

## A Fluorimetric Enzyme Assay for the Diagnosis of Sanfilippo Disease type D (MPS IIID)

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**Summary:** 4-Methylumbelliferyl- $\alpha$ -*N*-acetylglucosamine 6-sulphate was synthesized and shown to be a substrate for the lysosomal *N*-acetylglucosamine-6-sulphate sulphatase (GlcNAc-6S sulphatase). Fibroblasts and leukocytes from 3 different Sanfilippo D patients showed < 1% of mean normal GlcNAc-6S sulphatase activity. The enzymatic liberation of the fluorochrome from 4-methylumbelliferyl- $\alpha$ -*N*-acetylglucosamine 6-sulphate requires the sequential action of the GlcNAc-6S sulphatase and  $\alpha$ -*N*-acetylglucosaminidase. A normal level of  $\alpha$ -*N*-acetylglucosaminidase activity was insufficient to complete the hydrolysis of the reaction intermediate 4-methylumbelliferyl- $\alpha$ -*N*-acetylglucosaminide formed by the GlcNAc-6S sulphatase. A second incubation in the presence of excess  $\alpha$ -*N*-acetylglucosaminidase is needed to avoid underestimation of the GlcNAc-6S sulphatase activity.

Sanfilippo disease (mucopolysaccharidosis type III, MPS III) comprises four different autosomal recessive subtypes (for review see Neufeld and Muenzer 1989) and is clinically characterized by severe central nervous system degeneration, but only mild somatic abnormalities. Owing to different defects in the degradation of heparan sulphate, the patients store excessive amounts of this mucopolysaccharide in various tissues.

MPS III type D (McKusick 252940) is caused by a deficiency of *N*-acetylglucosamine-6-sulphate sulphatase (GlcNAc-6S sulphatase (EC 3.1.6.14); Kresse et al 1980). So far 9 patients have been reported (Gatti et al 1982; Matalon et al 1982; Coppa et al 1983; Kaplan and Wolfe 1987; Siciliano et al 1991). GlcNAc-6S sulphatase is routinely assayed using radiolabelled oligosaccharides derived from heparan sulphate (Kresse et al 1980; Freeman and Hopwood 1992).

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In this paper we describe a novel, simple fluorimetric assay for GlcNAc-6S sulphatase, using 4-methylumbelliferyl- $\alpha$ -D-glucosaminide (MU- $\alpha$ GlcNAc-6S) and show its usefulness in the diagnosis of MPS IIID.

## MATERIALS AND METHODS

4-Methylumbelliferyl- $\alpha$ -D-N-acetylglucosamine 6-sulphate (MU- $\alpha$ GlcNAc-6S) was synthesized from MU- $\alpha$ GlcNAc, which was prepared as described (Voznyi 1991). To a solution of 0.31 g MU- $\alpha$ GlcNAc in 10 ml abs. pyridine, 0.46 g trityl chloride was added and the mixture was kept at room temperature for 72 h. The mixture was cooled to 0°C; benzoyl chloride (0.5 ml in 5 ml dry chloroform) was added and after 20 h at room temperature, chloroform was added to dilute. The reaction products were then washed with water and saturated NaHCO<sub>3</sub>, and concentrated. The residue was treated with trifluoroacetic acid (0.5 ml 90% in 5 ml chloroform) for 1 h. Evaporation of volatile components and purification by silica chromatography (Zsiska and Meyer 1991) yielded 3,4,-O-benzoylated MU- $\alpha$ GlcNAc (compound 2; 0.4 g, 83%);  $[\alpha]_D = +124^\circ$  ( $c = 0.7$ , chloroform). To a solution of compound 2 (0.12 g in 1.2 ml DMF), 0.22 g freshly prepared Et<sub>3</sub>NSO<sub>3</sub> complex was added. After 24 h at room temperature, the mixture was concentrated and purified by silica chromatography (Zsiska and Meyer 1991). This yielded the Et<sub>3</sub>N salt of 6-sulphated compound 2 (compound 3; 0.11 g, 70%);  $[\alpha]_D = +91^\circ$  ( $c = 0.5$ , chloroform), m.p. 220–224°C. To a solution of compound 3 (77 mg in 0.7 ml THF), 0.4 ml 1 mol/L NaOH was added and after 48 h stirring the mixture was neutralized with acetic acid, concentrated, applied to a silica column, eluted with toluene–ethanol–triethylamine (6:4:0.3) and crystallized from ethylacetate–ethyl ether to yield the Et<sub>3</sub>N salt of MU- $\alpha$ GlcNAc-6S ( $M_r$  559).

