Identification of β -N-acetylhexosaminidase A in mouse tissues with the fluorogenic substrate 4-methylumbelliferyl- β -N-acetylglucosamine 6-sulphate

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β -N-Acetylhexosaminidase from mouse tissue was separated into its constituent isoenzymes on DEAE-cellulose and its activity was monitored with 4-methylumbelliferyl- β -N-acetylglucosamine and 4-methylumbelliferyl- β -N-acetylglucosamine 6-sulphate. Forms corresponding to the human isoenzymes A (acidic), B (basic) and an 'intermediate' form were present in mouse liver and spleen, whereas in kidney the B and 'intermediate' forms predominated, with A present only as a minor component. In brain the 'intermediate', A and C activities were detected. Testis had predominantly A activity, whereas epididymis, the tissue with the highest specific activity of β -N-acetylhexosaminidase, had an abundance of the 'intermediate' form, but was almost entirely lacking in the A form.

INTRODUCTION

Human β -N-acetylhexosaminidases A and B have been studied intensively and their complete amino acid sequences have now been deduced from the deoxy-nucleotide sequences of the cloned cDNAs for the α - and β -subunits (Myerowitz *et al.*, 1985; O'Dowd *et al.*, 1985; Korneluk *et al.*, 1986). By contrast, there is comparatively little known about the subunit structures of the enzymes from other species. The pig epididymal β -N-acetylhexosaminidases are an exception to this, and here, although there are acidic (A) and basic (B) forms of the enzyme, both appear to have closely similar subunit compositions (Parkes *et al.*, 1984) and it is not really evident whether the M_r -70000 polypeptide corresponds to the α - or β -subunits observed in the human β -N-acetylhexosaminidases.

There is good evidence that β -N-acetylhexosaminidase, and a number of other glycosidase activities in rodent tissues, is regulated by testosterone (Conchie & Findlay, 1959), but, with the exception of β -glucuronidase (Palmer *et al.*, 1983), virtually nothing is known about the structures of the enzymes. Clearly, a description of the enzymology of β -N-acetylhexosaminidase in rodent tissues is desirable for analysis of the effects of testosterone at the molecular level, but there is nothing, apart from elution profiles of the enzyme from rat kidney (Robinson *et al.*, 1967), testis (Majumder *et al.*, 1975) and urine (Halman *et al.*, 1984) on ion-exchange columns, to suggest the presence of more than a single form of rodent β -N-acetylhexosaminidase. There is no evidence, direct or indirect, for the presence of two gene products in the structure of rodent β -N-acetylhexosaminidases.

Inbred mice are the mammals of choice for the study of gene regulation because they offer the potent combination of classical and molecular-genetic approaches to the analysis of gene expression. Strain differences have been observed in the total activity of mouse spermatozoal β -

N-acetylhexosaminidase (Self *et al.*, 1978), but nothing is known of the constituent isoenzymes. The presence of the α -subunit of β -N-acetylhexosaminidase in humans confers an ability to hydrolyse the ganglioside G_{M2} and also the synthetic substrates *p*-nitrophenyl- β -N-acetylglucosamine 6-sulphate and 4-methylumbelliferyl- β -N-acetylglucosamine 6-sulphate (4-MeUmbGlcNAc-6-SO₄) (Fuchs *et al.*, 1983; Inui & Wenger, 1984; Ben-Yoseph *et al.*, 1985; Kytzia & Sandhoff, 1985). Hydrolysis of the latter gives a simple and sensitive assay for the presence of α -subunits in intermediate forms of human hexosaminidase (Beccari *et al.*, 1987) and in principle may reveal the presence of active α -subunits in β -N-acetylhexosaminidases of other species. In the present paper we report on the separation of β -N-acetylhexosaminidase isoenzymes from mouse tissues by ion-exchange chromatography on DEAE-cellulose and the identification of a form of the enzyme corresponding to human β -N-acetylhexosaminidase A based on its ability to hydrolyse 4-MeUmbGlcNAc-6-SO₄.

EXPERIMENTAL

Materials

4-Methylumbelliferyl β -N-acetylglucosaminide (4-MeUmbGlcNAc) and an impure preparation of 4-MeUmbGlcNAc-6-SO₄ were purchased from Koch-Light Laboratories, Haverhill, Suffolk, U.K. The cation-exchanger DEAE-cellulose DE-52 was from Whatman, Maidstone, Kent, U.K. Dowex 50 (X2; 400 mesh) was supplied by Sigma Chemical Co, Poole, Dorset, U.K. All other reagents were of analytical grade.

