Biochimica et Biophysica Acta, 327 (1973) 114–120

BBA 67052

A SIMPLE METHOD FOR THE PURIFICATION OF COMMERCIAL NEURAMINIDASE PREPARATIONS FREE FROM PROTEASES

M. W. C. HATTON AND E. REGOECZI
Department of Pathology, McMaster University, Hamilton, Ontario (Canada)

(Received May 21st, 1973)

SUMMARY

A simple method is described for the further purification of commercially prepared neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) (*ex Clostridium perfringens* filtrate). A single-step ion-exchange chromatography process renders the enzyme free from proteolytic enzymes and much contaminating protein. Consequently when ^{125}I-labelled proteins, such as fibrinogen, casein, caeruloplasmin, are used as substrates, no proteolytic activity is observed. In contrast, the commercial sample is shown to cleave trichloroacetic acid-soluble ^{125}I-labelled peptides from these three proteins. In addition the purified neuraminidase preparation is subjected to both polyacrylamide gel electrophoresis and Sephadex gel filtration under various conditions. Heat stability studies show the enzyme to retain approx. 28% activity after 3 h at 58 °C.

INTRODUCTION

Neuraminidases (acylneuraminyl hydrolase, EC 3.2.1.18) are an important tool in protein chemistry and cell biology for the study of glycoproteins. Because of its good yield, stability and non-dependence on divalent cations, neuraminidase from *Clostridium perfringens* is probably the most widely used enzyme of this kind.

Depending on the growth conditions, *Clostridium* strains release varying amounts of proteolytic activity into the culture medium[1], and unfortunately the purification of neuraminidase on a commercial scale by a well established technique[2] often fails to yield a product that is not proteolytic. Consequently it is frequently difficult to decide whether a change following desialylation of a glycoprotein is due to modification of the carbohydrate structure, the polypeptide chain, or both.

Since the number of laboratories privileged to prepare pure neuraminidase from their own bacterial culture is limited, there seems to be a need for a simple and efficient method to purify commercial preparations. Such a technique is presented below.

MATERIALS AND METHODS

Source of neuraminidase

Neuraminidase Type VI (Sigma Chemical Co. Ltd, St. Louis, Mo., U.S.A.) was used throughout the study. Most of the results reported below were obtained with lot No. 92C-8090. 1 unit of enzyme activity has been previously defined[2].

Purification of commercial neuraminidase

Amberlite IRC-50 (chromatographic grade; B.D.H. Ltd) resin was washed first with 0.2 M NaOH and then 0.2 M HCl before equilibration with 0.01 M sodium phosphate buffer (pH 6.1). 10 units of neuraminidase were dissolved in 10 ml of the same buffer and allowed to run slowly into a column of resin (6.5 cm × 1.5 cm). The column was washed with 0.01 M phosphate (pH 6.1) until no further protein emerged. Neuraminidase activity was eluted with 0.2 M Na_2HPO_4 containing 3% NaCl. The active fractions were pooled and dialysed exhaustively against 0.05 M ammonium acetate (pH 6.0) prior to freeze-drying and storage at −70 °C.

Estimation of neuraminidase activity

An extract from human mucin (approx. 3 mg/ml) prepared by the method of Pineo *et al.*[3] was used as a substrate. When buffered at pH 5.2 with 0.1 M sodium acetate, 0.4 ml substrate was exposed to 0.1 ml enzyme for 15 min at 37 °C. This incubation procedure was used throughout with the exception of the Amberlite IRC-50 elution profile (see Fig. 1). Sialic acid was determined by the method of Aminoff[4].

Polyacrylamide gel electrophoresis

A procedure described previously was used[5] at pH 8.3 and the method of Reisfeld *et al.*[6] at pH 4.3.

Determination of heat stability

A sample of purified enzyme (2 ml in 0.2 M phosphate (pH 6.0) containing approx. 0.01 unit/ml) was held at 57–58 °C for up to 3 h. Samples of 0.1 ml were taken at various intervals for assay.

