HUMAN *N*-ACETYL-β-HEXOSAMINIDASES: HYDROLYSIS OF *N,N'*DIACETYLCHITOBIOSE BY A LOW MOLECULAR WEIGHT ENZYME

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1. Introduction

N-Acetyl-β-hexosaminidase (EC 3.2.1.30) is commonly assayed by estimating the aglycone liberated from a suitable aryl-*N*-acetyl-β-hexosaminide. This practice has much to commend it since assays based on these substrates are rapid and sensitive and may be used to detect the enzyme activity in solution or after electrophoresis in a variety of media. So widespread is the use of these synthetic glycosides that the description of an enzyme as a hexosaminidase invariably alludes to the use of a nitrophenyl or methylumbelliferyl *N*-acetylhexosaminide for its detection. *N*-Acetyl-β-hexosaminidase has a broad aglycone specificity [1] and activities measured with synthetic substrates are often projected as having relevance to the hydrolysis of natural substrates. There is some justification for this since highly purified *N*-acetyl-β-hexosaminidase is also active towards a trisaccharide from hyaluronic acid [2] globoside and asialo GM_2 [3,4].

Two main forms of *N*-acetyl-β-hexosaminidase are present in normal human tissues [5–7]. These are kinetically indistinguishable when synthetic substrates are used but this similarity masks a more fundamental difference in the specificity of these enzymes which is revealed in the GM_2 gangliosidosis. The accumulation of ganglioside GM_2 in the tissues of patients with Tay-Sachs disease appears to be due to the absence of hexosaminidase A, the elevated levels of hexosaminidase B being unable to hydrolyse the storage product [7]. By analogy with Sanfillipo syndrome type B in which the lack of *N*-acetyl-α-glucosaminidase results in the storage of heparan sulphate [8] the almost complete absence of *N*-acetyl-β-hexosaminidase in Sandhoff's disease [9] might be expected to result in the storage of those glycosaminoglycans containing the β-*N*-acetylhexosaminide linkage. It is generally agreed, however, that there is no storage of glycosaminoglycans [10,11] in Tay-Sachs disease or Sandhoff's disease. One explanation for the absence of glycosaminoglycan storage in Sandhoff's disease could be the presence of a hexosaminidase capable of hydrolysing oligosaccharides but undetected by the synthetic substrates. Such an enzyme has not so far been found but this is not altogether surprising since there has been no attempt to isolate hexosaminidases while monitoring their activity towards natural substrates. Glycolipids and oligosaccharides are difficult to obtain in any quantity and since the activity of highly purified hexosaminidase towards some of these substrates is low, activities in crude extracts or column eluates might be beyond the limits of detection.

The disaccharide *N,N'*diacetylchitobiose was chosen as a substrate because it is the most readily available *N*-acetyl-β-glucosaminyl disaccharide being recovered in reasonable yields from acid hydrolysates of chitin. Chitin oligosaccharides give low blanks in the Morgan–Elson reaction used to assay free *N*-acetylglucosamine liberated as a result of enzyme action. An advantage of using the disaccharide as a substrate rather than higher members of the homologous series is that two *N*-acetylglucosamine residues are produced for each bond hydrolysed.

In the experiments reported here *N,N'*diacetylchitobiose (chitobiose) has been used as a substrate for *N*-acetyl-β-hexosaminidase from human liver. The

presence of a low molecular weight enzyme (chitobiase) is described which is active towards the disaccharide but inactive towards 4-methylumbelliferyl (4MU) *N*-acetyl-β-glucosaminide.

2. Materials and methods

2.1. *Materials*

Human liver was obtained post mortem and stored at −15°C until required. Chitin was obtained from Eastman Kodak Ltd., Kirby, Liverpool and 4-methylumbelliferyl-2-acetamido-2-deoxy-glucopyranoside from Koch Light Laboratories Ltd., Colnbrook, Bucks. Charcoal was supplied by BDH Ltd., Poole, Dorset and Celite 535 was a gift from Johns Manville Ltd., London.

2.2. *Preparation of N,N'diacetylchitobiose*

Chitin was ground to a fine powder in a Moulinex coffee grinder and 20 *g* stirred into 350 ml concentrated hydrochloric acid. After standing at 4°C for 2 hr the suspension was heated at 40°C for 2.5 hr, cooled on ice and titrated to pH 1.0 with 50% sodium hydroxide [12]. Care was taken to ensure that the temperature was kept below 40°C during neutralisation. The residue of undigested chitin and crystalline sodium chloride was removed by filtration and the bulk of monosaccharides and salt separated from the oligosaccharides by passing the filtrate through a 4.5 X 75 cm column of charcoal–celite.

