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PURIFICATION OF NEURAMINIDASE FROM INFLUENZA VIRUSES BY AFFINITY CHROMATOGRAPHY

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Summary

The neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) of the influenza virus recombinant strain (HON2) was solubilized with detergents and isolated by affinity chromatography. The neuraminidase could be purified to a single high molecular weight glycoprotein when assayed under non-reducing conditions on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme showed an increase in specific activity from 2.46 to 189 μM *N*-acetylneuraminic acid released per min per mg protein and the recovery represented 123% of the activity of intact virus particles. The enzyme could be purified from laboratory preparations of virus or from outdated influenza virus vaccine. Viral neuraminidases purified by this technique were stable at pH 6.0 for several hours.

Introduction

The neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) of influenza virus appears to be a tetrameric glycoprotein with a total molecular weight of about 240 000—250 000 [1—3]. The viral neuraminidase exists as 'spike' on the surface of the influenza virion and possesses a hydrophylic area which includes the active site and a 'hydrophobic foot' which is inserted into the phospholipid bilayer surrounding the inner core of the influenza virion. Purification of influenza viral neuraminidase must be preceded by solubilization either by the addition of detergents or by proteolytic cleavage of the hydrophylic portion from the small 'hydrophobic foot' (for review see ref. 4).

Utilizing the affinity column bearing the inhibitor, *N*-(*p*-aminophenyl)oxamic acids, Cuatrecasas and Illiano [5], found a 2.2-fold enrichment of neuraminidase activity from particulate influenza. In this report, the purification of influenza viral neuraminidase with this column was greatly increased by solubilization of the enzyme from the viral envelope with the anionic detergent,

sodium dodecyl sulfate (SDS), followed by stabilization of activity and solubility with Triton X-100.

Materials and Methods

Preparation of virus. The X-7 recombinant strain of influenza virus was grown in the allantoic sac of embryonated eggs and purified as reported previously [2]. In addition, two outdated lots of influenza vaccine were employed as sources of neuraminidase. X-38 (NEq1N2) vaccine was donated by Lederle Laboratories of Pearl River, N.Y. Aichi (H3N2) vaccine was a gift from Merrell-National Laboratory of Swiftwater, Pa.

Preparation of virus for affinity chromatography. Viral preparations were dissolved in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl at a protein concentration of approx. 1.0 mg/ml as determined by the Lowry assay [8]. 20—50 ml of the viral preparation was disrupted with 1.0% SDS and mixed for 15 min at 37°C (X-7 strain) or room temperature (X-38 and Aichi strains). Triton X-100 was added to a final concentration of 10% and the pH adjusted to 5.0 by overnight dialysis at 4°C versus the adsorbing buffer (0.05 M sodium acetate, 2 mM $CaCl_2$, 0.2 mM EDTA, and 0.1% Triton X-100).

Preparation of the column and chromatography of the neuraminidase. The affinity column adsorbent was prepared according to the original procedure of Cuatrecasas and Illiano [5]. The affinity adsorbent was placed in a 2.5-cm diameter column to a bed height of 10 cm. The buffers employed for adsorption and elution were those described [5], modified by the addition of 0.1% Triton X-100. The adsorbing buffer was adjusted to pH 5.0 rather than the original pH 5.5. A flow rate of 180 ml/h was maintained with a peristaltic pump. All fractions were assayed for protein [8] and neuraminidase activity [9,10].

Lowry assay in the presence of Triton X-100. One or two drops of 10% SDS were added to all Triton X-100-containing samples (0.1 ml volume), with mild heating, before addition of reagents for the Lowry assay [8]. This procedure eliminated the need for solvent extraction of the Triton X-100 before proceeding with this assay.

Neuraminidase assay. Neuraminidase activity was assayed with 5—10 μl aliquots of the column fractions in a final volume of 0.25 ml buffer, (0.05 M sodium acetate, 2 mM $CaCl_2$, and 0.2 mM EDTA, pH 6.0, with 0.1% Triton X-100). Fetuin, the substrate, was added to a concentration of 0.25% and the samples were incubated for 15 min at 37°C. Released neuraminic acid was measured according to the Warren procedure [9] as modified by Aminoff [10].

Concentration of column fractions. The viral protein and neuraminidase fractions were concentrated by ultrafiltration in an Amicon unit (model 52) using PM-10 membranes. Neuraminidase activity remained nearly constant under these conditions. The non-neuraminidase-containing protein fractions were subsequently separated into the major polypeptide components by SDS gel filtration [11].

SDS polyacrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis was performed as previously described [2]. To eliminate Triton X-100, protein samples were precipitated with four volumes of acetone at 0°C. The

gels were stained for protein with Coomassie Blue in 7% acetic acid. Glycoproteins were visualized by the periodic acid-Schiff technique [12].