Determination of molecular weight by gel filtration

A column (53 cm × 2.2 cm) of Sephadex G-200 was equilibrated with either 0.125 M phosphate buffer (pH 7.3) or 0.1 M sodium acetate (pH 5.5). Samples of purified neuraminidase (Peak II; see Fig. 1) were incubated at alkaline (pH 9.0) or acidic (pH 5.0) conditions for up to 5 min prior to loading onto the column and eluting at pH 7.3 and 5.5, respectively. ^{131}I-Labelled albumin and ^{125}I-labelled immunoglobulin G (IgG) were added to the sample as column markers. Assays were performed on the eluted fractions to determine the position of neuraminidase.

Proteolytic activity of neuraminidase preparations

Proteolysis was measured by studying the release of radioactive peptides from iodine-labelled proteins after incubation with neuraminidase. The following proteins were used: casein (Hammarsten, B.D.H. Ltd; further prepared as a substrate by the

method of Rick[7]); human fibrinogen (Kabi Ltd, Grade L); human transferrin and albumin (Behringwerke A.G.). IgG was prepared by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography. Human caeruloplasmin and bovine tendon collagen were kindly donated by Dr J. Bienenstock and Dr R. Rathbone (Department of Pathology, McMaster University), respectively. After trace-labelling with ^{125}I using the iodine monochloride method[8], the concentration of each labelled preparation was adjusted with carrier protein solution in 0.1 M ammonium acetate (pH 7.2) to the value given in the results. A volume (0.4 ml) was incubated for 18 h at 37 °C with 0.1 ml of neuraminidase preparation or 0.1 M ammonium acetate (pH 7.2). After counting the incubates, 1 ml 20% (w/v) trichloroacetic acid was added, centrifuged at 3000 rev./min for 10 min at 20 °C, and known aliquots of the supernatant were recounted. Supernatant activities were expressed as percentage of the protein-bound radioactivity originally present in the samples.

Proteolytic action on gelatin

Gelatin (B.D.H. Ltd) was prepared as a 2% solution in 0.1 M Tris–HCl (pH 7.2). For incubation with neuraminidase preparations, 0.3 ml gelatin was reacted with 0.2 ml enzyme preparation for 1 h at 37 °C. Then the samples were placed in the cold (2 °C) for 30 min and those samples which had or had not gelled were recorded.

RESULTS

Chromatography of neuraminidase on Amberlite IRC-50 resin

A typical elution profile is shown in Fig. 1. Using the starting conditions described above, nearly all neuraminidase activity was adsorbed by the Amberlite IRC-50 resin. By contrast, gelatinase activity and approx. 66% of the total protein were washed through the column (Peak I). Some neuraminidase activity also emerged

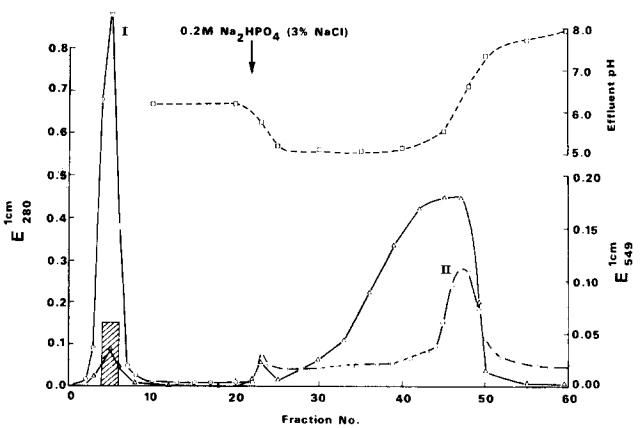

Fig. 1. Fractionation of commercial neuraminidases on Amberlite IRC-50 resin (6.5 cm × 1.5 cm). The symbols indicate: ○—○, absorbance (280 nm); △—△, neuraminidase activity (549 nm) of 0.1 ml fraction incubated with 0.1 ml mucin and 0.3 ml 0.1 M sodium acetate buffer (pH 6.0) at 37 °C for 15 min; □—□, effluent pH; hatched area, gelatinase activity. The fraction volume was 5.5 ml and further elution details are given in the text.