Total leukocytes were isolated from heparinized blood as described previously (van Diggelen et al 1990) and frozen until used. Skin fibroblasts were cultured according to routine procedures in Ham's F10 medium supplemented with 10% fetal bovine serum and antibiotics. The cells were harvested with trypsin 7 days after the last subculture and stored at –70°C. Fibroblasts from patients with Sanfilippo types A, B, C, Sanfilippo type D (84RD287, 84RD288 and 91RD415) or multiple sulphatase deficiency (91RD153) were obtained from the European Human Cell Bank, Rotterdam, the Netherlands (W.J. Kleijer). Leukocytes from a proven Sanfilippo D patient were kindly provided by Drs J.E.M. Groener and B.J.H.M. Poorthuis (Leiden).

For the standard N-acetylglucosamine-6-sulphate sulphatase (GlcNAc-6S sulphatase) assay, homogenates were prepared by sonication of cell material in 0.25% (w/v) Triton X-100 in water. Reaction mixtures consisted of 5  $\mu$ l homogenate (20  $\mu$ g protein for fibroblasts or 30  $\mu$ g for leukocytes) and 5  $\mu$ l 10 mmol/L MU- $\alpha$ GlcNAc-6S in 0.2 mol/L sodium acetate buffer, pH 5.6, containing 20 mmol/L lead acetate. MU- $\alpha$ GlcNAc-6S will become available from Moscerdam Substrates (for inquiries: O.P. van Diggelen, Rotterdam). The reaction mixtures were incubated for 17 h (fibroblasts) or 24 h (leukocytes) at 37°C, after which 20  $\mu$ l twice-concentrated McIlvain's phosphate–citrate buffer, pH 4.7, and 10  $\mu$ l (16  $\mu$ g protein) of a partially purified lysosomal enzyme preparation from bovine testis (Verheijen et al 1982) was added and a second incubation of 6 h at 37°C was carried out. For certain

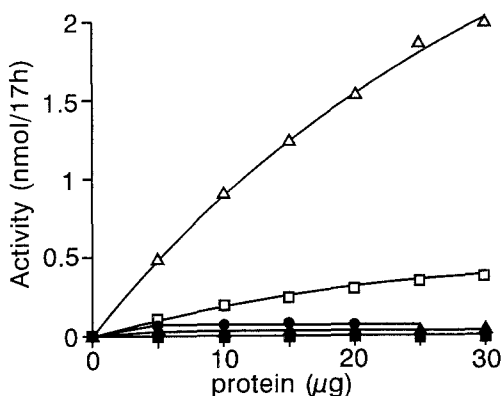
experiments homogenates were dialysed for 17 h against 0.9% NaCl. Reactions were terminated by the addition of 200  $\mu$ l 0.5 mol/L  $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ , pH 10.7, and the fluorescence of 4-methylumbelliferone (MU) was measured on a Fluoroskan (Titertek) fluorimeter.

Protein was determined as described previously (van Diggelen et al 1990).

## RESULTS

The potential of 4-methylumbelliferyl- $\alpha$ -D-N-acetylglucosamine 6-sulphate (MU- $\alpha$ GlcNAc-6S) as substrate for the GlcNAc-6S sulphatase was tested. GlcNAc-6S sulphatase activity was barely detectable in undialysed homogenates, and a very low but significant activity, which did not increase with increasing amounts of protein, could be detected in dialysed fibroblast homogenates (Figure 1). Addition of lead acetate to reaction mixtures containing undialysed homogenates resulted in much higher activities that increased almost linearly with the amount of protein (Figure 1, open symbols). Addition of lead acetate to dialysed homogenates resulted in activities similar to those obtained in undialysed homogenates (not shown).

Liberation of MU from MU-GlcNAc-6S by GlcNAc-6S sulphatase requires the sequential action of two enzymes: desulphation by GlcNAc-6S sulphatase followed by hydrolysis by  $\alpha$ -N-acetylglucosaminidase. To avoid the second step becoming rate limiting, a second incubation (6 h) in the presence of extra added  $\alpha$ -N-acetylglucosaminidase (22 nmol/6 h; specific activity: 234 nmol/h per mg protein) was carried out routinely. This was the minimum amount of  $\alpha$ -N-acetylglucosaminidase that could in 6 h quantitatively hydrolyse any amount of MU- $\alpha$ GlcNAc between 10 and 2000 pmol. This exogenous enzyme source, a glycoprotein fraction of bovine origin, also contained GlcNAc-6S sulphatase activity (11 nmol/6 h), however, this activity did not interfere since it could be inhibited completely by the high concentration of phosphate used in the standard assay (substrate blanks, also incubated with exogenous enzyme were only 30–40 pmol). Omission of the second



**Figure 1** Effect of lead acetate on GlcNAc-6S sulphatase activity in normal leukocyte (squares) and fibroblast homogenates (triangles). Open symbols, with lead acetate (10 mmol/L); solid symbols, without lead acetate. Reaction conditions as in standard assay except for amount of protein. Solid circles, dialysed fibroblast homogenate without lead acetate

step resulted in underestimation of GlcNAc-6S sulphatase in leukocytes by 98% and in fibroblast by 70–80%.

