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Note

Cytotoxic contaminants in commercial *Clostridium perfringens* neuraminidase preparations purified by affinity chromatography

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Neuraminidase (sialidase, N-acetylneuraminate glycohydrolase, EC 3.2.1.18) produced by bacteria of the orders *Eubacteriales* and *Pseudomonadales* is frequently employed in studies on the properties of mammalian cell surfaces and cell surface sialic acid. The enzyme from *Clostridium perfringens*, CP NAN-ase*, is particularly important in this connection because its activity is Ca^{2+}-independent. Since commercial preparations of purified CP NAN-ase may contain cytotoxic contaminants[1], interpretations of experiments performed on whole cells with such preparations are open to question. For this reason, a published method for the further purification of microbial neuraminidases by affinity chromatography on Seph–Gly–Gly–Tyr–NAPOA[2] has attracted general attention although data on purity of the resulting enzymes were not presented. More recently Rood and Wilkinson[3], using a Seph–Gly–Tyr–NAPOA gel instead of the Seph–Gly–Gly–Tyr–NAPOA recommended by Cuatrecasas and Illiano[2], have reported that cytotoxic contaminants of CP NAN-ase, *i.e.* hemagglutinin, hemolysin and phospholipase C cannot be separated from CP NAN-ase by the latter method. They recommended application of the material to the affinity columns at pH 7.5 instead of 5.5, claiming that at this pH non-specific adsorption largely is overcome, and only NAN-ase is adsorbed. The present communication describes observations concerning the purity of commercially available NAN-ase preparations and attempts at further purification by affinity chromatography as described by Cuatrecasas and Illiano[2] and Rood and Wilkinson[3]. We conclude that although these methods are not suitable for the purification of CP NAN-ase to homogeneity, they can nevertheless be useful in removing much of the cytotoxic contaminants found in commercial CP NAN-ase preparations.

MATERIALS AND METHODS

Preparations and use of columns

Seph–Gly–Gly–Tyr–NAPOA was prepared as described by Cuatrecasas and

* Abbreviations used are: CP NAN-ase, *Clostridium perfringens* neuraminidase; VC NAN-ase, *Vibrio cholerae* neuraminidase; Seph–Gly–Gly–Tyr–NAPOA, Sepharosyl-glycylglycyltyrosyl-[N-(*p*-aminophenyl)oxamic acid]; Seph–Gly–Tyr–NAPOA, Sepharosyl-glycyltyrosyl-[N-(*p*-aminophenyl)oxamic acid].

Illiano[2] with the following modifications: N-(*p*-nitrophenyl)oxamic acid, which is no longer available commercially, was prepared in collaboration with Dr. N. Barton by the procedure of Aschan[4]; catalytic hydrogenation of the compound to the corresponding *p*-aminophenyl derivative was carried out in methanolic HCl. Seph–Gly–Tyr–NAPOA was prepared in the identical manner except that Gly–Tyr was used instead of Gly–Gly–Tyr. By amino acid analysis of acid hydrolysates, the Seph–Gly–Gly–Tyr derivative contained 15 μmoles and the Seph–Gly–Tyr derivative 17 μmoles of peptide per ml of packed gel. Three to four ml of packed gel were used per column. Eluting buffers were applied at a flow-rate of 12 ml/h and 2-ml fractions were collected.

Enzyme assays

The following enzymatic activities were assayed under appropriate conditions giving a reaction rate that was constant with time and proportional to protein concentration: (a) NAN-ase activity was determined by the method of Cassidy *et al.*[5] with 1 mg of bovine submaxillary mucin (Worthington, Freehold, N.J., U.S.A.) per incubation mixture as substrate; (b) protease activity was determined by incubating the enzyme (0.05–0.5 mg protein) with 3 mg of Azocoll (Calbiochem, La Jolla, Calif., U.S.A.) for 15–30 min and, after brief centrifugation, measuring the absorbance at 520 nm of the supernatant fluid. Phospholipase C activity was assayed by the method of Pastan *et al.*[6] with soybean lecithin as substrate and 1 m*M* Ca^{2+}.