in Peak I but it represented only approx. 5% of the total. The use of a smaller column (4.5 cm × 1.5 cm) allowed a greater quantity of activity (approx. 17%) to escape adsorption. After washing with 0.01 M sodium phosphate (pH 6.1) to remove traces of contaminants 0.2 M Na_2HPO_4 containing 3% NaCl eluted the active enzyme (Peak II). The recovery of neuraminidase activity was 70–80% of the load after pooling Peak II. Dialysis, freeze-drying and storage at −70 °C did not significantly destroy activity.

Assessment of proteolytic activity in Peaks I and II and in commercial neuraminidase

Labelled proteins. In Table I is given a summary of the percentage of substrate released as trichloroacetic acid-soluble peptides during enzyme incubation at 37 °C for 18 h. No proteolytic activity was detected using this method on human serum albumin, transferrin and rabbit IgG, each at 5 mg/ml, and bovine collagen (1.7 mg/ml at pH 6.2) by any of the neuraminidase preparations. However, the commercial sample and Peak I significantly lysed fibrinogen and casein, and to a lesser extent caeruloplasmin. No proteolytic activity was detected in Peak II samples.

TABLE I

PROTEOLYTIC ACTIVITY OF COMMERCIAL NEURAMINIDASE, PEAKS I AND II FROM AMBERLITE IRC-50 CHROMATOGRAPHY ON CERTAIN ^{125}I-LABELLED PROTEINS

For each assay 0.1 unit of enzymic activity was taken for both commercial neuraminidase and Peak II. The $E\ ^{1\ cm}_{280\ nm}$ of Peak I solution was 4.9.

Labelled protein	*Concn (mg/ml)*	*% Radioactivity in trichloroacetic acid supernatant*		
		Commercial neuraminidase	*Peak I*	*Peak II*
Caeruloplasmin	2.5	2.6	0.3	0.0
Casein	5.0	5.0	3.3	0.0
Fibrinogen	5.0	6.6	4.2	0.0

Gelatin. A 2% gelatin solution incubated with both commercial neuraminidase (0.1 unit/ml) and Peak I was found to have lost the ability to gel when placed at 2 °C for 30 min. A similar incubation with Peak II (0.1 unit/ml) did not affect gelling.

Some properties of Peak II neuraminidase

Heat stability. The rate of inactivation is shown in Fig. 2 with approx. 28% activity remaining after 3 h exposure of the enzyme to 57–58 °C.

Polyacrylamide gel electrophoresis. Electrophoresis was used to determine the degree of purification achieved by ion-exchange chromatography. Although distinct gel patterns were obtained for both the commercial sample and Peak I (Fig. 3), the gels of Peak II despite relatively large loading of protein were only weakly stained or even blank. Staining was attempted with both 0.25% Coomassie Brilliant Blue in 7% acetic acid and 1% Amido Black in 7% acetic acid for various times (1–6 h) and temperatures (37 or 70 °C). It is therefore concluded that the purified *Cl. perfringens* enzyme does not easily stain with these two protein dyes.

Molecular weight. In Fig. 4 the elution profile of an alkaline pH-treated sample

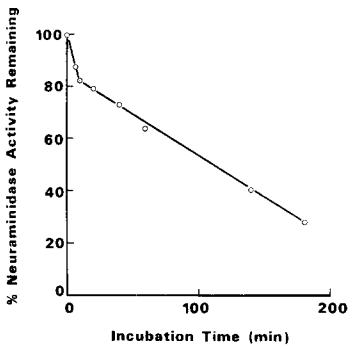

Fig. 2. Heat inactivation of Peak II neuraminidase incubated at pH 6.0 and 57–58 °C prior to assay.

of Peak II from a Sephadex G-200 column is shown. The neuraminidase activity eluted as a peak of mol. wt approx. 47 000 with a faster running shoulder which was computed at approx. 100 000. A sample of Peak II which did not receive alkaline treatment was run in 0.1 M sodium acetate (pH 5.5) and appeared as a major peak in the 100 000 region.