Results

Affinity chromatography of the viral neuraminidase

Early attempts to isolate influenza viral neuraminidase by affinity chromatography employed SDS throughout the buffer systems. Although the neuraminidase of the A_2 subtype (N2) was relatively stable 1% SDS at pH 7.0, inactivation of the enzyme occurred when the pH was lowered to 5.0 for adsorption to the column. When Triton X-100 was employed as the disrupting agent excellent recovery of neuraminidase activity was obtained. However, other viral proteins coeluted with the neuraminidase and the fold purification was relatively low. Thus the use of SDS for solubilization of the neuraminidase and Triton X-100 for maintenance of activity appeared optimal. Triton X-100 was added to a high concentration (10%), presumably displacing the SDS from the micelles maintaining the neuraminidase in solution after disruption of the viral envelope. The use of concentrations of Triton X-100 below 10% resulted in low yields of enzyme. If the level of SDS was increased to 20% for disruption of more concentrated viral preparations, additional Triton X-100 (up to 20%) was required for maximum recovery of neuraminidase activity.

The enzyme was purified according to the procedure of Cuatrecasas and Illiano [5]. A slightly more acidic buffer was used for the adsorption cycle, pH 5.0, rather than 5.5, resulting in increased column capacity. The chromatographic pattern for the isolation of X-7 viral neuraminidase is shown in Fig. 1.

The neuraminidase eluted sharply on elevation of pH, centered at about pH 7.0. Only a small amount of protein was associated with the enzyme, the bulk of the protein passed directly through the column. SDS-polyacrylamide gel electrophoresis of the total viral protein and purified enzyme is shown in Fig. 2. The enzyme was purified to a single high molecular weight component with a molecular weight in excess of 200 000. This component had previously been

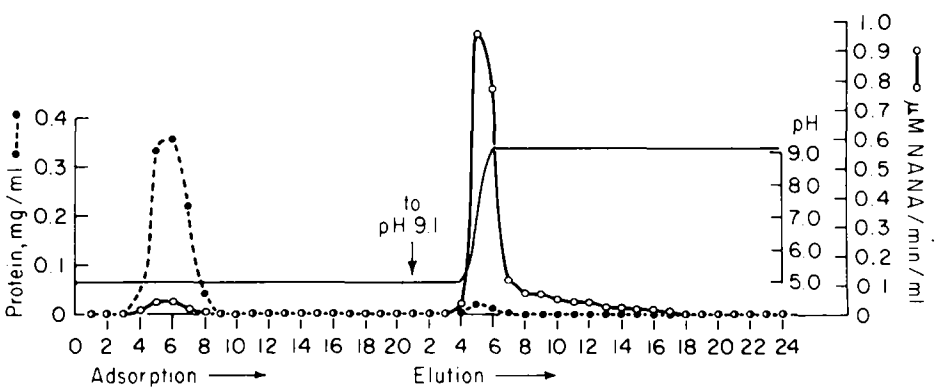

Fig. 1. Affinity chromatography of influenza virus of the X-7 strain (HON2). Adsorption of neuraminidase from the viral preparation was performed at pH 5.0; neuraminidase was eluted by elevation to pH 9.1. Elution of protein is shown by the broken line (●- - - - -●). Elution of neuraminidase activity is shown by the solid line (○———○).

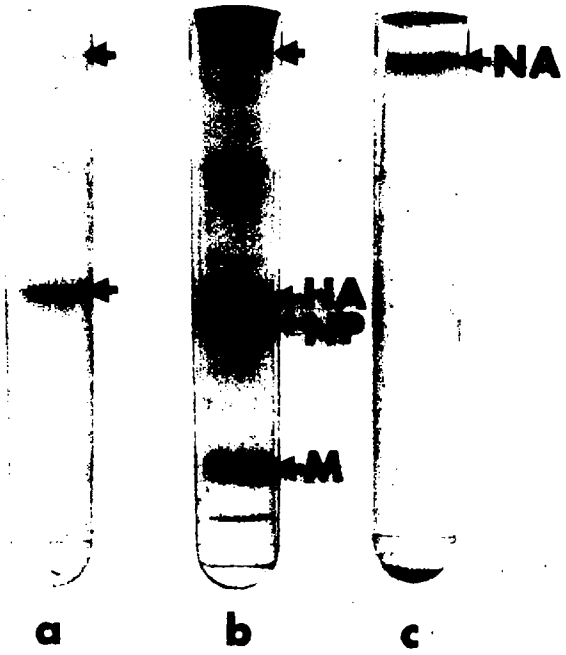

Fig. 2. SDS-polyacrylamide gel electrophoresis of total viral protein (a and b) and neuraminidase purified by affinity chromatography (c) for the X-7 influenza virus preparation shown in Fig. 1. SDS-polyacrylamide gels b and c were stained for protein with Coomassie Blue, gel a was stained for carbohydrate using periodic acid-Schiff's reagent. The glycoproteins NA (neuraminidase) and HA (hemagglutinin) are shown in gel a. The non-glycosylated proteins NP (nucleoprotein) and M (matrix protein) are shown on gel b in addition to neuraminidase and hemagglutinin. Electrophoresis was conducted without prior reduction of the proteins. The direction of electrophoresis is downward.

