ACTIVITY OF *N*-ACETYL-β-D-HEXOSAMINIDASES A AND B FROM HUMAN LIVER TOWARDS VARIOUS OLIGOSACCHARIDES AND A GLYCOPEPTIDE CORE DERIVED FROM GLYCOPROTEINS

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Received 26 September 1977

1. Introduction

Despite the current intensive study of human *N*-acetyl-β-hexosaminidases A and B, there is still much to be learned about their substrate specificities and natural functions. The absence of hexosaminidase A in Tay Sachs disease [1] and of both hexosaminidase A and B in Sandhoff disease [2] result in massive fatal accumulation of gangliosides and these findings have stimulated interest in the role of hexosaminidases in glycolipid metabolism. Experiments performed in vitro have shown that hexosaminidases A and B will hydrolyse asialo-GM_2 [3] and it appears that only hexosaminidase A will hydrolyse GM_2, although the rate of hydrolysis of this substrate is extremely low [4].

There is also evidence for the involvement of hexosaminidases in glycosaminoglycan metabolism. A heptasaccharide from chondroitin sulphate is hydrolysed by normal fibroblast extracts, but not by an extract of fibroblasts from a patient with Tay Sachs disease [5]. Storage of glycosaminoglycans in fibroblasts from a patient with Sandhoff disease can be corrected by the addition of exogenous hexosaminidase from normal fibroblasts to the cells in culture [6].

N-Acetyl-β-hexosaminidases may also be involved in glycoprotein metabolism and the presence of oligosaccharides containing mannose and *N*-acetylglucosamine in the urine of patients with Sandhoff disease [7] gives support to this view. However, there has been no direct demonstration that *N*-acetyl-β-hexosaminidases A and B will hydrolyse glycoproteins or their degradation products.

We have purified hexosaminidases A and B from human liver and tested their ability to hydrolyse terminal β-GlcNac from a variety of oligosaccharides derived from acetolysis of ovomucoid and from an asialo-agalacto-glycopeptide of human serotransferrin. In this paper, we assess the role of these enzymes in the degradation of glycoproteins.

2. Materials and methods

2.1. *Materials*

Human liver was obtained post-mortem and stored at −15°C until required. Sephadex G-75, Con-A Sepharose and CNBr-activated Sepharose 4B were purchased from Pharmacia, London W5, DEAE-cellulose and CM-cellulose were obtained from Whatman, Maidstone, Kent. 4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (4MUGlcNac) was supplied by Koch Light, Colnbrook, Bucks. *N,N'*-Diacetyl-chitobiose was a gift from l'Industrie Biologique Française, Clichy. The disaccharide β-GlcNac (1 → 6)-Man was a gift from Dr J. Defaye, Grenoble. The other oligosaccharides were isolated after acetolysis [8] of a glycopeptide obtained from avian ovomucoid [9]. The asialo-agalacto-glycopeptide was

obtained after sequential digestion with neuraminidase and β-galactosidase of the glycopeptide isolated from human serotransferrin [10,11].

Buffer constituents and other reagents were of analytical grade and purchased from Fisons Scientific Apparatus, Loughborough, England.

2.2. *Purification of enzymes*

Human liver was homogenised in 10 mM sodium phosphate buffer, pH 6.8, and centrifuged at 12 000 × *g* for 30 min at 4°C. Unless otherwise specified, 10 mM sodium phosphate buffer, pH 6.8, was used throughout the purification. The supernatant was brought to 60% saturation by adding solid $(NH_4)_2SO_4$ and was stirred at 4°C overnight. The resulting precipitate was centrifuged as before and redissolved in a minimum volume of buffer. The solution was dialysed against several changes of buffer until sulphate could no longer be detected in the diffusate. Con-A Sepharose (40 ml) was washed with 400 ml buffer containing 0.5 M NaCl, and then gently stirred with the dialysed liver extract for 1 h at 4°C. The gel was packed into a 50 ml syringe barrel fitted with a porous plastic sinter and was washed with buffer containing 0.5 M NaCl until the E_{280} was almost zero. Adsorbed glycoproteins were eluted with 0.25 M α-methyl mannoside in buffer. The eluate was concentrated against buffer by vacuum dialysis at 4°C.