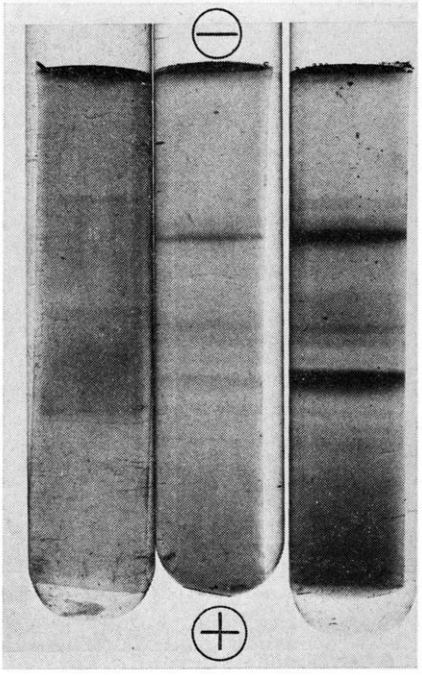

Fig. 3. Polyacrylamide gel electrophoresis of commercial neuraminidase, Peaks I and II at pH 8.3. The gels (7.5% acrylamide) were run for 35 min at 4 mA/gel and stained in 0.25% Coomassie Brilliant Blue in 7% acetic acid for 2 h at 70 °C. From left to right: Peak II concentrate (approx. 40 μg protein loaded); Peak I (20 μg protein); commercial neuraminidase (80 μg protein).

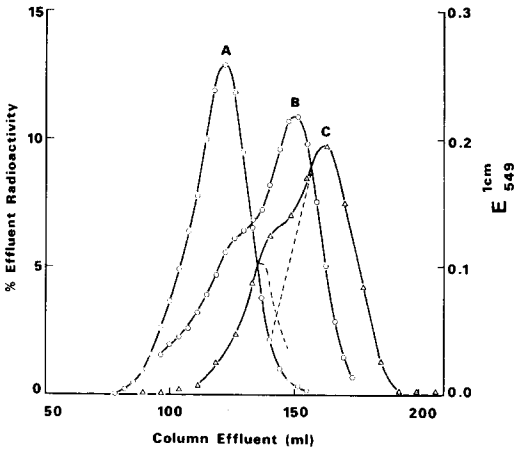

Fig. 4. Sephadex G-200 gel filtration of Peak II neuraminidase after exposure to 1 M glycine–NaOH buffer (pH 9.0) for 1 min at 15 °C. The eluant was 0.125 M sodium phosphate at pH 7.3. Column markers were ^{131}I-labelled IgG(A) and ^{125}I-human serum albumin (B). The shoulder on the albumin peak is attributed to the presence of polymeric forms[14]. Neuraminidase activity (C) is represented by $E_{549\ nm}^{1\ cm}$ and a computed estimate of two component peaks is shown by the dashed lines.

DISCUSSION

Earlier observations by Kraemer[9] demonstrated the need for commercial neuraminidase preparations derived from *Cl. perfringens* filtrate to be free from impurities. Kraemer showed such preparations to contain cytotoxic, haemolytic and phospholipase activities which were independent of the neuraminidase property. The presence of proteolytic activity in commercial samples is therefore hardly surprising. Indeed, in samples which we have received over the last 18 months contamination with proteolytic activity could invariably be detected.

