Short Communications

An Improved Method for Purifying Sialidase

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An adsorbent specific for sialidase (EC 3.2.1.18) was prepared by coupling a glycoprotein containing glycosidically linked sialic acid to Sepharose. This adsorbent does not display the non-specific adsorption that occurs in previously reported methods.

The first affinity adsorbent for purifying sialidase was described by Cuatrecasas & Illiano (1971). This material has been shown to adsorb a number of other enzymes, including many of the major bacterial toxins present in filtrates from *Clostridium perfringens*, when the latter was used as a source of sialidase (Rood & Wilkinson, 1974). This adsorbent contains a sialidase inhibitor of as yet uncertain action (an *N*-substituted oxamic acid) linked to Sepharose by a tripeptide spacer arm. The adsorption of sialidase and contaminating proteins to this material appears to be non-specific. There is, however, some uncertainty as to whether the adsorption arises from ion-exchange phenomena (Rood & Wilkinson, 1974) or hydrophobic effects (Huang & Aminoff, 1974).

The interest in a sialidase preparation free of contaminating activities stems from the very wide use of this enzyme as a tool in the investigation of the role of sialic acids (Spiro, 1970). The affinity adsorbent now described here has the advantage of being easily and cheaply prepared. It is based on a substrate of the enzyme and appears not to adsorb many of the contaminating enzymes which have caused problems for previous workers. The insolubilized glycoprotein is also being used in inhibitor studies of sialidase.

Experimental

Sialidase type V (lot. no. N-2876, an $(\text{NH}_4)_2\text{SO}_4$ fraction from *Cl. perfringens* filtrate) was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Freeze-dried human α_1 -acid glycoprotein was bought from the Protein Fractionation Centre, Ellen's Glen, Edinburgh, U.K. *Collocalia* mucoprotein (from bird's nest) was obtained from a Chinese emporium (Loon Fung General Provisions, Gerrard Street, London W.1, U.K.).

Sepharose 4B was activated as described by March *et al.* (1974); 2 g of CNBr were used for 20 ml of settled Sepharose. The activated Sepharose was added to a filtered solution of 100 mg of α_1 -acid

glycoprotein in 40 ml of 0.2 M-NaHCO₃ buffer, pH 9.4. After 22 h at 4°C, the Sepharose was extensively washed with the coupling buffer and finally washed and allowed to sediment repeatedly from suspension in 1-litre amounts of 2 M-NaCl. The gel was stored at 4°C in distilled water containing 0.2% sodium azide.

Free or enzymically released *N*-acetylneuraminic acid was assayed as described by Warren (1959). Samples of the coupled Sepharose treated with 0.05 M-H₂SO₄ at 80°C for 1 h to hydrolyse glycosidically linked *N*-acetylneuraminic acid gave high readings in the Warren (1959) assay, possibly owing to agarose hydrolysis products. Because of this, the amount of bound *N*-acetylneuraminic acid was determined by incubating the coupled Sepharose with two additions of 0.17 unit of neuraminidase at 37°C for 2 h. A minimum value of 0.56 μmol of *N*-acetylneuraminic acid/ml of settled Sepharose was obtained by this method. The α_1 -acid glycoprotein contained about 4% (w/w) of *N*-acetylneuraminic acid.

Assay of sialidase activity. The substrate for sialidase was a ficin digest of *Collocalia* mucoprotein (Aminoff, 1961). The assay solution contained 500 μg of bound *N*-acetylneuraminic acid/ml of 0.05 M-sodium acetate buffer, pH 5.0 at 37°C.

Assay of proteinase activity. Proteinase activity was measured by incubating 0.1 ml portions of enzyme fractions with 1 ml of 1% (w/w) casein in 0.1 M-sodium borate buffer, pH 7.6, for 2 h at 37°C. Solutions were deproteinized with 10% (w/v) trichloroacetic acid and the supernatants assayed by the method of Lowry *et al.* (1951). Activity was indicated by the extinction at 750 nm; 1 unit of proteinase corresponds to 1 mequiv. of tyrosine produced/min.

Assay of phospholipase C activity. Phospholipase activity was determined as described by Pastan *et al.* (1968) with egg phosphatidylcholine as a substrate; 1 unit of activity corresponds to 1 μmol of phosphatidylcholine hydrolysed/min.

Assay of haemagglutinin activity. This was by the standard pattern test (W.H.O. Expert Committee on Influenza, 1953), by using serial dilutions of test fractions in 0.25 ml volumes in W.H.O. trays (obtained from Arnold Horwell, London N.W.6, U.K.). Chicken erythrocytes (0.5%, v/v) were used and 1 mM-NiCl₂ was added to the saline solutions as suggested by Rood & Wilkinson (1974). The activity of haemagglutinin in units/ml was the reciprocal of the end-point dilution in the assay.