Methods

Purification of 4-MeUmbGlcNAc-6-SO₄. 4-MeUmbGlcNAc-6-SO₄ was purified from an impure preparation of monosulphated MeUmbGlcNAc as described by Beccari *et al.* (1987).

Abbreviations used: MeUmb, methylumbelliferyl; GlcNAc, β -N-acetylglucosamine; SO₄, sulphate.

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Table 1. Specific activities of β -N-acetylhexosaminidase in mouse tissues using 4-methylumbelliferyl β -N-acetylglucosaminide and 4-methylumbelliferyl β -N-acetylglucosamine-6-sulphate as substrates

Tissue (n)	10 ⁴ × Specific activity (± S.D.) (μmol/mg of protein)		
	4-MeUmb-GlcNAc (a)	4-MeUmb-GlcNAc-6-SO ₄ (b)	(b)/(a)
Liver (3)*	7.0–12.0	1.6–2.9	0.24
Spleen (6)	54.0 ± 10.0	11.8 ± 1.7	0.22
Kidney (6)	34.8 ± 5.6	2.3 ± 0.33	0.07
Brain (6)	123 ± 9.3	30.0 ± 2.6	0.24
Testis (6)	11.9 ± 1.5	3.0 ± 0.4	0.25
Epididymis (6)	241 ± 59	7.0 ± 4.7	0.03

* Values for liver are the range of activities.

Assay of β -N-acetylhexosaminidase activity. Enzyme activity was determined by using the two fluorogenic substrates 4-MeUmbGlcNAc and 4-MeUmbGlcNAc-6-SO₄, as described previously (Beccari *et al.*, 1987). One unit of activity is the amount of enzyme that hydrolyses 1 μmol of substrate/min under the conditions described above.

Enzyme preparations. Mouse tissues (BALB/c) were removed from freshly killed animals and stored at –20 °C until required. Extracts (10%, w/v) were prepared by homogenizing tissues in 10 mM-sodium phosphate buffer, pH 6.0. The supernatant recovered after centrifugation at 8000 rev./min (r_{av} , 10 cm) for 20 min at 4 °C in a Sorvall RC-5B centrifuge was dialysed against 10 mM-sodium phosphate buffer, pH 6.0.

Ion-exchange chromatography. A sample of each enzyme preparation (2.0 ml) was loaded on to a column of DE-52 (4 ml) equilibrated with 10 mM-sodium phosphate buffer, pH 6.0. Unretained protein was eluted with the column buffer and then a linear gradient of NaCl (0–0.5 M in 60 ml of column buffer) was applied. Finally the column was eluted with 1.0 M-NaCl in the same buffer. Protein in the eluates was monitored at 280 nm.

RESULTS AND DISCUSSION

All the mouse tissues we surveyed were able to hydrolyse both 4-MeUmbGlcNAc and 4-MeUmbGlcNAc-6-SO₄ (Table 1), although there was an 8-fold difference in the ratios of the two activities in these tissues. Elution profiles of β -N-acetylhexosaminidase from mouse tissues on DEAE-cellulose DE-52 revealed a complex, but reproducible, pattern of peaks when assayed with 4-MeUmbGlcNAc (Fig. 1). The profiles from spleen and liver were the most closely similar. Both had a proportion of activity that was unretained by the column and in both the greater part of the activity was eluted in the salt gradient. This separation of the total activity into a relatively basic fraction (B) and more acidic components (A) is similar to that presented by the

Fig. 1. Separation of liver and spleen β -N-acetylhexosaminidases on DEAE-cellulose

Extracts of (a) spleen (containing 20 mg of protein) and (b) liver (400 mg of protein) were applied to DEAE-cellulose columns (4 ml) equilibrated with 10 mM-sodium phosphate buffer, pH 6.0, and eluted with the same buffer until the peak of unretained protein had been collected. Protein retained by the column was eluted with a linear gradient of NaCl (-----). At the end of the gradient the column was eluted with 0.5 M-NaCl (10 ml) and then 1.0 M-NaCl (15 ml). Fractions (2 ml) were assayed for activity towards 4-MeUmbGlcNAc (●) and 4-MeUmbGlcNAc-6-SO₄ (○) as described in the Experimental section.

