STUDIES ON N-ACETYLE-β-GLUCOSAMINIDASE FROM CARDIAC MUSCLE

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ABSTRACT

Lysosomal N-Acetyl-β-D-glucosaminidase was purified to homogeneity by Blue-Sepharose and ConA-Sepharose Chromatography followed by affinity chromatography on immobilised N-acetylgalactosamine column. The enzyme was separated into A- and B-forms by ion exchange chromatography. Both the forms were homogeneous and glycoprotein in nature. The enzyme even in the homogenate stage could not hydrolyze N-acetylgalactosamine derivatives. It was inhibited by pHMB and contained 7 and 8 mole of free SH groups per mole of A- and B-form respectively. The aminogroups of the molecules are necessary for biological activity.

INTRODUCTION

N-ACETYL-D-GLUCOSAMINE (glcNAc) is an essential component of mammalian glycoproteins and proteoglycans. It is associated with polypeptide-linked and other oligosaccharide chains in— α and β-linkages. The β-linkages with oligosaccharide chains may be cleaved by mammalian β-hexosaminidases which also cleave β-linked N-acetyl-D-galactosamine (galNAc). There is no report of a single lysosomal enzyme of mammalian origin which can hydrolyze β-glcNAc linkage alone. During our investigations on the involvement of lysosomal enzymes in the tropical endomyocardial fibrosis (EMF), we have characterised from normal cardiac muscle, a lysosomal enzyme which cleaves β-glcNAc linkages but not β-galNAc ones. The purification and physicochemical properties of mammalian β-hexosaminidases have been reported in detail[1]. There is very little information on the structural aspects of the enzyme. The present study describes the purification, properties and effect of chemical modifications on biological properties of normal cardiac muscle β-D-glucosaminidase (E.C.3.2.1.30).

MATERIALS AND METHODS

Maleic anhydride was obtained from Sigma Chemicals Co., St. Louis, USA. Polyethylene glycol (PEG)-6000 was purchased from local sources. The sources of the bio-chemicals and other reagents have been described earlier[2,3]

Enzyme assay

The assay system for the enzyme contained 200 μ mole of citrate-phosphate buffer, pH 4.5, 100 μg bovine serum albumin, 0.5 μmol of *p*-nitrophenyl-β-N-acetylglucosamine and suitable amount of enzyme in a total volume of 0.5 ml. The reaction mixture was incubated for 15 min at 37°C. The reaction was stopped and the colour was measured according to method described earlier[2]. One unit of enzyme was defined as the amount required to liberate 1 nmol of *p*-nitrophenol per min at 37°C. Other glycosidases were assayed as before[2].

Affinity columns: Cibacron Blue F3GA dye was coupled to Sepharose CL-4B according to the method of Boehme *et al*[4]. Concanavalin A (ConA) and 6-amino caproic acid were separately immobilised to cyanogen bromide-activated Sepharose 4B as described earlier[2]. *p*-Aminophenyl-β-N-acetylgalactosamine was coupled to the carboxyl group of immobilised 6-aminocaproic acid according to the method of Lisman and Overdijk[5].

Polyacrylamide gel Electrophoresis: The polyacrylamide gel electrophoresis was carried out in 5% and 7.5% gel at pH 4.3 in β-alanine-acetic acid and at pH 8.3 in Tris-HCl buffer according to Davis[6]. The gels were destained as described earlier[2].

The molecular weight of the protein was estimated by gel filtration on Sephadex G-200 according to Andrews[7]. The standard proteins used were cytochrome C (12,500), soybean trypsin inhibitor (21,500), chymotrypsinogen (25,000), ovalbumin (45,000), bovine serum albumin (67,000), aldolase (158,000) and catalase (240,000). The total carbohydrate content was estimated by phenol-sulphuric acid method using galactose as standard[8]. Monosaccharide constituents were analysed as described by Banerjee and Basu[9]. The protein was estimated by the method of Lowry *et al*[10]

with crystalline bovine serum albumin as standard. The free sylphydryl (SH) group was measured by direct Ellman's reaction[11] and the total SH groups by the method of Habeeb[12].

Cysteine and *p*-hydroxy mercuribenzoate (pHMB) at different concentrations were preincubated alone or together according to details in table 3.

Chemical modification of the aminoacid and carbohydrate residues

The amino groups were modified by treatment with trinitrobenzene sulphonic acid (TNBS) according to Fields[13]. The enzyme (25 μg protein) in 1 ml was incubated with 1.5 mM TNBS in the presence of 100 μmol of sodium borate buffer, pH 8.5 for 5 min at 25°C.

Maleylation of the protein was carried as described by Butler *et al*[14]. In a typical experiment 100 μg protein in 0.5 ml of 0.1 M borate buffer, pH 8 was mixed with 1.5 mg of solid maleic anhydride and incubated for 16 hr at 24°C. It was then dialyzed against 0.05 phosphate buffer, pH 7 for 18 hr with 3 changes.