Assay of haemolysin activity. The activity of haemolysin was measured by the lysis of washed rat erythrocytes incubated in 1 ml of 0.01 M-phosphate buffer, containing 0.85% (w/v) NaCl, 0.25% (v/v) cells, 1 μ mol of dithiothreitol and 0.1 ml of test fraction, at pH 7.2 and 37°C. The reaction was slowed by rapid chilling and the absorbance of the supernatant measured at 559 nm after centrifugation; 1 unit of haemolysin activity was defined as that causing 50% haemolysis in 10 min.

Results and discussion

Before purification, the sample of *Cl. perfringens* filtrate had the following activities: sialidase, 0.4 unit/mg; proteinase, 0.03 unit/mg; phospholipase C, 0.005 unit/mg; haemolysin, 128 units/mg; haemagglutinin, 256 units/mg.

Approx. 2 ml of Sepharose-glycoprotein was placed on to 1 ml of Sephadex G-10 in a 10 ml plastic syringe barrel. This column was equilibrated with 0.02 M-sodium acetate buffer, pH 5.0, containing

0.1 M-KCl and 0.02% sodium azide at 4°C. Then 8.4 mg of the crude sialidase was applied to the column in 1 ml of the equilibration buffer and washed on to the column until no significant protein (E_{280}) emerged. The flow rate was 30 ml/h. The protein peak coincided with the elution of the dark-brown colour characteristic of this fraction of *Cl. perfringens* filtrate. Sialidase was immediately eluted with 0.1 M-sodium borate buffer, pH 8.5, containing 0.1 M-KCl. The most active fractions were pooled. These contained 44 μ g of protein of specific activity 60 units/mg, representing a purification, based on protein, of 200-fold. The recovery, based on total units, was about 80%. Some activity escaped adsorption in the equilibration buffer.

Fig. 1 shows the elution profile of the affinity column. The major contaminating activities were eluted with the equilibration buffer. This is especially gratifying for haemagglutinin, since many sialic acid-containing glycoproteins are known to be potent inhibitors of viral haemagglutination (Gottschalk, 1966).

In a prior experiment, 0.1 M-NaHCO₃ buffer, pH 9.1, containing 0.1 M-KCl failed to elute sialidase, but 0.1 M-sodium borate, pH 8.5, caused quantitative elution. This weakening of the affinity of the column for sialidase in the presence of borate may arise from the formation of a ligand-borate complex. The polyhydroxyl chain of *N*-acetylneuraminic acid is a possible candidate for this. Similar complex-formation has been proposed to explain the specific elution by borate of other enzymes from columns containing covalently linked carbohydrate (Barker *et al.*, 1972).

Fig. 1. Affinity chromatography of sialidase on Sepharose- α_1 acid glycoprotein

Protein (8.4 mg) was applied in 1 ml of 0.02 M-sodium acetate buffer, pH 5.0, containing 0.1 M-KCl. The column was washed with the same buffer. At point A the column was eluted with 0.1 M-borate buffer, pH 8.5. (a) ●, Sialidase activity (units/ml); —, protein (E_{280}); ▲, $10^{-3} \times$ haemagglutinin activity (units/ml); ■, proteinase activity (E_{750}). (b) ○, $10^3 \times$ Phospholipase C activity (units/ml); △, $10^{-3} \times$ haemolysin activity (units/ml).

Purity of the protein. Pooled fractions from several column runs were essentially free of the contaminating activities mentioned. The protein appears homogeneous on gel electrophoresis, but stains very poorly with Coomassie Brilliant Blue R. The original material gave 20 distinct bands and diffuse areas of stain.

Since the affinity ligand (*N*-acetylneuraminic acid) is released by prolonged incubation with sialidase, some care must be exercised with the use of the column. For example, in an experiment where the small column described was deliberately overloaded (400mg of protein), the sialidase activity was all eluted in the equilibration buffer, but retarded from the protein peak. Presumably, even at 4°C the very large excess of sialidase released most of the bound *N*-acetylneuraminic acid. This problem can be overcome by a choice of larger columns, appropriate to the size of the sample applied. It is likely that much larger amounts of α_1 -acid glycoprotein could be coupled to the Sepharose by increasing the amount of CNBr used in the coupling step. I have found that, for large amounts of the *Cl. perfringens* fraction used here, a single run on a Sephadex G-100 column (at pH 6.0

with 0.2M-KCl as eluent) before affinity chromatography as described removes up to 80% of the contaminating protein.

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