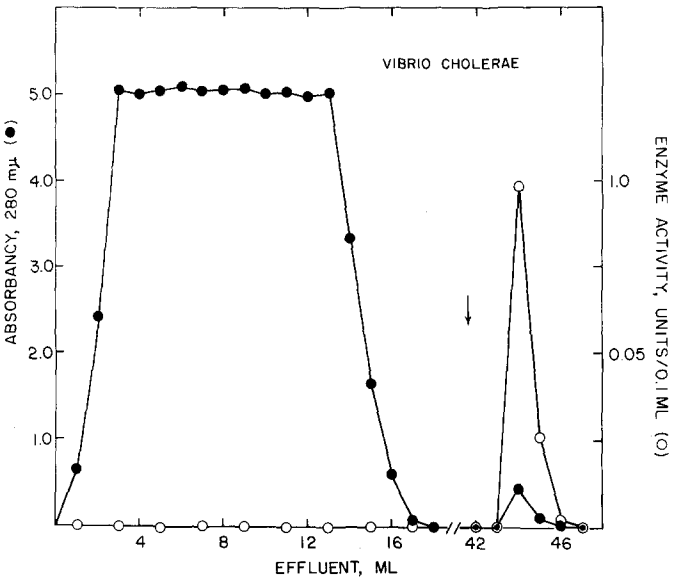


Figure 2. Affinity chromatography of neuraminidase from Vibrio cholerae using the derivative shown in Scheme I. 140 mg of a crude filtrate of Vibrio cholerae (Sigma) were suspended in 12 ml of 0.05 M sodium acetate buffer, pH 5.5, containing 2 mM $CaCl_2$ and 0.2 mM EDTA, centrifuged for 20 minutes at 2000 rpm and dialyzed for 18 hours at 4° against the same buffer. The sample, containing 92 mg of protein was chromatographed by procedures identical to those described in Figure 1. The detailed data are summarized in Table I.

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can be used to concentrate dilute solutions of enzyme. Re-chromatography of the purified enzymes on the same columns does not result in further purification or loss of significant enzymatic activity. The adsorbents can be used repeatedly without apparent loss of effectiveness. The purification of Vibrio cholerae enzyme is particularly striking since the sample applied on the column was a crude filtrate of the bacterium.

The pH (5.5) of the buffer used in chromatography is quite important since at pH 6.5 the enzyme (from the three sources) emerges even without altering the conditions of the buffer, although its migration is retarded with respect to the major protein fraction. It is interesting that despite reported differences in the catalytic pH optimum of the enzymes obtained from different sources (8,9), the optimum pH for adsorption to the affinity column is about 5.5 for all three enzymes tested.

It is notable that intact influenza virus adsorbs strongly to the affinity column (Table I). The virus is desorbed from the column more readily than the enzymes from Clostridium perfringens and Vibrio cholerae. The virus emerges at the front of the eluting buffer, and the pH of the tube containing the eluted virus is 8.1; in contrast, the bacterial enzymes emerge in a solution having a pH of 9.0. The eluted virus is readily sedimented by centrifugation (25,000 x g for 30 minutes), and it can be re-chromatographed and eluted quantitatively. These procedures appear to be relatively innocuous to the virus particles.

These results indicate that neuraminidase exists in a highly superficial portion of the viral particle. The retention of effective interactions between a particulate fraction and a small ligand properly immobilized to a solid support provides further encouragement for the application of the principles and methodology of affinity chromatography to the purification of complex and particulate biological structures (10) such as viruses, bacteria, immunocompetent cells (11), membrane hormone receptors (12) and enzyme complexes, specific ribosomal complexes (13), and multi-enzyme complexes.

The simple techniques described here should readily permit removal of contaminating neuraminidases from other enzyme preparations. The ease with which purification is achieved should also permit more widespread and safer use of this enzyme as a topographic probe of glycoproteins. The use of purified enzymes of different origin and substrate specificity should prove useful in such studies. For example, the enhancement of glucose transport of isolated fat cells in response to insulin is modified selectively but in different degrees by the three enzymes described in this report (2). Preliminary results of studies of the chemical and physical properties of the purified bacterial enzymes confirm earlier observations suggesting the existence of aggregated molecular forms (8,14) which appear to be at least in part dissociated by detergents and sulfhydryl reagents.

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