shown to possess neuraminidase activity [2]. A summary of the purification data is presented in Table I. The specific activity of the neuraminidase preparation increased from 2.46 to 189 μM *N*-acetylneuraminic acid released per min per mg, an enrichment of 77-fold. The neuraminidase activity was associated with 1.6% of the total protein. This value is considerably lower than the 7% of

TABLE I

NANA, *N*-acetylneuraminic acid.

	Neuraminidase activity		Protein		Specific activity (μM NANA/ min per mg)	Purification (-fold)
	Total (μM NANA/ min)	Yield (%)	Total (mg)	Yield (%)		
SDS-disrupted X-7 virus + Triton X-100	40.7	—	16.5	—	2.46	—
Non-adsorbed virus fraction (protein neuraminidase)	2.46	4.7%	10.0	60.6	—	—
Enzyme fraction (neuraminidase)	49.9	123.0%	0.26	1.6	189	77

total protein given in the literature for the neuraminidase content of the virion, although this value is known to vary among strains [4]. Both the degree of purity of the virus preparation and activation occurring with solubilization of the neuraminidase would affect the fold purification.

Generally, 20—50 mg viral protein was applied per chromatographic cycle. Quantities exceeding these resulted in oversaturation of the column and neuraminidase activity would be associated with the non-adsorbed protein. This capacity appeared to be considerably below that found by Cuatrecasas and Illiano [5] who employed columns of much smaller dimensions. The addition of Triton X-100 to the buffers and/or the existence of neuraminidase-detergent micelles, creating a relatively large active molecule which would not be accessible to internal binding sites within the agarose particles, might affect apparent column capacity.

The neuraminidase had to be in the active state for adsorption to the column to occur. If the enzyme was inactivated during preparation for chromatography, none of the high molecular weight protein was adsorbed to the column.

Purification of neuraminidase from vaccines

Influenza viral neuraminidase could also be isolated from outdated vaccine lots with a high degree of purity as shown in Fig. 3. Two separate purifications are shown for each vaccine strain. The specific activity of ten preparations of neuraminidase from X-38 vaccine averaged 25.4 μM/min per mg (range of 8.0—49.5 μM/min per mg). The average increase in specific activity was 10.6 (range of 3.5—21.4).

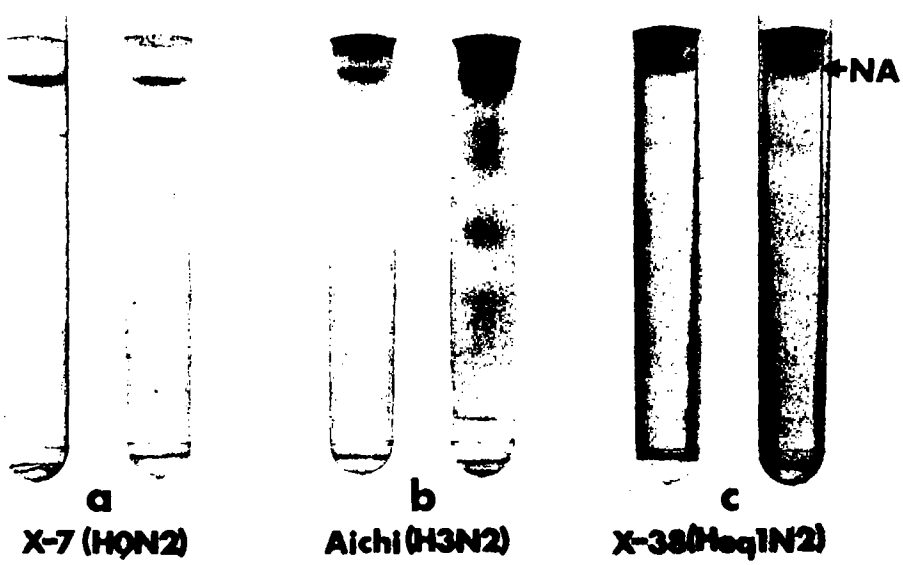

Fig. 3. Comparison of neuraminidases isolated by affinity chromatography from different strains. Neuraminidase was purified from laboratory grown X-7 (HON2) virus or outdated lots of vaccine (Aichi (H3N2) and X-38 (Heq1N2)). Each SDS polyacrylamide gel represents a separate isolation of the enzyme. All three pairs of isolated neuraminidases belong to the N2 subtype but each is coupled with a separate subtype of hemagglutinin. Staining for protein was performed with Coomassie Blue.