Iodoacetic acid at a concentration (0.02 M) was preincubated at 25°C with 25 μg protein for 60 min in 1 ml of either 50 μM sodium phosphate buffer, pH 7 or 50 μM sodium borate buffer, pH 8. Iodoacetamide at 0.02 M concentration was preincubated at 25°C in borate buffer, pH 8 containing 25 μg protein in 0.01 ml for 60 min.

Periodic acid was treated according to Spiro[15]. Twenty μg protein in 1 ml of 0.1 M citratephosphate buffer, pH 4.5 was preincubated separately in 0.03 M and 0.1 M periodic acid at 4°C in dark for 120 min. The enzyme samples, after chemical modification, were dialyzed against 500 volumes of 0.02 M phosphate buffer, pH 6 for 16 hr at 4°C with 3 changes. The residual activities were measured under standard conditions with suitable control.

Enzyme Purification

Human heart muscles were collected at autopsy within 6–8 hr after death. The tissue was brought to the laboratory in ice, washed thoroughly and cut into small pieces and kept at −20°C in polythene bags until the time of experiment. All operations were carried out at 0–4°C unless otherwise mentioned.

Frozen tissue (50 g) was homogenised with 250 ml of 0.02 M phosphate buffer, pH 7 containing 0.1 M NaCl in a Sorvall-Omnimixer for 2 min at full speed. The homogenate was stirred for 30 min and centrifuged at 20,000 g for 20 min in SORVALL RC-5 B centrifuge. The clear supernatent was made 25% (w/v) saturated with PEG-6000 and the pH adjusted to 7 with dilute ammonia solution. After stirring for 30 min the suspension was centrifuged as before. The precipitate was collected and dissolved in minimum volume of 0.02 M phosphate buffer, pH 7 containing 0.1 M NaCl. This solution was mixed with Blue-Sepharose (80 mg protein/ml gel) and kept for 30 min. The Blue-Sepharose gel was previously equilibrated with same buffer. The gel-protein suspension was centrifuged at 2,000 g for 5 min at 25°C and the supernatant was collected. The gel was washed twice with the same buffer by centrifugation (5 ml buffer/ml of gel) and the supernatant was collected. The pooled supernatant was centrifuged at 20,000 g for 20 min and the clear supernatant was passed through a ConA-Sepharose column (2.5 × 8 cm) equilibrated with 0.05 M phosphate buffer, pH 7 containing 1 M NaCl at a flow rate of 15 ml/hr. The column was washed with the same buffer at 25°C till the effluent had an absorbance of less than 0.05 at 280 nm. The enzyme was eluted at 25°C with the same buffer containing 0.5 M α-methyl-D-glucoside and 10 ml fractions were collected. The active fractions (3 to 30) were pooled and dialyzed for 16 hr against 100 volumes of 0.02 M phosphate buffer, pH 6 with 3 changes. The dialyzed solution was lyophilized to 10–12 ml.

The lyophilised solution (3 ml) was then passed through a Sepharose-caproyl aminophenyl-β-N-acetyl galactosamine column (1.2 × 10 cm). The column was equilibrated and washed with 0.02 M phosphate buffer (pH 6) till the absorbance at 280 nm became less than 0.05 at a flow rate of 10 ml/hr. The active fractions (4 to 8) were eluted with 0.02 M phosphate buffer, pH 7.4 containing 0.15 M NaCl and 0.05 M N-acetyl glucosamine and 2 ml fractions were collected. The pooled active fraction from all the batches were concentrated by ultrafiltration through YM-10 membrane and dialyzed for 16 hr with 3 changes against 60 volumes of 0.02 M phosphate buffer, pH 7.

DEAE-Sephadex A-50 column (1.1 × 7 cm) was equilibrated with 0.02 M phosphate buffer, pH 7 at a flow rate of 10 ml/hr. The dialyzed enzyme was passed through this column and washed with the same buffer containing 0.05 M NaCl till absorbancy at 280 nm was less than 0.05. The breakthrough and washings were pooled together and concentrated by ultrafiltration and designated as B-form. The column was then eluted with equilibrating buffer containing 0.2 M NaCl and 2 ml fractions were collected. The eluted enzyme fractions (3 to 11) were pooled and concentrated as before and designated A-form.