Hemolysis determination

Hemolysis was measured at 540 nm by the method of Okada *et al.*[7] and was expressed as percentage of complete hemolysis. Complete (100%) hemolysis was obtained by addition of one drop of concentrated ammonia to a suspension of erythrocytes in water 10 min before termination of a 1-h incubation.

Polyacrylamide gel electrophoresis

The procedure described by Weber *et al.*[8] was followed. The gel (7 × 0.5 cm diameter) was prepared from 7.5% acrylamide, 0.2% N,N'-methylene-bis(acrylamide), 0.1% SDS, 0.15% N,N,N',N'-tetramethylene diamine and 0.06% ammonium persulfate in 0.1 *M* sodium phosphate buffer, pH 7.2. The electrophoresis buffer was 0.1 *M* sodium phosphate, pH 7.2 and 0.1% SDS. Samples were dissolved in 0.01 *M* sodium phosphate buffer, pH 7.0 containing 1% SDS and 1% 2-mercaptoethanol and boiled for 5 min. Staining and destaining was executed as described[8]. Electrophoresis was carried out at 7 mA per gel until the bromophenol marker (final concentration 0.005%) reached the bottom of the gel (about 5 h).

Protein was determined by the method of Hartree[9].

RESULTS AND DISCUSSION

Clostridium perfringens neuraminidase

The experiments reported here were done with Worthington NEUP preparation, lot 54B385. Sigma (St. Louis, Mo., U.S.A.) Type V preparations which were reported to be about 100 times more potent as a cytotoxic and hemolytic agent[1] were not investigated.

TABLE I

PROTEASE AND NEURAMINIDASE ACTIVITY OF FRACTIONS OBTAINED BY AFFINITY CHROMATOGRAPHY OF *CLOSTRIDIUM PERFRINGENS* NEURAMINIDASE UNDER VARIOUS CONDITIONS

Samples of CP NAN-ase in either 0.05 *M* acetate buffer, pH 5.5 or in 0.05 *M* Tris–HCl buffer, pH 7.5 were applied to the particular column and washed with the same buffer until the absorbance at 280 nm was zero. The column was then washed with $NaHCO_3$ buffer, pH 9.1. The respective column fractions were combined to give F_1 (starting buffer column-wash) and F_2 ($NaHCO_3$-column effluent which was immediately acidified to pH 6.0). F_1 and F_2 were then lyophilized, the dry residues were dissolved in water and dialyzed for 3–4 h against 0.03 *M* sodium acetate buffer, pH 5.0 for NAN-ase determination or against 0.03 *M* Tris–HCl buffer, pH 7.5 for other enzyme assays.

Sample	*Protein*		*Protease*		*NAN-ase*	
	mg	*Recovery (%)*	*Sp. act.**	*Recovery (%)*	*Sp. act.***	*Recovery (%)*
(a) Seph–Gly–Gly–Tyr–NAPOA; 0.05 *M* acetate buffer, pH 5.5						
Total	2.6	100	2.9	100	7.6	100
F_1	0.5	23	0.1	4	trace	
F_2	1.3	50	2.7	94	12.9	82
(b) Seph–Gly–Gly–Tyr–NAPOA: 0.05 *M* Tris–HCl buffer, pH 7.5						
Total	2.6	100	1.1	100	7.1	100
F_1	2.1	80	1.3	89	0.6	4
F_2	0.3	11	0.8	6	40.8	89
(c) Seph–Gly–Tyr–NAPOA; 0.05 *M* Tris–HCl buffer, pH 7.5						
Total	5.6	100	0.9	100	6.0	100
F_1	4.5	78	1.1	91	0.8	7
F_2	0.3	5	0.8	4	33.8	100

* Absorbance at 520 nm per mg of protein per 30 min at 37°.
** Number of μmoles of N-acetylneuraminic acid formed per mg of protein per min at 37°.

