The Purification and Properties of a β -*N*-Acetylhexosaminidase from *Trichomonas foetus*

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A β -*N*-acetylhexosaminidase was purified 800-fold from extracts of *Trichomonas foetus* by affinity chromatography on a column of *N*-(ϵ -aminohexanoyl)-2-acetamido-2-deoxy- β -D-glucopyranosylamine bound to CNBr-activated Sepharose. The enzyme has a dual specificity for the *p*-nitrophenyl β -D-glycosides of *N*-acetylglucosamine and *N*-acetylgalactosamine. The parent sugars are both competitive inhibitors. The enzyme has a mol.wt. approx. 150000 and a pH optimum of 6.2. It is suggested that the same active site catalyses both activities and that no part is played by the 4-hydroxyl group in substrate binding, but it is involved in determining the catalytic rate.

Extracts of the flagellate *Trichomonas foetus* will degrade the carbohydrate moiety of the human tumour index substance, carcinoembryonic antigen (R. G. Edwards, P. Thomas & J. H. Westwood, unpublished work). This glycoprotein of mol.wt. approx. 200000, contains approx. 60% by weight of carbohydrate, made up of galactose, mannose, *N*-acetylglucosamine (2-acetamido-2-deoxyglucose) and sialic acid units (Westwood *et al.*, 1974).

Extracts of *T. foetus* contain β -glucosidases (β -D-glucoside glucohydrolase, EC 3.2.1.21), galactosidases (α -D-galactoside galactohydrolase, EC 3.2.1.22, and β -D-galactoside galactohydrolase, EC 3.2.1.23), mannosidases (α -D-mannoside mannohydrolase, EC 3.2.1.24), hexosaminidases (2-acetamido-2-deoxy-D-hexoside acetamidodeoxyhexohydrolase, EC 3.2.1.52 and EC 3.2.1.49), fucosidases (α -L-fucoside fucohydrolase, EC 3.2.1.51) (Watkins, 1959; Harrap & Watkins, 1970) and neuraminidases (acylneuraminyl hydrolase, EC 3.2.1.18) (Romanovska & Watkins, 1963). We have been interested in the isolation of individual glycosidases from such extracts for use in conjunction with sequential Smith degradations (i.e. application in sequence of periodate oxidation, borohydride reduction and acid hydrolysis) (Goldstein *et al.*, 1965) in studying structure in relation to the antigenic activity of carcinoembryonic antigen.

The purification of human hexosaminidases A and B by affinity chromatography by using *N*-(ϵ -amino-hexanoyl)-2-deoxy- β -D-glucopyranosylamine bound to CNBr-activated Sepharose has been reported (Geiger *et al.*, 1974). We now describe a similar purification of one of the β -*N*-acetylhexosaminidases from *T. foetus* and report on some properties of the enzyme.

Experimental

Materials

N-Acetylglucosamine, *N*-acetylgalactosamine (2-acetamido-2-deoxy-D-galactose) and the *p*-nitrophenyl glycosides of 2-acetamido-2-deoxy- β -D-glucose, 2-acetamido-2-deoxy- α -D-glucose, 2-acetamido-2-deoxy- β -D-galactose, 2-acetamido-2-deoxy- α -D-galactose, β -D-glucose, α -D-mannose, and α - and β -D-galactose, were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. CNBr-activated Sepharose 4B and Blue Dextran 2000 were purchased from Pharmacia Chemicals, Uppsala, Sweden. Hog gastric mucin was a gift from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K., and was purified by precipitation from aqueous solution with ethanol. Bovine serum albumin for use as a standard for protein determination was purchased from Armour Pharmaceutical Co. Bio-Gel A (1.5m) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. All other chemicals were of analytical reagent grade. Cells of *T. foetus* were obtained as a frozen paste from the Microbiological Research Establishment, Porton Down, Salisbury, Wilts., U.K.