RESULTS AND DISCUSSION

The N-acetyl-β-D-glucosaminidase A-form of normal cardiac muscle was purified 650-fold, while the B-form was purified 300-fold (table 1). One of the steps in the purification procedure was treated with Blue-Sepharose gel. This step is introduced to remove albumin from the tissue extract which in turn increased the specific activity from 30 to 41. This treatment enabled a cleaner separation by ConA-Sepharose affinity chromatography. Both the forms of purified enzyme were completely free from other lysosomal glycohydrolase activities. The purity of the enzyme was judged by polyacrylamide gel electrophoresis under varying conditions. A-form showed one single band at acid and alkaline pH. B-form showed one single band at acidic pH, while it did not move under alkaline pH. Recently Battari *et al*[16] reported a rat liver cytosolic endo-β-glucosaminidase with pH optimum of 6.5. The present report is the first one of a lysosomal glycohydrolase cleaving exclusively β-glcNAc derivative. The physiological function of this enzyme in normal cardiac muscle is not known.

Table 1 *Purification of N-Acetyl-β-D-Glucosaminidase*

Steps	Total protein (mg)	Total activity (units)	Specific activity
Homogenate	5626	32,147	5
Supernatant	1470	30,833	21
PEG precipitation	956	28,985	30
Blue-sepharose			
Supernatant	654	26,712	41
ConA-sepharose	28	17,262	617
Gal-Nac-sepharose	3	5,250	1705
DEAE-sephadex			
A	0.74	2,394	3281
B	0.24	354	1475

pH optimum and kinetic constants

A-form of the enzyme had optimum activity at pH 4.5 in citrate-phosphate buffer, B-form had a broad pH optimum of 4.2 to 4.6 in the same buffer. But both forms were inactive towards galNAc derivative. The K_m values with glcNAc derivative were 0.55 and 0.58 mM for A-form and B-Form respectively. The V_{max} value calculated was 54.4 nm/min/mg protein for A-form while that of B-form was 252 nm/min/mg protein.

Effect of temperature

Both the forms were preincubated at pH 4.5 with 100 μg bovine serum albumin in citrate-phosphate buffer at temperature between 25°C and 55°C for varying periods of time. Aliquots were withdrawn at 10 min intervals and the enzymic activities were assayed under standard conditions. A-form completely loses its activity after 30 min at 55°C while B-form retains 90% of its activity at the same temperature upto 240 min. This effect is similar to that observed with mammalian β-hexosaminidase[17,18].

Inhibition studies

The effect of inhibitors on enzyme activity was examined by incubating the enzyme with various inhibitors for 15 min at 25°C and then assayed under standard conditions. Table 2 summarises the types of inhibition and inhibition constants. The glcNAc, galNAc and acetate are competitive inhibitors whereas glucosamine and galactosamine are very poor non-competitive inhibitors. Similar types of inhibition were observed with β-hexosaminidase of brain[17,18] and normal human urine[9]. Acetate was equally effective in inhibiting the reaction with respect to β-glucosaminidase activity. K_i was of the same order as glcNAc and competitive in nature (table 2). Acetate has very little structural resemblance with true substrate/product. It had been observed in the case of β-hexosaminidase A from normal human urine[19] that it is the acetic acid rather than acetate which inhibits the enzyme activity, probably as acetic acid binds with the hydrophobic region of the enzyme.

p-Nitrophenyl-β-D-galNAc at 4×10^{-4} M and 6×10^{-4} M was preincubated for 15 min at 25°C with

Table 2 *Effect of inhibitors*

Inhibitors	N-Acetyl-β-D-glucosaminidase A-Form (K_i mM)	B-Form (K_i mM)	Type of inhibition
Acetate	2.8	2.6	C
N-Acetyl galactosamine	0.56	0.58	C
N-Acetyl glucosamine	2.5	3.0	C
Galactosamine*	—	—	NC
Glucosamine*	—	—	NC

C = Competitive; NC = Noncompetitive

*Values were not calculated as very high concentrations of inhibitors were required even to obtain 50% inhibition. The slope of the lines obtained with (500 mM) galNAc and (500 mM) glcNAc showed the type of inhibition.

both the forms of enzyme (6 μg protein) and then assayed under standard conditions. The enzyme activity was inhibited by 55% and 65% respectively. But the above β-galNAc derivative could not be utilised as substrate. The K_i for free galNAc was 4–5 times lower as compared to that of glcNAc and acetate. An observation relevant to this is on the use of immobilised β-glcNAc as affinity ligand. It has been observed that the enzyme did not bind to this column. The same enzyme preparation bound to immobilised β-galNAc as affinity ligand and was eluted with buffer containing glcNAc but not NaCl alone.

Molecular weight

The molecular weights of both the forms were estimated by gel filtration on Sephadex G-200. The molecular weight of A-form was 110,000 dalton and that of B-form was 130,000 dalton.

Carbohydrate content

Total carbohydrate content was 6.5% and 6.2% for A- and B-forms respectively. A- and B-forms contained galactose, mannose, N-acetyl glucosamine and N-acetyl galactosamine.