The column was prepared by pouring a slurry of equal weights of charcoal and celite in water over a 5 cm plug of celite in the bottom of the column. Before use the column was eluted with 200 ml of 0.2 M sodium acetate and then several litres of distilled water. The filtrate was applied and the column eluted with water (1.5 litres) until the eluate was chloride free when tested with saturated silver nitrate. Oligosaccharides were eluted in a linear alcohol gradient which reached 30% in 4 litres. Fractions (25 ml) were assayed for reducing sugar using a Technicon Autoanalyser and monitored by TLC on 0.25 mm layers of Silica gel G developed in *n*-propanol: water 70:30 (v/v). Saccharides were detected by spraying with a solution of 0.2 N potassium permanganate in 4 N sulphuric acid and warming gently for a few minutes until white spots appeared on a light brown background [13]. The peak containing predominantly disaccharide was concentrated to 10 ml and run on a 2.6 X 79 cm column of Sephadex LH 20 in water [14]. Those fractions which gave a single spot of chitobiose on TLC were taken to dryness. Yield 625 mg (3% from chitin) m.p. 204–206 $[\alpha]_D^{20}$ = +27.1 for a fresh solution in water.

2.3. *Enzyme assays*

N-acetyl-β-hexosaminidase was assayed by the method described by Dance et al. [6]. Chitobiase was assayed by incubating 100 μl of enzyme solution at 37°C with 100 μl 10 mM chitobiose in McIlvaine sodium phosphate citrate buffer pH 3.5. The reaction was stopped after a suitable incubation period lasting up to 2 hr by adding 100 μl 0.8 M potassium tetraborate pH 9.1 and boiling for 3 min in capped tubes. After cooling in ice water 3 ml *p*-dimethylaminobenzaldehyde reagent was added [15] and the colour allowed to develop at 37°C for 10 min before reading the extinction at 545 nm.

Substrate blanks were included with each assay and when crude extracts were used enzyme blanks were included in which the enzyme was added after the addition of tetraborate to the buffered substrate. When assays were performed on concentrated extracts, denatured protein was removed by centrifugation before reading the extinction.

2.4. *Column chromatography*

Chromatography on DEAE-cellulose was performed on 0.9 X 25 cm columns in 0.01 M sodium phosphate buffer pH 7 [16]. The sample applied to the column was 10 ml of a 20% liver extract prepared in the same buffer. Samples for gel permeation chromatography were prepared from 20% aqueous homogenates of liver which had been centrifuged at 7500 *g* for 30 min at 4°C. Ultrafiltration [17] of 50 ml supernatant against 0.01 M sodium citrate pH 4.5 reduced the volume to 5 ml and undissolved protein was removed by centrifugation at 12 000 *g* for 30 min at 4°C. Concentrated extract (3 ml) was run on a 2.6 X 35 cm column of Sephadex G100 equilibrated in 0.01 M sodium citrate pH 4.5.

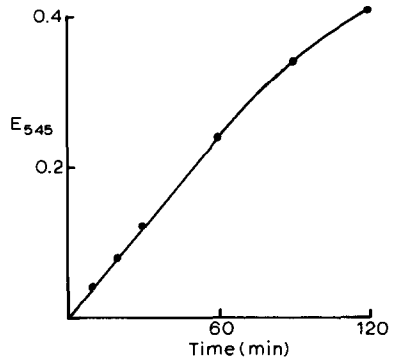

Fig. 1. The effect of incubation time at 37°C on the release of *N*-acetylglucosamine from chitobiose by a human liver extract. Details of the assay are given in the Materials and methods section.

3. Results

Preliminary experiments on 20% aqueous extracts of human liver showed that the release of *N*-acetylglucosamine from chitobiose was linear with time for 1 hr, the rate decreasing between 1 and 2 hr, fig. 1. The substrate concentration (5 mM in the incubation mixture) was chosen to converse the substrate and although the enzyme was not saturated it was sufficient to give readily detectable activities. The rate of product formation was proportional to the dilution of a 20% extract over a four-fold range. The pH activity curve in phosphate citrate buffer had a broad peak with a maximum at pH 3.5. Chitobiose and *N*-acetyl-β-hexosaminidase were assayed in five samples of normal liver. The results, table 1, show

Table 1
Chitobiase and *N*-acetyl-β-hexosaminidase activities in extracts of human liver.