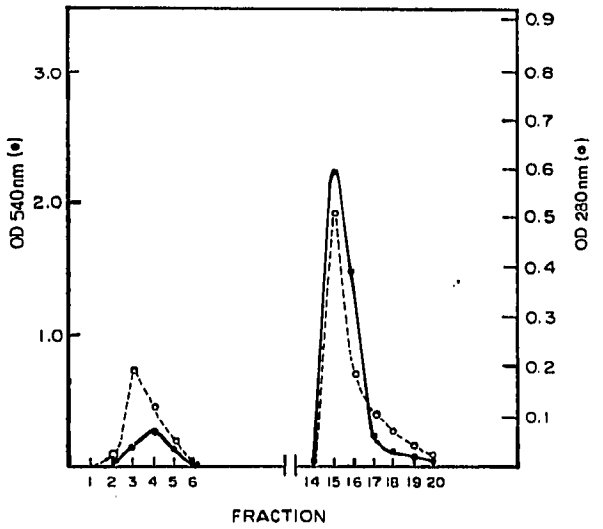

Fig. 1. Affinity chromatography of CP NAN-ase on Seph-Gly-Gly-Tyr-NAPOA at pH 5.5. Two ml of NEUP CP NAN-ase containing 3.2 mg protein in 0.05 *M* acetate buffer, pH 5.5 were applied to a column containing the adsorbent which had been equilibrated with the same buffer. Protein (○) was determined by absorbance at 280 μm; hemolysin (●) determinations were performed on aliquots of 100 μl.

As seen in Table I (a), application of the enzyme to the Seph–Gly–Gly–Tyr–NAPOA column at pH 5.5 led to a non-specific adsorption of protein with 50% of the applied protein and 94% of the protease activity recovered in the NAN-ase-containing Fraction 2. Similar results were obtained with "hemolysin" activity which heavily contaminates the NEUP preparations. Thus, as illustrated in Fig. 1, most of the applied hemolysin was adsorbed to the column at pH 5.5 and was subsequently eluted along with NAN-ase at pH 9.1. Attempts to demonstrate the presence of phospholipase C in the NEUP preparation were negative (limit of detection: 2×10^{-4} enzyme unit per ml of effluent; one unit is the amount of enzyme required to catalyze the hydrolysis of 1 μmole of lecithin per min in a standard reaction mixture). Kraemer[1], who also could not show the breakdown of several purified lecithins by a different lot of NEUP NAN-ase reported, however, that if erythrocyte ghosts were used as substrate, the breakdown of phosphatidyl choline was readily demonstrable by thin-layer chromatography. Therefore, we examined the behavior of phospholipase C from *Cl. perfringens* (Worthington) on the Seph–Gly–Gly–Tyr–NAPOA column and found that this enzyme also is adsorbed to the column at pH 5.5 and subsequently eluted at pH 9.1.

Much less non-specific adsorption was observed when enzyme samples were applied to the affinity columns at pH 7.5, as recommended by Rood and Wilkinson[3]. As illustrated in Table I (c), only 5% of the applied protein (containing 100% of the NAN-ase activity) and 4% of protease activity were adsorbed at pH 7.5 and subsequently eluted from the Seph–Gly–Tyr–NAPOA column at pH 9.1.

