EFFECT OF TRYPTOPHAN MODIFICATION ON THE ACTIVITY OF BACTERIAL AND VIRAL NEURAMINIDASE

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1. Introduction

Neuraminidases (mucopolysaccharide *N*-acetylneuraminyl hydrolase, EC 3.2.1.18) have been isolated from bacteria, vertebrate tissues and the envelope of certain animal viruses [1]. In contrast to the extensive studies concerning the immunological aspects of the viral enzyme, very little work has been done on its chemical properties.

A comparative study has therefore been initiated on the structure–function relationships of neuraminidases from viral and bacterial origin.

This communication deals with the results obtained on specific modification of tryptophan residues of the enzyme.

2. Materials and methods

Influenza A_2/Asia/57 virus was grown in the allantoic sac of chicken embryos and concentrated from the allantoic fluid by differential centrifugation (or precipitation with polyethylenglycol 6000) [2]. Purification was achieved by equilibrium centrifugation in a preformed linear sucrose gradient (5 to 50% w/w in phosphate buffered saline) using the Beckman Ti 15 zonal rotor. Solubilized viral neuraminidase was purified by DEAE-cellulose chromatography on Cellex D (Calbiochem) as described in the legend to fig. 1.

Protease activity was determined using Azocoll (Calbiochem) as substrate.

Clostridium perfringens neuraminidase preparations were obtained from Schwarz-Mann or Sigma and further purified by affinity chromatography as described by Cuatrecasas and Illiano [4].

The absorbent used was a kind gift of Dr. F. Dorner, Department of Immunochemistry. Neuraminidase activity was measured with fetuin or bovine *N*-acetylneuraminyllactose (sialolactose) as substrate. Liberated sialic acid was assayed according to Aminoff [5].

Modification studies [6]: Increasing amounts of *N*-bromosuccinimide (fresh aqueous solution) were added to the enzyme solution in 0.1 M acetate buffer pH 5.0.

Koshland's reagent (2-hydroxy-5-nitrobenzylbromide) was dissolved in acetone (0.1 M stock solution or dilutions in acetone), and added to the buffered enzyme solution. The final mixture contained 10% acetone and up to 0.01 mM/ml reagent. Residual neuraminidase activities after the modifications were tested either directly (with appropriate controls for the reagents used) or after removal of reagents and low molecular weight reaction products on columns of Sephadex G-25 equilibrated with acetate buffer pH 5.0 or phosphate buffered saline.

3. Results

3.1. *Purification of neuraminidase*

Purification of neuraminidase released from the virion by treatment with detergent and ether and subsequent pronase digestion was achieved by chromatography on DEAE-cellulose. Fig. 1 shows a typical elution profile.

Using this chromatographic purification step neuraminidase is not only freed from viral or non-viral impurities but also from the pronase used for solubilization. Pronase is found in the void volume of