Sample	Chitobiase nmoles/min/g wet wt.	Hexosaminidase nmoles/min/g wet wt.
1	39.0	120
2	35.5	112
3	34.5	92
4	24.5	42
5	32.3	82
Mean	33.1	89.6

that the synthetic substrate was hydrolysed two to three times as rapidly as chitobiose. The ratio of the two activities varies by a factor of two in the samples assayed.

Chromatography of human liver extracts on DEAE-cellulose gave two characteristic peaks of approximately equal hexosaminidase activity. The B form was unretained under these conditions and was eluted as a double peak with the breakthrough protein. Form A was then eluted in a linear salt gradient, fig. 2. Chitobiose was eluted with the breakthrough protein as a sharp peak with a shoulder and then as a low spread activity in the salt gradient-dropping to zero along with the tail of hexosaminidase A.

Gel permeation chromatography of a liver extract on Sephadex G 100 in 0.01 M sodium citrate buffer pH 4.5 gave a single peak of hexosaminidase activity which appeared just behind the blue dextran used to indicate the void volume, fig. 3. The elution profile for chitobiose was more complex and possibly arises from partially resolved molecular weight variants of enzymes with activity towards the disaccharide. A rather flat peak comprising about a quarter of the total chitobiase activity had the same distribution as hexosaminidase. A small shoulder of chitobiase was eluted on the tail of the hexosaminidase and preceded the main chitobiase peak which was without detectable activity towards the synthetic substrate.

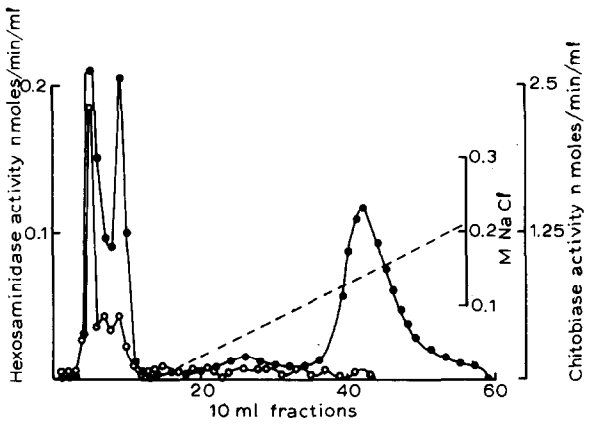

Fig. 2. The elution profile of *N*-acetyl-β-hexosaminidase (●-●-●) and chitobiase (o-o-o) from DEAE-cellulose at pH 7.0 10 ml 20% liver extract were applied to a 0.9 X 25 cm column of ion exchanger in 0.01 M sodium phosphate buffer pH 7.0. After elution of the unretained protein a linear sodium chloride gradient was used to elute hexosaminidase A.

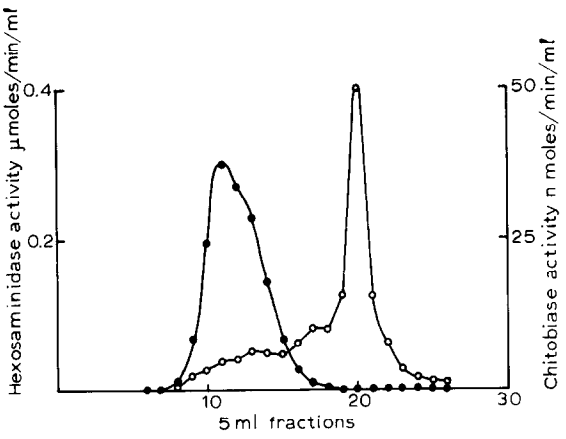

Fig. 3. Separation of *N*-acetyl-β-hexosaminidase (●-●-●) and chitobiase (o-o-o) by gel permeation chromatography of a liver extract on Sephadex G 100. The column was eluted with 0.01 M sodium citrate buffer pH 4.5

An estimate of molecular weight for chitobiase made by comparison with the elution of markers of known molecular weight from the same column gave values from around 25 000 in 0.01 M sodium citrate pH 4.5 to 50 000 in sodium phosphate pH 7.0

4. Discussion

Human liver extracts hydrolyse chitobiose and 4MU *N*-acetyl-β-glucosaminide at comparable rates although neither was assayed under conditions that would give maximum velocity. About a quarter of the chitobiase activity is associated with hexosaminidase, mol. wt. 130 000 [3] and the remainder is due to an enzyme with mol. wt. 25 000 to 50 000.

