# STUDIES ON A NEURAMINIDASE FROM *STREPTOMYCES GRISEUS*

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## 1. Introduction

Neuraminidase (*N*-acetylneuraminate glycohydrolase EC 3.2.1.18) has been detected in bacteria, vertebrates and viruses [1], but there are few reports of its detection and isolation from non-pathogenic microorganisms [2,3]. Much is known about the enzymatic and immunological properties of neuraminidases but little information is available on their structures. In this communication we describe the purification of neuraminidase from *Streptomyces griseus* by affinity chromatography. We have determined the amino acid composition of the pure enzyme which is a glycoprotein and have carried out its selective chemical modification, demonstrating that arginine residues are essential for full enzymic activity.

## 2. Materials and methods

### 2.1. *Culture conditions*

*Streptomyces griseus* MB395-A5 was shake-cultured under aerobic conditions for 72 h at 30°C as previously described [2] except that $\alpha_1$-acid glycoprotein (10 mg/litre) was added to the medium to increase the yield of neuraminidase [4]. After the neuraminidase in the culture fluid had been purified by conventional means [2], a final affinity chromatographic step using fetuin bound to cyanogen bromide-activated Sepharose was employed [5,6] when a two-fold purification was obtained (fig.1).

### 2.2. *Assays and molecular weight determinations*

Protein concentrations were determined by the method of Lowry [7]. Neuraminidase assay: substrate (0.4 ml) ($\alpha_1$-acid glycoprotein 4 mg/ml or sialyl lactose 1 mg/ml in 0.02 M sodium acetate, pH 5.3), neuraminidase solution (0.1 ml) and acetate buffer (0.5 ml) were incubated for 50 min at 37°C and then heated at 100°C for 2 min. The NANA released was then determined [8].

After the final affinity chromatographic step, the neuraminidase did not degrade *N,N*-dimethyl casein [9] and hence was judged to be free of proteases. The molecular weight of the neuraminidase was between 32 000 (gel filtration after [10]) and 36 500 (polyacrylamide gel electrophoresis [11] when the enzyme ran as a single sharp band). For both determinations, ribonuclease, cytochrome *c*, soya bean trypsin inhibitor, chymotrypsinogen and bovine serum albumin were used as standards. Treatment of the polyacrylamide gel after electrophoresis with periodate/dansyl hydrazide [12] or periodate /Schiff's reagent [13] showed that a carbohydrate band was coincident with the enzyme band indicating that the neuraminidase is a glycoprotein.

### 2.3. *Chemical studies*

The values for the amino acid content of neuraminidase are given in table 1 and are the mean of 4 determinations. The N-terminal amino acid of the enzyme after performic acid oxidation was alanine using the procedure of Gray [14].

To neuraminidase (1.0 mg) in 0.02 M sodium phosphate, pH 8.6 (2.0 ml) at room temperature was added 2,4-pentane dione to make a final concentration of 50 mM. At various times, a sample (0.2 ml) was withdrawn and applied to a Sephadex G-25 column (1 × 6 cm) which was eluted with 0.02 M sodium acetate buffer, pH 5.3. The specific activity

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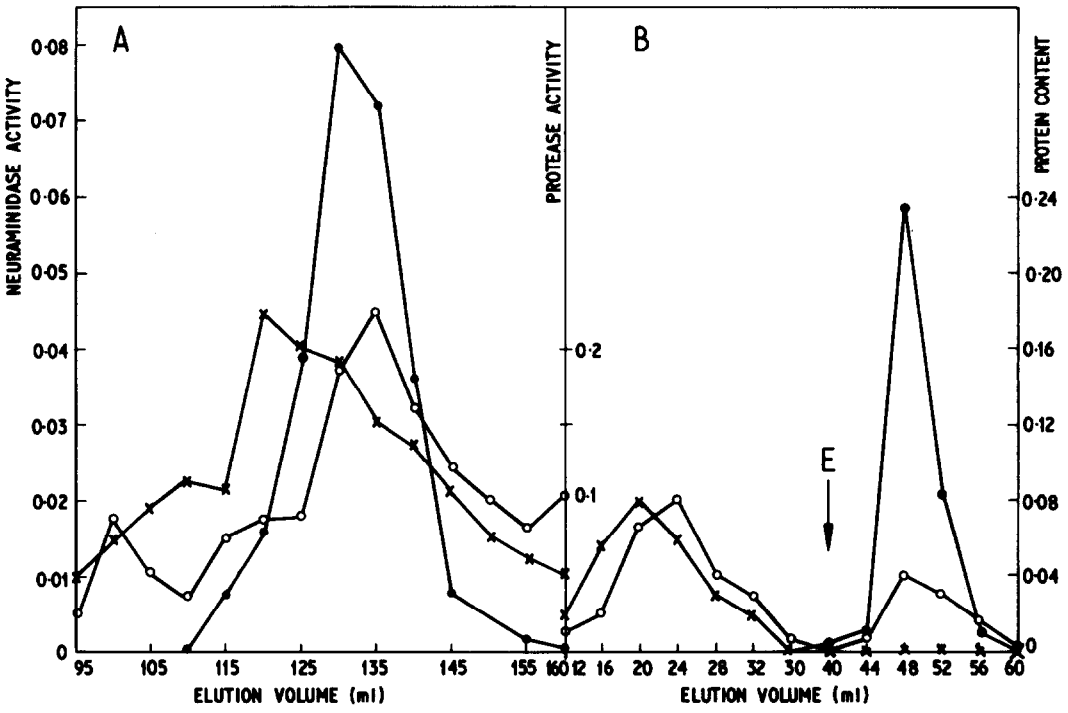


Fig.1. Neuraminidase purification. (A) Gel filtration on Sephadex G-100 after purification as previously described [2]. (B) Affinity chromatography on a fetuin–Sepharose column (1.2 × 12.5 cm). The neuraminidase-containing fraction after step A was concentrated by ultrafiltration (Bio-Fiber 50) to 1.2 ml and containing 1.92 mg protein. This was applied to the affinity column which was then eluted with 0.02 M sodium acetate buffer pH 5.3. Neuraminidase was eluted after changing the buffer at point E to 0.1 M sodium borate pH 8.5. (–○–○–) protein content (mg/ml) [7]. (–x–x–) protease activity (mol. bonds cleaved/0.1 ml) [9]. (–•–•–) neuraminidase activity (μmol NANA released/0.1 ml).