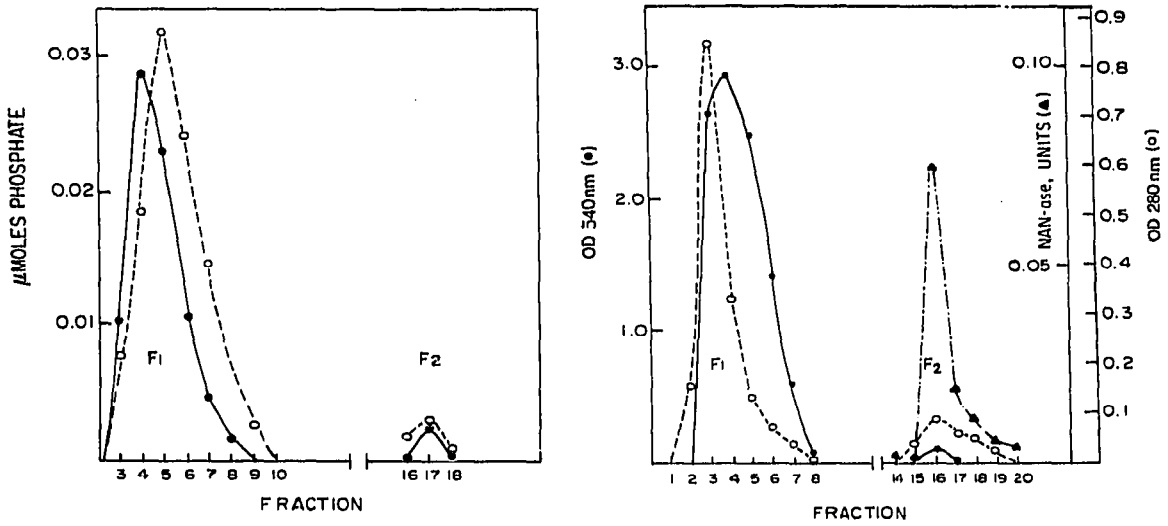

Fig. 2. Affinity chromatography of *Clostridium perfringens* phospholipase C at pH 7.5 on Seph–Gly–Gly–Tyr–NAPOA (●) and Seph–Gly–Tyr–NAPOA (○) columns. Two mg of phospholipase C (Worthington) in 3 ml of Tris–HCl buffer, pH 7.5 were applied to the particular column. Ten-μl aliquots were used for assay.

Fig. 3. Affinity chromatography of CP NAN-ase on Seph–Gly–Gly–Tyr–NAPOA at pH 7.5. Conditions of experiment were the same as those in Fig. 1 except that 0.05 *M* Tris–HCl buffer, pH 7.5 was used instead of acetate buffer. Protein (○) was measured by absorbance at 280 nm; hemolysin (●) and NAN-ase (▲) determinations were performed on 100 μl and 10 μl, respectively.

Similarity in the performance of the two columns having a Gly–Gly–Tyr and a Gly–Tyr arm, respectively, is illustrated in Table I (b and c) and in Fig. 2. [The cost of Gly–Tyr (Sigma) is about 1/20 of that of Gly–Gly–Tyr (Fox, Los Angeles, Calif., U.S.A.).] Most of the cytotoxic activities investigated: protease (Table I), hemolysin (Fig. 3) and phospholipase C (Fig. 2) did not adsorb to either column at pH 7.5.

The SDS–polyacrylamide gel electrophoresis patterns of commercial NAN-ase preparations and of the two fractions (F_1 and F_2) obtained by affinity chromatography of CP NAN-ase under different conditions are shown in Fig. 4. Although cytotoxicity of the resulting preparation (F_2) is greatly reduced by changing the pH of the starting buffer to pH 7.5, the state of "purified" enzyme does not approach homogeneity.

Vibrio cholerae neuraminidase

The purity of two different lots of VC NAN-ase (Calbiochem, lots 400389 and 400807) were investigated. The enzyme is available in 1-ml ampoules containing ap-