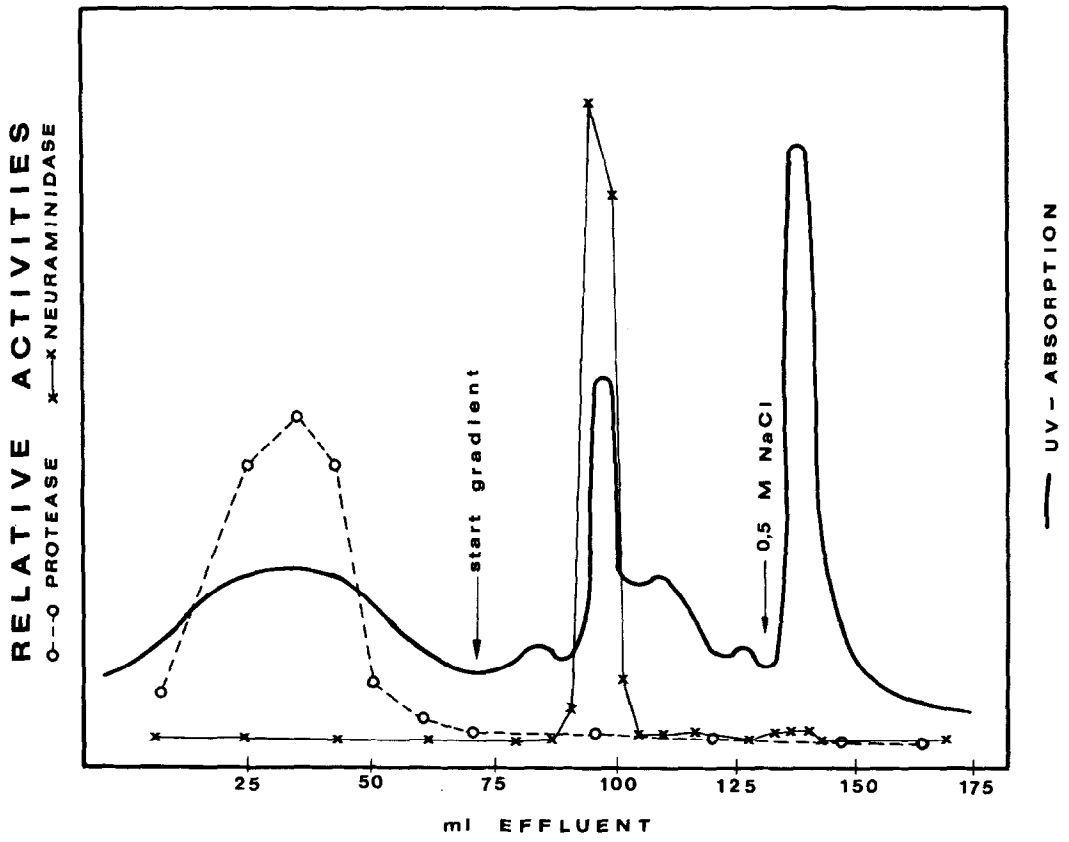

Fig. 1. Chromatography of viral fragments on DEAE-cellulose. Fifty ml of purified influenza A_2 concentrate was treated with Tween 80 and ether and subsequently digested with Pronase [3]. The column (Cellex D, Calbiochem, 1.6 × 20 cm) was equilibrated with 0.01 M Tris pH 7.5. Elution was started with this buffer and followed by a linear gradient to 0.2 M NaCl in the same buffer. After completion of the gradient elution was continued with 0.5 M NaCl. Protein concentration was monitored by UV-absorption (——) and the fractions were tested for their neuraminidase (x–x–x) and protease (o–o–o) activities.

the column whereas neuraminidase is retained quantitatively by the ion exchange resin.

3.2. *Oxidation of tryptophan with N-bromosuccinimide*

After incubation with *N*-bromosuccinimide complete loss of enzymatic activity of neuraminidase from viral as well as bacterial origin was observed. For complete inactivation an approx. 4-fold molar excess of reagent was required. Interestingly enough inactivation of neuraminidase could also be observed when the intact virus particle was incubated with the oxidizing agent.

3.3. *Alkylation with Koshland's reagent*

Enzymatic activity was found to be abolished by addition of this reagent to isolated enzyme preparations or intact influenza virus. However a much larger molar excess (approx. 1000-fold) was required for complete inactivation.

3.4. *Protection of tryptophan by substrate*

The influence of added substrate on the degree of inactivation with *N*-bromosuccinimide was studied. Table 1 summarizes the results obtained. Fetuin as well as the low molecular weight substrate sialolactose protect neuraminidase to a certain extent against inactivation by *N*-bromosuccinimide. As expected, no protection is observed by addition of the non-substrate protein albumin.

Table 1
Protection of tryptophan by substrate.

Additions during oxidation	Inactivation (%)	Protection (%)
None	100	–
0.01 mg Sialolactose	46	54
0.1 mg Sialolactose	0	100
0.006 mg Fetuin	86	14
0.06 mg Fetuin	0	100
0.2 mg Bovine serum albumin	100	0

Enzyme dilutions in acetate buffer (0.1 M, pH 5.0) were incubated with the minimal excess of *N*-bromosuccinimide required for complete inactivation in the presence and absence of different substances. After 30 min at 4° substrate concentrations were adjusted to 0.1 mg sialolactose and 0.3 mg fetuin, respectively, and the residual enzymatic activities determined at 37°.

4. Discussion

From chemical modification studies of intact influenza virus using iodine and fluorodinitro benzene, Hoyle [7] has excluded tryptophan as a part of the active site of viral neuraminidase. However, the results of the present communication strongly suggest an essential role of tryptophan in either the active site region or in maintaining the intact structure of the protein molecule necessary for activity of the enzyme.

This was concluded from experiments with purified influenza and *Cl. perfringens* neuraminidase using the site specific reagents *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzylbromide in the presence and absence of added substrate.

Preliminary experiments [8] have shown that nitration or acetylation of tyrosine residues, as well as oxidation or alkylation of cysteine residues do not effect enzymatic activity. Under these circumstances both reagents used in this study can be regarded as specific for tryptophan [6].

The similarity of modification patterns observed in isolated and virion bound enzyme are in agreement with the postulated superficial location of the enzyme on the surface of the virus. In the case of alkylation with Koshland's reagent steric hindrance is very likely an important factor for the slow reaction observed.

Studies on the importance of other amino acid residues (especially amino and imidazol groups) for the enzymatic activity and on the immunological properties of the modified products obtained are in progress.

Acknowledgements

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