The most interesting feature of the elution from Sephadex G 100 is the presence of the chitobiase peak which is completely lacking hexosaminidase activity. The existence of this enzyme, which has not previously been detected, could not have been anticipated on the basis of assays performed with the synthetic substrate.

Most of the chitobiase is unretained by DEAE-cellulose at pH 7.0 and is eluted along with hexosaminidase B. However, the ratio of the two activities varies considerably across the profile and it seems likely that the chitobiase activity represents the low molecular weight enzyme and activity contributed by hexosaminidase B. Due to the instability of hexosaminidase A under the conditions of the chitobiose assay, 1 hr at pH 3.5, it is not possible to assess what proportion of the activity can be ascribed to the A and B forms of hexosaminidase.

Enzymes which are capable of binding and hydrolysing *N*-acetyl-β-hexosaminides other than the aryl derivatives might have a sufficiently broad specificity to permit the hydrolysis of chitobiose. Lysozyme may be discounted since it has very low activity towards chitobiose in the absence of the chitin tetrasaccharide [18] and moreover it has a molecular weight of about 15 000 [19]. Hyaluronidase behaves quite differently from chitobiase on gel permeation and ion exchange chromatography since it is excluded from Sephadex G 100 [20] and is retained by DEAE-cellulose at pH 6.4 [21].

It seems possible that the enzyme described here as chitobiase might hydrolyse *N*-acetyl-β-hexosaminidase linkages involving other sugars such as those found in oligosaccharides formed during the degradation of glycosaminoglycans. Although the evidence for the hydrolysis of such oligosaccharides by highly purified hexosaminidase is persuasive [2] it does not preclude the existence of a specific oligosaccharase lacking activity towards synthetic substrates. Indeed the differential loss of oligosaccharase and hexosaminidase observed on freeze drying an enzyme preparation from beef liver [2] might be taken as evidence for them being at least partially separate.

The detection of a low molecular weight enzyme active towards chitobiose but undetected by 4MU *N*-acetyl-β-hexosaminide emphasises the need to use natural substrates if the enzymes involved in the hydrolysis of *N*-acetyl hexosamine-containing carbohydrates are to be unequivocally identified.

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References

[1] Walker, P.G. (1966) in: the Amino Sugars (Balazs, E.A. and Jeanloz, R.W., eds.) Vol. IIB, 155–169, Academic Press, New York and London.
[2] Weissmann, B., Hadjioannou, S. and Tornheim, J. (1964) J. Biol. Chem. 239, 59–63.
[3] Sandhoff, K. and Wassle, W. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1119–1133.
[4] Wenger, D.A., Okada, S. and O'Brien, J. (1972) Arch Biochem. Biophys. 153, 116–129.
[5] Robinson, D. and Stirling, J.L. (1968) Biochem. J. 107, 321–327.
[6] Dance, N., Price, R.G., Robinson, D. and Stirling, J.L. (1969) Clin. Chim. Acta 24, 189–197.
[7] Okada, S. and O'Brien, J.S. (1969) Science 165, 698–700.
[8] O'Brien, J.S. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1720–1722.
[9] Sandhoff, K. (1968) Life Sci. 7, 283–288.
[10] O'Brien, J.S., Okada, S., Ho, M.W., Fillerup, D.L. and Veath, M.L., Federation Proc. 30, 956–969.
[11] Applegarth, D.A. and Bozoian, G. (1972) Clin. Chim. Acta 39, 269–271.
[12] Rupley, J.A. (1964) Biochim. Biophys. Acta 83, 245–255.
[13] Powning, R.E. and Irzykiewicz, H. (1967) J. Chromatog. 29, 115–119.
[14] Capon, B. and Foster, R.L. (1970) J. Chem. Soc. Sect. C. 1654–1655.
[15] Reissig, J.C., Strominger, J.L. and Leloir, L.F. (1955) J. Biol. Chem. 217, 959–966.
[16] Stirling, J.L. (1972) Biochim. Biophys. Acta 271, 154–162.
[17] Robinson, D., Price, R.G. and Dance, N.E. (1967) Biochem. J. 102, 525–532.
[18] Kravchenko, N.A. (1967) Proc. Roy. Soc. B. 167, 429–430.
[19] Canfield, R.E., Kammerman, S., Sobel, J.H. and Morgan, F.J. (1971) Nature New Biol. 232, 16–17.
[20] Aronson, N.N. and Davidson, E.A. (1967) J. Biol. Chem. 242, 437–440.
[21] Bowness, J.M. and Tan, Y.H. (1968) Biochim. Biophys. Acta 151, 288–290.