Table 1
Amino acid content of neuraminidase[a]

| Amino acid | No. residues[b] | Amino acid | No. residues[b] |
|---|---|---|---|
| Asp | 23.3 | Val | 18.9 |
| Thr | 17.3 | Met | 5.4 |
| Ser | 17.9 | Ileu | 12.4 |
| Glu | 38.6 | Leu | 19.3 |
| Pro | 38.6 | Tyr | 7.0 |
| Gly | 39.7 | Phe | 8.3 |
| Ala | 21.1 | His | 9.0 |
| Cys | 1.3 | Lys | 17.3 |
| | | Arg | 9.9 |

[a] Determined by Dr J. E. Fox, Macromolecular Analysis Service, Birmingham University

[b] Calculated for 32 000 mol. wt

of the neuraminidase-containing fractions was determined as described above. The column eluate was made 0.2 M in hydroxylamine and incubated at 37°C for 3 h. The solution was dialysed against several changes of 0.02 M sodium acetate buffer, pH 5.3 (500 ml) and assayed for neuraminidase activity (fig.2).

When neuraminidase (500 μg) was incubated with phenyl gloxal (500 μg) in *N*-ethylmorpholinium acetate buffer (1 ml, 0.1 M, pH 8) 60% of the enzymic activity was lost after 20 min at 37°C, while 90% activity was lost after 120 min. In similar experiments with [7-$^{14}$C]phenylgloxal [15], 6–7 moles of phenylglyoxal were incorporated after 120 min/neuraminidase molecule (32 000 mol. wt). When the experiments with [$^{14}$C]phenylgloxal were carried out in the presence of *N*-acetylneuraminic acid (0.01 M), less than one mole of phenylgloxal was incorporated/enzyme molecule.

Neuraminidase (2 mg) was incubated with recrystallised 2,4,6-trinitrobenzene sulphonic acid trihydrate (2 ml, 5 mM in 0.1 M sodium phosphate buffer, pH 7.6) [16]. After 5 h, an aliquot (0.2 ml) was removed and applied to a Sephadex G-25 column which was eluted with 0.02 M sodium acetate buffer, pH 5.3. No diminution of the specific activity of the enzyme was observed.

When neuraminidase (0.5 mg) was incubated with diethyl pyrocarbonate (1 ml, 1 mM in 0.1 M sodium phosphate buffer, pH 7.0) for 50 min at room temperature [17] followed by dialysis against 0.02 M sodium acetate buffer, pH 5.3, very little loss of specific activity occurred. Similarly photo-oxidation of the enzyme with Rose Bengal for 1 h [17] caused little change in specific activity.

## 3. Discussion

We have found that while the isolation procedure described by Kunimoto et al. [2] gives neuraminidase of reasonable purity, considerable further purification can be achieved using a column of fetuin bound to cyanogen bromide-activated Sepharose [5,6]. By this means, approx. 1 mg of pure enzyme can be obtained from a 10 litre culture of *S. griseus*. The enzyme has a low content of sulphur-containing amino acids, particularly cysteine which is present to an extent of 1–2 residues/ 32 000 mol. wt. This was confirmed by the methods of Rohrbach et al. [18] and Cavallini et al. [19] when values of 1.1 and 1.8, respectively, were obtained. It is significant that the *S. griseus* neuraminidase is not affected by *p*-chloromercuribenzoate unlike the enzyme from *Clostridium perfringens* [2].

The *S. griseus* neuraminidase is inactivated by 2,4-pentanedione; after 80 h only 4% of the original activity remained. Treatment of this inactivated enzyme with hydroxylamine gave only 7% of the original activity. In blank reactions, when the neuraminidase was incubated with the reaction buffers alone, 96% of the original activity remained after 80 h. 2,4-Pentanedione reacts with arginine and lysine residues in proteins [20]; treatment of the inactivated enzyme with hydroxylamine regenerates lysine residues leaving only arginine residues modified. The uptake of 6–7 phenylglyoxal residues/enzyme molecule means that a maximum of 3 arginine residues are modified as Takahashi [21] has shown that two molecules of the reagent combine with each arginine residue. We find that the neuraminidase is unaffected by 2,4,6-trinitrobenzene sulphonic acid, a lysine selective reagent [16]. Moreover, neither treatment of the enzyme with diethyl pyrocarbonate nor its photo-oxidation in the presence of Rose Bengal, both histidine modifying processes, have any significant effect on the enzymic activity. These latter observations are in contrast to those made with influenza virus neuraminidase, when the presence of an essential histidine was demonstrated [22].

Our results indicate that arginine residues are essential for full activity of *S. griseus* neuraminidase. The presence of a free terminal α-carboxylic acid group in a substrate is a strict requirement of neuraminidases [23] and some of these arginine residues may be involved in binding the substrate to the enzyme as the number of phenylglyoxal residues bound decreases significantly in the presence of *N*-acetylneuraminic acid. Further work is in progress on the nature of the active site of the enzyme.

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