Tests used for the detection of proteolytic contaminants in neuraminidase preparations vary widely and comparisons are difficult. However, haemoglobin[2], α-globulin and others[10] have been used as substrates and no proteolytic activity detected. The present data, based on studies with seven labelled proteins, would seem to indicate that some proteins are more sensitive to *Cl. perfringens* protease(s) than are others. Clearly, the introduction of a uniform substrate would be highly desirable. Because of the high sensitivity of the Aα-chain of fibrinogen to proteolytic enzymes[11], this protein lends itself well for the purpose.

Preliminary inhibition studies showed that the proteolytic activity in neuraminidase from *Cl. perfringens* is not abolished by serum protease inhibitors, crystalline soya bean trypsin inhibitor (1 mg/ml) or diisopropyl phosphofluoridate (10^{-3} M) using fibrinogen as a substrate. In the presence of EDTA (0.1 M) gelatinase activity was successfully inhibited but unfortunately 90% of neuraminidase activity on mucin was also lost. In contrast, the chromatographic method described above represents a simple and effective technique of rendering neuraminidase preparations free of proteases. At least 95% of the neuraminidase activity can be adsorbed on modest-sized columns of Amberlite IRC-50 with a concomitant increase of purity of the recovered enzyme.

The ability of neuraminidases to polymerize is well recorded. Schramm and Mohr[12] estimated the mol. wt of the enzyme from *Vibrio cholerae* to be 10 000–20 000 at pH 8.5 by ultracentrifugal studies, and higher molecular weights were observed under other conditions. However, Pye and Curtain[13] reported the same enzyme to have a mol. wt of approx. 90 000 at pH 6.7. From the present study with the *Cl. perfringens* enzyme, it is possible that polymerisation also occurs under certain conditions. Thus after brief exposure of the enzyme to pH 9.0 a mixture of active forms is observed after gel filtration at pH 7.3 with mol. wt approx. 50 000 and 100 000, respectively. In comparison incubation at pH 5.0 and subsequent gel filtration at pH 5.5 yields a single active peak with mol. wt approx. 100 000.

During this study an unsuccessful attempt was made to iodinate neuraminidase using iodine monochloride. Less than 1% of the radioactivity became associated with the enzyme and 80% of the fraction which did label dissociated during a 5-day period of storage at −10 °C. This behaviour suggests that the label was adsorbed rather than properly substituted into the protein. Neuraminidase may therefore be devoid of or have only few tyrosyl residues which are not accessible for labelling.

ACKNOWLEDGEMENTS

These investigations were supported by a grant (No. MT-4074) by the Medical Research Council of Canada and by a Senior Research Fellowship awarded to M.W.C.H. by the Canadian Heart Foundation.

REFERENCES

1 Mansson, I. and Smith, L. D. S. (1962) *Acta Pathol. Microbiol. Scand.* 55, 342–348
2 Cassidy, J. T., Jourdian, A. W. and Roseman, S. (1965) *J. Biol. Chem.* 240, 3501–3506
3 Pineo, G. W., Regoeczi, E., Hatton, M. W. C. and Brain, M. C. (1973) *J. Lab. Clin. Med.* 82, 255–266
4 Aminoff, D. (1961) *Biochem. J.* 81, 384–392
5 Hatton, M. W. C. (1973) *Biochem. J.* 131, 799–807
6 Reisfeld, R. A., Lewis, U. J. and Williams, D. E. (1962) *Nature* 195, 281–283
7 Rick, W. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), p. 812, Academic Press, New York and London
8 McFarlane, A. S. (1958) *Nature* 182, 53
9 Kraemer, P. M. (1968) *Biochim. Biophys. Acta* 167, 205–208
10 Blumberg, B. S. and Warren, L. (1961) *Biochim. Biophys. Acta* 50, 90–101
11 Gaffney, P. J. (1971) *Nat. New Biol.* 234, 281–282
12 Schramm, G. and Mohr, E. (1959) *Nature* 183, 1677–1678
13 Pye, J. and Curtain, C. C. (1961) *J. Gen. Microbiol.* 24, 423–425
14 Rossing, N. and Jensen, H. (1967) *Clin. Sci.* 32, 89–99