Methods

Assay of enzyme activities. Routine assays with the *p*-nitrophenyl glycosides as substrates were carried out as follows. The enzyme solution (0.025 ml) was added to a solution (0.2 ml) of the appropriate *p*-nitrophenyl glycoside (0.5 mg/ml) in 0.05 M-sodium phosphate buffer (pH 7.0) and incubated at 37°C for 15 min (or 17 h for the α -mannosidase and β -glucosidase assays). The reaction was terminated by the addition

of 1 M- Na_2CO_3 (1 ml) and the release of *p*-nitrophenol was measured spectrophotometrically at 400 nm. For the determination of kinetic constants the initial rate of *p*-nitrophenol formation was monitored at 400 nm by using a Pye Unicam SP. 500 monochromator with a Gilford model 220 absorbance indicator, the output of which was connected to a Honeywell strip chart recorder. The full-scale deflexion of the recorder was set to an absorbance of 0.1. Cell compartments were thermostatically maintained at 30°C. Substrate solution (0.1 ml) was added to 0.2 M-sodium phosphate buffer (pH 7.0, 0.8 ml) and the reaction was initiated by the addition of enzyme solution (0.1 ml). The rate of *p*-nitrophenol formation was recorded during 5 min. Specific activities were determined from extrapolated V_{max} values and expressed as μmol of *p*-nitrophenol released/min per mg of protein. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

As *p*-nitrophenyl α -L-fucoside is not a substrate for the fucosidase of *T. foetus* (Watkins, 1959), fucosidase activity was measured by incubating hog gastric mucin (0.1 ml; 10 mg/ml) with the enzyme solution (0.1 ml) in 0.05 M-sodium phosphate buffer (pH 7.0) at 37°C for 16 h. The reaction was stopped by the slow addition, with vigorous shaking, of ethanol (1 ml), and the precipitated glycoprotein was removed by centrifugation. The supernatant (0.2 ml) was assayed for free fucose by the method of Dische & Shetles (1948).

Preparation of *T. foetus* extracts. The procedure was essentially that described by Watkins (1966) for the preparation of cell-free extracts.

Determination of molecular weights. Molecular weights were determined by gel filtration (Andrews, 1965) by using a column (1.6 cm $\times$ 100 cm) of Bio-Gel A (1.5 m) equilibrated with 0.05 M-sodium phosphate buffer (pH 7.0). The column was calibrated with haemocyanin (mol.wt. 4.5×10^6), thyroglobulin (mol.wt. 669 000), ferritin (mol.wt. 448 000), fibrinogen (mol.wt. 330 000), human immunoglobulin G (mol.wt. 160 000) and bovine serum albumin (mol.wt. 67 000 monomer 134 000 dimer) as protein standards. The void volume was determined by using Blue Dextran 2000 and the inclusion volume by using pyridoxal 5'-phosphate.

Polyacrylamide-gel electrophoresis. This technique was performed in the Shandon analytical polyacrylamide-gel-electrophoresis apparatus. Gel concentrations were 8.5% and electrophoresis was carried out in a Tris-glycine buffer system at pH 8.6 (Davis, 1964). Gels were stained for protein by using a 0.1% solution of Coomassie Brilliant Blue in 20% (w/v) trichloroacetic acid. Enzyme activity was located on the gels by equilibrating individual gels with 0.2 M-sodium phosphate buffer (pH 7.0) for 20 min at 37°C followed by incubation with a solution (1 mg/ml) of

the appropriate *p*-nitrophenyl glycoside in the same buffer for up to 1 h at 37°C.

Preparation of the affinity column. *N*-(ϵ -Amino-hexanoyl)-2-acetamido-2-deoxy- β -D-glucopyranosylamine was prepared essentially by the method of Lotan *et al.* (1973), giving a product m.p. 186–189°C (decomp.), $[\alpha]_D^{25} +22.2^\circ$ (*c* 1 in water); literature m.p. 193–195°C (decomp.), $[\alpha]_D^{20} +23.3^\circ$ (*c* 1 in water). This product (10 mg) was coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The amount of ligand covalently bound to the Sepharose was determined by hydrolysis of the gel in 6 M-HCl (overnight) and measurement of the liberated 6-aminohexanoic acid on a Jeol amino acid analyser (JLC 6AH).

Purification of β -*N*-acetylhexosaminidase activities. Freeze-dried *T. foetus* extract (10 mg) was dissolved in 0.005 M-sodium phosphate buffer (pH 7.0, 0.5 ml) and applied to a column (3 cm $\times$ 1 cm) of the activated Sepharose (0.8 μmol of ligand bound/ml of settled gel). The column was washed with the phosphate buffer, and fractions (1.5 ml) were collected until all the material absorbing at 280 nm was eluted from the column. The column was then washed with 0.5 M-sodium phosphate buffer (pH 7.0), and fractions (1.5 ml) were collected until the eluate was free of β -*N*-acetylhexosaminidase activity. A final washing with 0.05 M-sodium phosphate buffer containing 0.04 M-*N*-acetylglucosamine was carried out until no further hexosaminidase was eluted from the column. All fractions were assayed for protein content (E_{280}), *N*-acetylglucosaminidase, *N*-acetylgalactosaminidase, β -galactosidase, α -mannosidase, β -glucosidase and α -L-fucosidase activities.