The concentrated eluate was applied to a column of Sephadex G-75 (80 × 5 cm) and eluted under gravity with buffer at 4°C. Fractions containing hexosaminidase were pooled and concentrated against buffer by vacuum dialysis at 4°C.

Concentrated eluate from the gel filtration step was applied to a column of DEAE-cellulose (30 × 2.5 cm) equilibrated in buffer at 4°C. The column was washed with the buffer until the E_{280} was almost zero and then activity retained by the column (*N*-acetyl-β-hexosaminidase A) was eluted with a linear salt gradient of 0–0.4 M NaCl in 800 ml of buffer.

N-Acetyl-β-hexosaminidase B, which is unretained on DEAE-cellulose under these conditions, was dialysed against 10 mM sodium citrate buffer, pH 4.4, and applied to a column of CM-cellulose (30 × 2.5 cm) equilibrated in this buffer. *N*-Acetyl-β-hexosaminidase B was eluted from the CM cellulose column with a linear salt gradient of 0–0.4 M NaCl in 800 ml of 10 mM sodium citrate buffer, pH 4.4.

N-Acetyl-β-hexosaminidases A and B were concentrated by vacuum dialysis against 10 mM sodium phosphate buffer, pH 6.0, in preparation for affinity chromatography. Affinity chromatography was carried out as described [12]. Before use with natural substrates, the activities of the purified enzymes were adjusted to 3.2 μmol/min/ml for hexosaminidase A and 2.9 μmol/min/ml for hexosaminidase B.

2.3. *Enzyme assays*

N-Acetyl-β-hexosaminidase was assayed fluorimetrically as described [13]. When oligosaccharides were used as substrates, enzyme (20 μl) was incubated with 80 μl oligosaccharide in phosphate-citrate buffer, pH 4.5. The concentration of substrate in the incubation mixture was 1 mM. Incubations were carried out at 37°C for up to 1 h.

For gas–liquid chromatographic (GLC) analysis the reaction was stopped by placing the tubes in a boiling water bath for three minutes.

2.4. *Sugar estimation*

Colorimetric estimation of free *N*-acetylglucosamine was by the method [14].

Free *N*-acetylglucosamine and mannose were also estimated by GLC analysis after removing the buffer constituents by ion-exchange chromatography. Samples corresponding to 220 μg total *N*-acetylglucosamine and 30 μg mesoinositol as an internal standard were placed in a test tube, lyophilised and dried over phosphorous pentoxide in a vacuum desiccator. The trimethylsilyl ethers were obtained by dissolving the dried sugars in a trimethylsilylating agent (hexamethyldisilazane/chlorotrimethylsilane/pyridine, 4 : 2 : 5, v/v/v) purchased from Sigma (St Louis, MO 63178, USA). Separations were carried out with glass columns (0.3 × 180 cm) containing 3% QF-1 on Chromosorb W-HMDS (100–200 mesh) in a gas chromatograph (Varian-Aerograph gas chromatograph, Model 2100). The chromatograph was temperature-programmed from 120–240°C at 2°C/min with a flow rate of nitrogen carrier gas of 20 ml/min. Characterisation of the disaccharides β-GlcNAc-(1 → 2)-Man; β-GlcNAc-(1 → 4)-Man and α-Man-(1 → 3)-Man was carried out by GLC according to the procedure [15].

3. Results

Suitable conditions for the enzymatic hydrolysis of the oligosaccharides were established using the colorimetric method to detect free *N*-acetylglucosamine and rates of hydrolysis were calculated as μmol GlcNAc released/min. The nature of the digestion products was determined by GLC analysis and the percentage of each substrate hydrolysed in 30 min was calculated. In table 1, the rates of hydrolysis of the oligosaccharides with a single terminal GlcNAc are given for comparison with the percentage hydrolysis calculated from the GLC results.

3.1. *Hydrolysis of oligosaccharides with a single terminal* N-*acetylglucosamine*

The configuration of the β-glycosidic bond linking GlcNAc to mannose (β-1,2 or β-1,4) has a marked effect on its susceptibility to hydrolysis by both *N*-acetyl-β-hexosaminidases A and B (table 1). The rate of hydrolysis of the β-1,2 linked disaccharide by *N*-acetyl-β-hexosaminidase B was seven times that of the β-1,4-linked disaccharide. In contrast, the corresponding trisaccharides, β-GlcNAc-(1→2)-α-Man-(1→3)-Man and β-GlcNAc-(1→4)-α-Man-(1→3)-Man, were both hydrolysed at about the same rate. The presence of the second mannose had almost no effect on the rate of hydrolysis of the β-1,4-linked GlcNAc, but reduced the rate of hydrolysis of the β-1,2-linked GlcNAc.