human β -N-acetylhexosaminidases A and B. As we have already noted with pig epididymal β -N-acetylhexosaminidases (Parkes *et al.* 1984), that superficial similarity does not necessarily mean that they have an arrangement of subunits corresponding to those of the human enzymes. Of the tissues we surveyed here, three, spleen and liver (Fig. 1) and kidney (Fig. 2a), had an appreciable proportion of the total activity that was unretained by the column, whereas this fraction was almost entirely lacking in brain (Fig. 2b), testis and epididymis (Fig. 3). The appearance of β -N-acetylhexosaminidase in unretained fractions might result from overloading of the column with protein, but we are satisfied that this is not so. Samples (eight each) of liver, kidney and spleen containing 20, 6 and 4 mg of protein respectively were run on DEAE-cellulose columns under conditions identical with those described in the Experimental section, and they showed results closely comparable with those shown in Figs. 1(a), 1(b) and 2(a). None of the unretained peaks were active towards 4-

Fig. 2. Separation of kidney and brain β -N-acetylhexosaminidases on DEAE-cellulose

Extracts of (a) kidney (containing 40 mg of protein) and (b) brain (10 mg of protein) were separated as described in the legend to Fig. 1. Fractions from the separation of brain β -N-acetylhexosaminidases were assayed for activity towards 4-MeUmbGlcNAc at pH 6.0 ($\blacktriangle$).

MeUmbGlcNAc-6-SO₄, which might have obtained if the columns had been overloaded.

With reference to the elution profiles of β -N-acetylhexosaminidase from spleen and liver (Fig. 1), we identified three partially resolved peaks of activity towards 4-MeUmbGlcNAc (labelled 1, 2 and 3) that were eluted in the salt gradient. In both these tissues peak 3 was the most abundant, peak 2 next and peak 1 the least abundant of the three. The activity towards 4-MeUmbGlcNAc-6-SO₄ in these tissues was eluted with the more acidic peaks of activity (2 and 3) detected with the non-sulphated substrate, and in liver there was an incipient separation into two forms. Kidney had the highest proportion of unretained activity, and the predominant form retained by the column was peak 1, followed in abundance by unresolved peaks 2 and 3. Activity towards 4-MeUmbGlcNAc-6-SO₄ was barely detectable in the eluate. In brain (Fig. 2b) the β -N-acetylhexosaminidase elution profile of the retained activity was relatively simple, with a predominant peak being eluted at a salt concentration similar to that of peak 1 of spleen and liver, and a shoulder corresponding to peak 2. Activity towards 4-MeUmbGlcNAc-6-SO₄ was eluted as a single sharp peak in the position of peak 2. β -N-Acetylglucosaminidase C is particularly abundant

Fig. 3. Separation of testicular and epididymal β -N-acetylhexosaminidases on DEAE-cellulose

Extracts of (a) testis (containing 120 mg of protein) and (b) epididymis (10 mg of protein) were separated as described in the legend to Fig. 1.

in brain (Hooghwinkel *et al.*, 1972) and may contribute to the activity profile at pH 4.5, even though its optimum is at more neutral pH values. When the eluate from brain was assayed at pH 6.0, there was a peak of activity, as expected, under peak 1, and another, with a higher activity at pH 6.0 than 4.5, which was eluted at a higher salt concentration. From its position in the elution profile and from the effect of pH on its activity, this latter peak is likely to be β -N-acetylglucosaminidase C.

There were considerable differences between the β -N-acetylhexosaminidases of testis and epididymis. Epididymis had a much higher specific activity than testis when 4-MeUmbGlcNAc was the substrate, but this difference was very much less apparent when 4-MeUmbGlcNAc-6-SO₄ was used. The predominant peak of β -N-acetylhexosaminidase in the extract of testis was peak 2, and this had activity towards both 4-MeUmbGlcNAc and 4-MeUmbGlcNAc-6-SO₄ (Fig. 3a). This peak was eluted in the same position as the less acidic form with activity towards 4-MeUmbGlcNAc-6-SO₄ that was observed in liver. The major form of β -N-acetylhexosaminidase in epididymis was eluted as a sharp peak of activity with a shoulder on the acidic side that eluted in position 2 (Fig. 3b). Very low activity was detected with 4-MeUmbGlcNAc-6-SO₄ and that was associated with the shoulder rather than the main peak. A notable feature of the elution profiles was the absence of activity towards 4-MeUmbGlcNAc-6-SO₄ associated with peak 1, which therefore resembles the 'intermediate' forms of human tissues (Beccari *et al.*, 1987).

We conclude, therefore, on the basis of their behaviour on DEAE-cellulose and their substrate specificities, that mouse tissues have β -N-acetylhexosaminidase isoenzymes analogous to the A, B and 'intermediate forms' seen in human tissues. Given the relatively close homology between murine and human genes, the most likely way that the various forms of mouse β -N-acetylhexosaminidases arise is through combinations of α - and β -gene products.

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