Results

A typical fractionation of the β -*N*-acetylhexosaminidase activities from *T. foetus* is shown in Fig. 1. For clarity only the assays for β -*N*-acetylglucosaminidase and β -*N*-acetylgalactosaminidase activities are shown. The column showed some evidence of overloading when more than 10 mg of the freeze-dried extract was applied, when some β -*N*-acetylhexosaminidase activity appeared in Fraction A. The recovery of β -*N*-acetylhexosaminidase activity from the column was greater than 90%. The column remained usable for at least 3 months when stored with 0.005 M-sodium phosphate buffer (pH 7.0) at 4°C during which time it was used for at least 10 fractionations without any marked deterioration. Fraction A contained most of the protein and considerable β -galactosidase activity, but little else. Fraction B contained α -*N*-acetylgalactosaminidase, β -galactosidase, β -glucosidase, α -mannosidase and α -L-fucosidase activities, all confined to the material absorbing at 280 nm, plus the broad peaks of β -*N*-acetyl galactosaminidase and β -*N*-acetylglucosaminidase

indicated in Fig. 1. Fraction C contained only the last two activities. In fraction B, the fractions containing hexosaminidase activity were combined and concentrated by vacuum dialysis with 0.05M-sodium phosphate buffer (pH 7.0). For kinetic analysis fraction C was extensively dialysed against 0.05M-sodium phosphate buffer (pH 7.0) until free of *N*-acetylglucosamine, which gave rise to the E_{280} seen for fraction C in Fig. 1. The total protein content of this fraction (Lowry *et al.*, 1951) was normally in the range 15–20 μ g. On examination of the fractions containing β -*N*-acetylhexosaminidase by gel electrophoresis, fraction B showed multiple bands (general protein stain) none of which was coincident with either the β -*N*-acetylglucosaminidase or the β -*N*-acetylgalactosaminidase activities. Fraction C, however, showed only a single band which stained with Coomassie Brilliant Blue and which was coincident with both the β -*N*-acetylglucosaminidase and the β -*N*-acetylgalac-

Fig. 1. Fractionation of the β -*N*-acetylhexosaminidase activities of *T. foetus* on a Sepharose affinity column

●, E_{280} ; ○, β -*N*-acetylglucosaminidase activity; △, β -*N*-acetylgalactosaminidase activity. Fraction A was eluted with 5mM-sodium phosphate buffer (pH 7.0); fraction B was eluted with a 0.5M-sodium phosphate buffer (pH 7.0); fraction C was eluted with a solution of 40mM-*N*-acetylglucosamine in 50mM-sodium phosphate buffer (pH 7.0). Further experimental details appear in the text.

tosaminidase activities. Fraction C hexosaminidase had an electrophoretic mobility higher than that of hexosaminidase in fraction B. Gel filtration of fraction B showed the hexosaminidase to have an average molecular weight of 280000, with some activity at higher molecular weight. The fraction-C hexosaminidase, however, behaved as a single species with mol.wt. 150000, and the ratios of β -*N*-acetylglucosaminidase to β -*N*-acetylgalactosaminidase activities were constant across the activity profile.

Kinetic investigations of fraction-C hexosaminidase showed a dual specificity towards the *p*-nitrophenyl β -D-glycosides of both *N*-acetylglucosamine and *N*-acetylgalactosamine, whereas *N*-acetylglucosamine and *N*-acetylgalactosamine competitively inhibited each activity. Assays were carried out at pH 7.0 to enable the production of nitrophenolate ion to be continually monitored and the kinetic constants calculated from double-reciprocal plots are shown in Table 1. Plots of activity against pH for each substrate were identical in shape and had an optimum at pH 6.2. Kinetic data comparable with those shown in Table 1 were also obtained at this pH. The fraction-C hexosaminidase had a specific activity (*p*-nitrophenyl β -*N*-acetylglucosaminide) of 53 units/mg of protein, and rechromatography on the affinity column showed little contamination with the fraction-B activity. The specific activity of the total *N*-acetylhexosaminidase activity in the *T. foetus* extract was 0.27 unit/mg of protein, but fraction C represented only 25% of the total enzyme activity giving an overall purification of 784-fold for this enzyme. The fraction-C enzyme was stable over a period of weeks at 4°C.