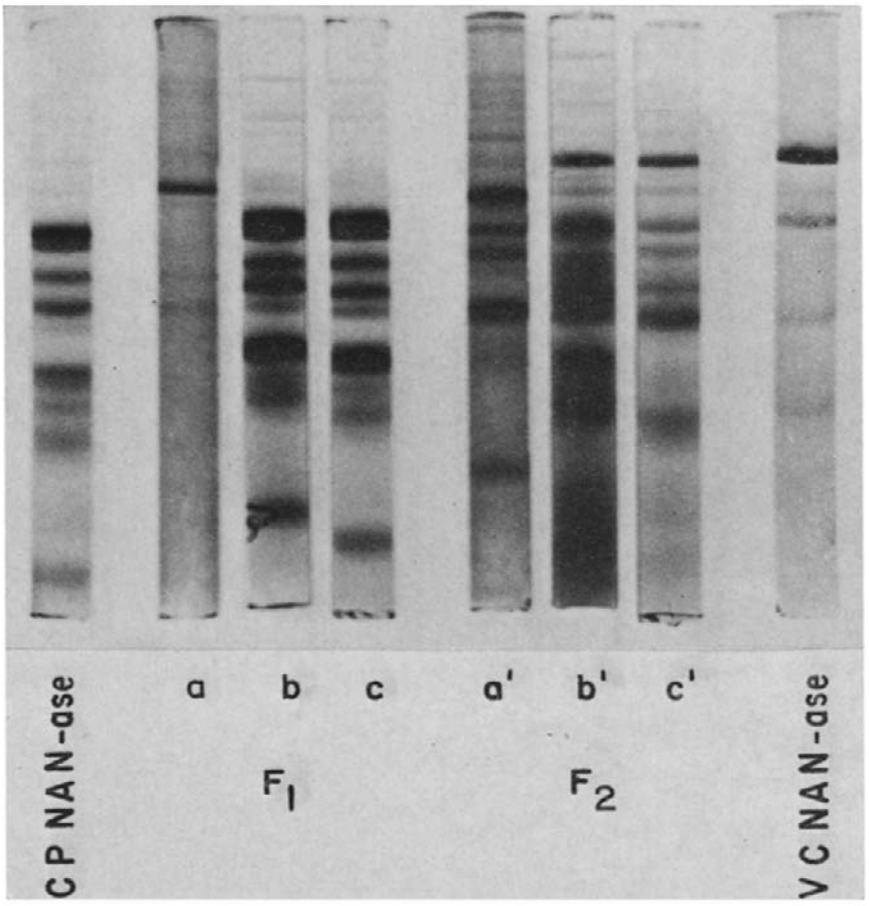

Fig. 4. SDS–polyacrylamide gel electrophoresis. a, a′ and b, b′ were fractionated on a Seph-Gly–Gly–Tyr–NAPOA column at pH 5.5 and 7.5, respectively; c and c′ were fractionated on a Seph–Gly–Tyr–NAPOA column at pH 7.5. The protein values in μg were: CP NAN-ase 57; a, 15; a′, 32; b, 40, b′, 88; c, 143; c′, 120; VC NAN-ase, 220.

proximately 100 μg of protein and 500 units of NAN-ase. Several millilitres of this solution were lyophilized, the dry residue was dissolved in water and dialyzed for 4 h against 0.03 *M* Tris–HCl buffer, pH 7.5. The SDS–polyacrylamide gel electrophoresis pattern of such concentrated preparation showed the presence of four protein bands (Fig. 4). However, the VC NAN-ase seemed to be free of cytotoxic contaminants and samples of the concentrated enzyme which contained up to 500 units of VC NAN-ase were negative when tested for protease, hemolysin and phospholipase C activities.

These findings indicate that the commercial NAN-ase preparations are contaminated with numerous proteins which, in the case of CP NAN-ase, are also highly cytotoxic. Although most of the cytotoxic contaminants can be removed by affinity chromatography at pH 7.5, the resulting neuraminidase preparations are still heterogeneous*. Since it is not possible to remove all contaminants by currently available affinity chromatography procedures, their presence should be taken into account in the interpretation of experiments which utilize NAN-ase to remove sialic acid as a probe of cell surface properties[11–14].

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* While this manuscript was prepared, Huang and Aminoff[10] reported their observations which implicate other *Cl. perfringens* glycosidases as possible contaminants of the "purified" CP NAN-ase preparations.