Using a different batch of *N*-acetyl-β-hexosaminidases A and B, prepared in an identical fashion, the ratio of the activities of the two enzymes towards β-GlcNAc-(1→4)-Man and β-GlcNAc-(1→2)-Man were very similar to those shown in table 1. On this occasion, the disaccharide β-GlcNAc-(1→6)-Man was subjected to hydrolysis by the enzymes. *N*-Acetyl-β-hexosaminidase A was 2.2 times and hexosaminidase B 1.8 times more active towards this substrate than they were towards β-GlcNAc-(1→2)-Man.

The influence of the subterminal sugar on the rate of hydrolysis of β-1,4-linked GlcNAc was assessed by incubating hexosaminidases A and B with β-GlcNAc-(1→4)-GlcNAc. The rates of hydrolysis of this and β-GlcNAc-(1→4)-Man were about the same.

3.2. *Hydrolysis of oligosaccharides with two terminal* N-*acetylglucosamines*

Branching of the oligosaccharide chains of ovomucoid occurs through substitution of a mannosyl group at positions 2 and 4. We tested the susceptibility of such branched oligosaccharides to hydrolysis by hexosaminidases A and B. There was no clear preference for the hydrolysis of β-1,2 or β-1,4 links in oligosaccharide E as both β-GlcNAc-(1→2)-Man and β-GlcNAc-(1→4)-Man were detected in the hydrolysate. Since the ratio of GlcNAc to Man released is 2:1, it appears that both β-1,2 and β-1,4 bonds are hydrolysed to the same extent (table 2).

When the enzymes were presented with a similar

Table 1
Hydrolysis of oligosaccharides with a single terminal GlcNAc by human *N*-acetyl-β-hexosaminidases A and B

Oligosaccharide	% Hydrolysis in 30 min		Enzyme (nmol/min/ml)	
	A	B	A	B
β-GlcNAc-(1→2)–Man	48	59	51	57
β-GlcNAc-(1→4)–Man	14	8	23	19
β-GlcNAc-(1→2)–α-Man-(1→3)–Man	7	10	15	15
β-GlcNAc-(1→4)–α-Man-(1→3)–Man	10	27	–	–
β-GlcNAc-(1→4)–GlcNAc	13	–	21	19

The rate of hydrolysis was determined by colorimetric estimation of GlcNAc released and the % hydrolysis which occurred was calculated from GLC analysis of the reaction products as described in the methods section

Table 2
Hydrolysis of oligosaccharides with two terminal *N*-acetylglucosamines by human hexosaminidases A and B

Oligosaccharide	Hexosaminidase A		Hexosaminidase B	
	% GlcNAc	% Man	% GlcNAc	% Man
E: β-GlcNAc-(1→2)↘ Man ↗ β-GlcNAc-(1→4)	25	13	26	10
K: β-GlcNAc-(1→2)↘ α-Man-(1→3)–Man ↗ β-GlcNAc-(1→4)	16	0	16	0
J: β-GlcNAc-(1→2)–α-Man-(1→3)↘ Man ↗ β-GlcNAc-(1→4)	10	0	12	0

The hydrolysis products released from branched oligosaccharides derived from ovomucoid by hexosaminidases A and B were identified by GLC analysis. Results are expressed as the percentage of *N*-acetylglucosamine and mannose released in 30 min

oligosaccharide (K) in which the substituted mannose was linked α-1,3 to mannose the extent of hydrolysis was reduced to 30%. Both β-1,2 and β-1,4 linked GlcNAc were removed since α-Man-(1→3)-Man was detected in the hydrolysates. The extent of hydrolysis of substrate J was similar to that of substrate K and α-Man-(1→3)-Man was again detected in the hydrolysate.