Discussion

This study has shown the presence of at least two separate β -*N*-acetylhexosaminidase activities in extracts of *T. foetus*, and one has been extensively purified in a single step by utilizing the principle of affinity chromatography thereby giving a purification factor of approx. 800. It is noteworthy that this purification illustrates what can be a major problem with the use of hydrophobic spacer arms such as

Table 1. Kinetic constants for the β -*N*-acetylhexosaminidase from *T. foetus* (fraction C) at pH 7.0

Values are means of three determinations. Abbreviations: GlcNAc, 2-acetamido-2-deoxy-D-glucose; GalNAc, 2-acetamido-2-deoxy-D-galactose.

Substrate	K_m (mM)	Relative V_{max}	K_i (GlcNAc) (mM)	K_i (GalNAc) (mM)
<i>p</i> -Nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside	0.25	100	1.07	0.51
<i>p</i> -Nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside	0.20	26.3	1.15	0.51

6-aminohexanoyl, in that non-biospecific adsorption between the spacer arm and proteins can occur, as well as the desired interaction [see O'Carra *et al.* (1974) and references therein]. Most of the protein eluted in fraction B was probably loosely bound by a non-specific interaction and hence was relatively easily removed by changing the ionic strength of the eluting buffer. Significantly, though, it was β -hexosaminidase which was most difficult to remove under these conditions, all other activities being eluted coincidentally with the small peak of material absorbing at 280 nm. The gel therefore exhibits some specificity towards these enzymes. On the other hand, fraction C almost certainly represents a true enzyme-inhibitor interaction, since the hexosaminidase activity could only be removed by including *N*-acetylglucosamine, a competitive inhibitor of the enzyme, in the eluting buffer.

The purified enzyme (fraction C) showed a dual specificity towards the *p*-nitrophenyl glycosides of β -*N*-acetylglucosamine and β -*N*-acetylgalactosamine. The K_m values for these substrates were similar, but the rate of hydrolysis of the glucosaminide was approximately four times that of the galactosaminide. This observation raises the possibility that whereas the 4-hydroxyl group is not required for the initial binding of the substrate it is a requirement for the orientation of the substrate once the enzyme-substrate complex has been formed and in this way affects the rate of hydrolysis of the glycoside.

Table 1 shows that the K_i values obtained with *N*-acetylglucosamine and *N*-acetylgalactosamine as inhibitors are independent of the substrate used. This evidence, along with the evidence that at all stages of the purification, including gel filtration, the ratio of the β -*N*-acetylglucosaminidase and the β -*N*-acetylgalactosaminidase activities remain constant suggest that the same enzyme active site is responsible for both activities. Further evidence has been obtained by using polyacrylamide-gel electrophoresis. The single band staining with Coomassie Blue and found in fraction C was always associated with both enzyme activities.

Gel electrophoresis of whole *T. foetus* extract showed the presence of two and what sometimes appeared to be three β -hexosaminidase activities. These activities could be detected by using either *p*-nitrophenyl β -*N*-acetylglucosaminide or *p*-nitrophenyl β -*N*-acetylgalactosaminide. Also present was an α -*N*-acetylgalactosaminidase activity with a low electrophoretic mobility; this enzyme, however,

could not be detected with *p*-nitrophenyl α -*N*-acetylglucosaminide. This activity is probably the α -*N*-acetylgalactosaminidase described by Watkins (1966) as being responsible for the destruction of blood-group A activity.

The effects of extracts of *T. foetus* and partially purified preparations of the enzyme from this organism on the glycoproteins responsible for blood-group activity have been studied extensively (Watkins, 1960, 1962, 1966) and has enabled information to be obtained about the antigenic properties of these molecules. The activity of the purified fraction-C hexosaminidase and the partially purified fraction-B activity towards glycoproteins and glycopeptides derived from the carcinoembryonic antigen may also provide information about the antigenic properties of this glycoprotein.

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