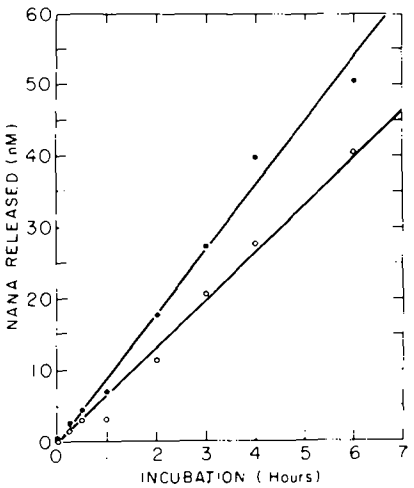

Fig. 4. Stability of influenza viral neuraminidase purified from X-7 strain (HON2) by affinity chromatography. Neuraminidase was incubated at 37° C for several hours either in the presence (●———●) or absence (○———○) of 0.1% Triton X-100 in 0.05 M sodium acetate buffer, pH 6.0, and 1 mM $CaCl_2$. The substrate was fetuin at a final concentration of 2.5%.

Stability of the purified neuraminidase

The neuraminidase purified by this technique was found to have excellent stability for several hours under the assay conditions at pH 6.0 (see Fig. 4). The enzyme as prepared contains 0.1% Triton X-100 but was diluted 400-fold. If Triton X-100 was included in the dilution buffer the rate of activity was greater than the rate for those samples with no detergent added. However, the enzyme appeared to be equally stable with or without additional Triton X-100.

Discussion

Reports have appeared on the non-specificity of the affinity column of Cuatrecasas and Illiano [5] for the adsorption of neuraminidase [13,14]. The successful purification of influenza viral neuraminidase might be attributed to two factors. The use of purified virus as the starting material diminishes the complexity of the protein preparation; one needs to separate the enzyme from only about six or seven proteins rather than the large number of proteins found in a cellular extract. Furthermore, the use of detergents may cause selective inactivation of other fractions, diminishing non-specific adsorption of other proteins.

This technique has been particularly successful for the purification of N2 neuraminidase, perhaps because these enzymes can be subjected to relatively rigorous conditions of detergent exposure without denaturation. The purification of B/Lee neuraminidase was not attempted; this enzyme is known to be especially resistant to denaturants [15,16]. Neuraminidase preparations from all influenza virus strains so far investigated could be "enriched" for the enzyme by passage over this column, although not all enzyme preparations could be purified to the same degree of purity as the N2 neuraminidase.

The development of this technique of affinity isolation for influenza viral neuraminidases (N2) results in the yield of a highly active, pure and stable product. Since detergents alone are used for solubilization of the enzyme, no proteolytic enzymes have been introduced which could subsequently contaminate the neuraminidase preparation. The purified viral neuraminidase should be useful to investigators studying the structure of glycoproteins or to those who wish to modify the surface of cells by the removal of neuraminic acid. Furthermore, the technique as developed should be applicable not only to the purification of other viral neuraminidases, but should also be of value for the purification of membrane-associated neuraminidase from other sources.

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References

1 Kendal, A.P. and Eckert, E.A. (1972) Biochim. Biophys. Acta 258, 484—495
2 Bucher, D.J. and Kilbourne, E.D. (1972) J. Virol. 10, 60—66
3 Wrigley, N.G., Skehel, J.J., Charlwood, P.A. and Brand, C.M. (1973) Virology 51, 525—529
4 Bucher, D.J. and Palese, P. (1975) in The Influenza Viruses and Influenza (Kilbourne, E.D., ed.) pp. 83—123, Academic Press, New York
5 Cuatrecasas, P. and Illiano, G. (1971) Biochem. Biophys. Res. Commun. 44, 178—184
6 World Health Organization (1972) Bull. W.H.O. 45, 119—124
7 Laver, W.G. and Kilbourne, E.D. (1966) Virology 30, 493—501
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
9 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
10 Aminoff, D. (1961) Biochem. J. 81, 384—392
11 Bucher, D.J. (1975) in The Negative Strand Viruses (Mahy, B.W.J. and Barry, R.D., eds.), Vol. 1, pp. 133—143, Academic Press, London
12 Zacharius, R.M., Zell, T.E., Morrison, J.H. and Woodlock, J.J. (1969) Anal. Biochem. 30, 148—152
13 Rood, J.I. and Wilkinson, R.G. (1974) Biochim. Biophys. Acta 334, 168—178
14 Huang, C.C. and Aminoff, D. (1974) J. Chomatogr. 371, 462—469
15 Laver, W.G. (1963) Virology 20, 251—262
16 Hoyle, L. and Almeida, J.D. (1971) J. Hyg. 69, 461—469