3.3. *Hydrolysis of a serotransferrin glycopeptide*

To assess the activity of hexosaminidase towards a glycopeptide, we incubated hexosaminidases A and B with an asialo-agalacto-glycopeptide fragment derived from serotransferrin and having the structure [11]:

β-GlcNAc-(1→2)–α-Man-(1→3)↘
β-Man-(1→4)–β-GlcNAc-(1→4)–β-GlcNAc–Asn–Lys
β-GlcNAc-(1→2)–α-Man-(1→6)↗

Hexosaminidases A and B hydrolysed this substrate, both releasing 4% of the terminal GlcNAc in 3 h.

4. Discussion

It is generally assumed that *N*-acetyl-β-hexosaminidases are involved in the catabolism of glycoproteins although this has not been tested directly on glycoprotein fragments likely to occur during degradation. Our results show that *N*-acetyl-β-hexosaminidases A and B are both active towards a variety of oligosaccharides that occur in glycoproteins and to a glycopeptide from serotransferrin. Of the substrates tested β-GlcNAc-(1→6)-Man was hydrolysed at the greatest rate. The relatively high activity that both enzymes had towards the substrate β-GlcNAc-(1→2)-Man was particularly notable since this is a characteristic linkage in glycoproteins [16]. However, this preference was not observed when trisaccharides with terminal β-1,2 and β-1,4-linked GlcNAc were used as substrates. It is possible that the proximity of the reactive hemiacetal group of mannose in the β-1,2-linked disaccharide may be a factor contributing to the high rate of hydrolysis. With the β-1,4-linked disaccharides the nature of the sub-terminal sugar did

not seem to be an important factor in the specificity of the enzymes, β-GlcNAc-(1→4)-Man and β-GlcNAc-(1→4)-GlcNAc being hydrolysed at about the same rate.

Both hexosaminidases A and B were less active towards branched oligosaccharides. The relatively high rate of hydrolysis of oligosaccharide E compared with K and J may again be influenced by the proximity of the hemiacetal group of mannose. In oligosaccharide E there is no preferential hydrolysis of either of the GlcNAc residues. We are unable as yet to assess whether this is so for oligosaccharides K and J, because we have not been able to identify with certainty which of the two trisaccharides remain after removal of a single GlcNAc residue. That oligosaccharides J and K are hydrolysed at similar rates suggests that the close proximity of the GlcNAc residues does not hinder hydrolysis.

N-Acetyl-β-hexosaminidases A and B have an almost identical pattern of activity towards the oligosaccharides tested and we may infer from this that the active sites of hexosaminidases A and B are very similar. There is convincing evidence for the existence of α-subunits unique to hexosaminidase A [17] but from our results the presence of these α-subunits does not seem to modify the substrate specificity of hexosaminidase A. It is possible of course that the presence of the α-subunits enhances the activity of the β-subunit towards both artificial and natural substrates without affecting its specificity and kinetic parameters. The question of whether the α-subunit itself possesses catalytic activity has not so far been satisfactorily resolved.

From our results it would seem that *N*-acetyl-β-hexosaminidases A and B could both be involved in the hydrolysis of oligosaccharides that arise during glycoprotein catabolism and also in the hydrolysis of glycopeptides with appropriate terminal sugars. We did not attempt to optimise the rates of hydrolysis of the substrates used in these experiments and the low rates of hydrolysis of the larger fragments do not necessarily reflect the rates to be expected in vivo. Locally high substrate concentrations or the presence of activation factors may increase the rate of hydrolysis.

Although *N*-acetyl-β-hexosaminidase A hydrolysed all the substrates that we tested, it does not seem to be necessary for the normal turnover of glycoproteins in vivo since products of glycoprotein catabolism do not accumulate in patients with Tay Sachs disease [7].

However, loss of both *N*-acetyl-β-hexosaminidases A and B in Sandhoff's disease results in urinary excretion [7,18] and liver accumulation [19] of oligosaccharides from glycoproteins. In contrast, products of glycosaminoglycan catabolism are not accumulated in either of these diseases [7] and may be degraded by enzymes other than *N*-acetyl-β-hexosaminidases A and B.

Acknowledgements

We thank Mr Y. Leroy for his skilful technical assistance. This investigation was supported in part by the Centre National de la Recherche Scientifique (Laboratoire Associé No. 217: Biologie physico-chimique et moléculaire des glucides libres et conjugués and RCP No. 425: Glucides et glycoconjugués). We are grateful to the MRC for a project grant and to the SRC for an European Short Term Fellowship to J.L.S.

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