All the lysosomal glycohydrolases known are glycoproteins. This phenomenon led us to treat the enzyme with periodate. When the enzyme was treated with 100 mM periodate for 2 hr at 4°C and pH 4.5 and then dialyzed for 16 hr against pH 6 buffer with 3 changes at 4°C, there was complete loss of activity for both the forms. But similar treatment with 30 mM periodate resulted in 60% loss of the activity. When the enzyme after the latter treatment was passed through ConA-Sepharose column only 70% of the applied activity was retained on the column, which was eluted with α-methylglucoside.

Effect of Chemical modification

The effects of chemical modifications are summarised in table 3. Cysteine alone has no effect. The enzyme retains full activity when cysteine and pHMB are preincubated together. Both forms are inhibited by 75% in the presence of 10^{-5} M pHMB. About 80% of the inhibited activity is recovered when cysteine (10^{-4} and 10^{-3} M) are added separately after preincubation. Similar effect was observed with normal human urinary B-hexosaminidase A and pHMB[19]. pHMB inhibited in a non-competitive fashion, with K_i values of 0.86×10^{-5} M for both the forms. This observation led us to estimate the free and total sulphydryl (SH) groups of both forms of β-glucosaminidase. A-form

Table 3 *Effect of chemical modification on the activity of β-glucosaminidase*

Modifier (M)	Percent residual activity A	B
None	100	100
TNBS	0	0
Maleic anhydride (1.5×10^{-5})	0	0
Cysteine (10^{-4})	100	100
pHMB (10^{-5})	25	25
Cysteine (10^{-4}) preincubated, then pHMB (10^{-5}) added and preincubated)	100	100
pHMB (10^{-5}) preincubated then cysteine (10^{-3} and 10^{-4}) added and preincubated)	60	60
Iodoacetamide (2×10^{-2})	100	100
Iodoacetic acid (2×10^{-2}, pH 7 and 8)	100	100
Periodate (10^{-1})	0	0
Periodate (3×10^{-2})	40	40

The details of the experiments are described in the text.

contained 7 moles of free SH-groups per mole of enzyme while the corresponding value for B-form was 8 moles. Total SH-groups of A-form were found to be 10 mole while that of B-form were 11 mole. Iodoacetamide (0.02 M) at pH 8 had no effect on the enzymic activities. Iodoacetic acid at similar concentration at pH 7 and 8 had no effect. There is complete loss of biological activities of both the forms when amino groups are modified with TNBS and maleic anhydride indicating that the amino groups are essential for biological activity.

There are certain similarities between mammalian β-hexosaminidase and the present enzyme. Both enzymes exist in two forms, which can be separated by ion-exchange chromatography and can be distinguished by the respective heat stability. But the present enzyme, unlike β-hexosaminidase, cannot hydrolyze β-galNAc-linkages.

ACKNOWLEDGEMENTS

This project was financed by a grant from Department of Science and Technology, New Delhi. The authors thank the Professor of Forensic Medicine, Trivandrum Medical College for the collection of autopsy tissue. We thank Dr P. S. Appukuttan for helpful suggestions.

5 December 1983

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ANNOUNCEMENTS

J. C. BOSE MEDAL OF THE INDIAN NATIONAL SCIENCE ACADEMY

The J. C. Bose Medal of the Indian National Science Academy has been awarded to Prof. V. Sasisekharan, Chairman of the Molecular Biophysics Unit of the Indian Institute of Science, Bangalore.

Prof. Sasisekharan was honoured for his pioneering work in life sciences. His work on polynucleotides conformation in relation to the DNA structure and other aspects of the macro-molecular structures of biological significance has been internationally recognised.

The molecular Biophysics Unit was started in 1971 as an interdisciplinary activity having collaboration with other departments. The thrust of the research in this area was aimed at challenging areas dealing with explanation of biological activity of various materials found in living systems in terms of their molecular structure. This unit has been recognised as the UGC Centre for Advanced Study in Biophysics since 1982. The previous Chairman of the unit was the celebrated scientist Dr G. N. Ramachandran, FRS the first to get the Albert Einstein Professorship.

SENSORS AND THEIR APPLICATIONS

The Instrument Science and Technology Group of the Institute of Physics in collaboration with the IEE, IMechE, IMC, IERE and the British Society for Strain Measurement, is organising the second conference and exhibition on Sensors and their Applications being held at the Boldrewood Conference Centre, Southampton from 10–12 September 1985.

The aim of the Conference is to provide a forum for scientists and technologists concerned with new ideas of application in the field of the sensors and should appeal to those in both industrial and academic sectors involved in the design, manufacture and application of sensors.

Further information on the Conference and exhibition can be obtained from the Meetings Officer, the Institute of Physics, 47 Belgrave Square, London SW 1X 8QX.