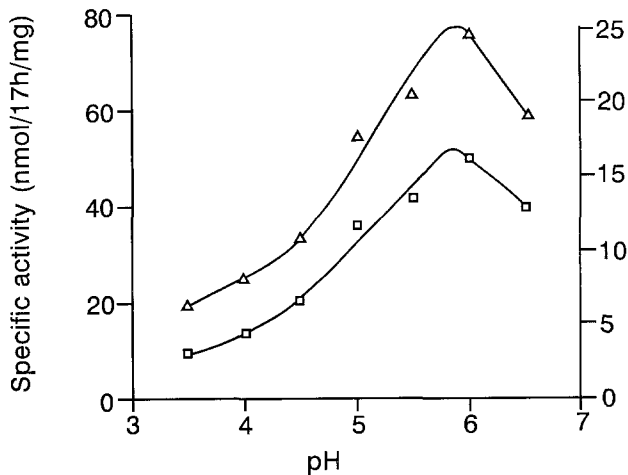
In the experiments shown below, the GlcNAc-6S sulphatase was assayed under standard conditions as described in Materials and Methods (except for the condition that was varied). The pH optimum of the GlcNAc-6S sulphatase from leukocytes and fibroblasts was pH 5.8 (Figure 2). We chose pH 5.6 for the standard assay because acetate buffer has insufficient buffering capacity above this pH. The apparent  $K_m$  was estimated to be 9 mmol/L for leukocytes and 6 mmol/L for fibroblasts (Figure 3). The sulphatase activity was approximately linear with time up to 24 h (Figure 4).

Under standard assay conditions the GlcNAc-6S sulphatase activities were determined in material from patients and controls. The three investigated patients, originating from the Netherlands and France, had no detectable GlcNAc-6S sulphatase activity in leukocytes or fibroblasts (Table 1). Fibroblasts from a patient with multiple sulphatase deficiency had 3% activity, whereas patients with Sanfilippo disease types A, B and C had normal GlcNAc-6S sulphatase activity (Table 1).

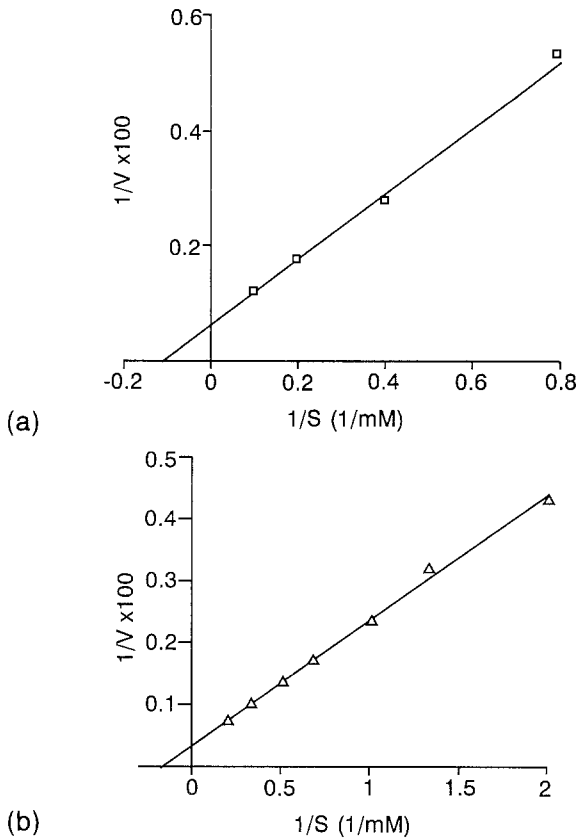
## DISCUSSION

We have defined conditions for the assay of GlcNAc-6S sulphatase using the fluorogenic substrate 5-methylumbelliferyl- $\alpha$ -D-N-acetylglucosamine 6-sulphate (MU- $\alpha$ GlcNAc-6S). Formation of the fluorochrome requires the sequential action of both GlcNAc-6S sulphatase and  $\alpha$ -N-acetylglucosaminidase. Underestimation of the sulphatase activity could be avoided by subjecting the reaction mixtures to a second incubation with excess  $\alpha$ -N-acetylglucosaminidase under condition where exogenous GlcNAc-6S sulphatase activity was inhibited completely by a high concentration of phosphate.

MU- $\alpha$ GlcNAc-6S was shown to be useful in the diagnosis of Sanfilippo type D disease. The residual activities in cells from the three MPS IIID patients we



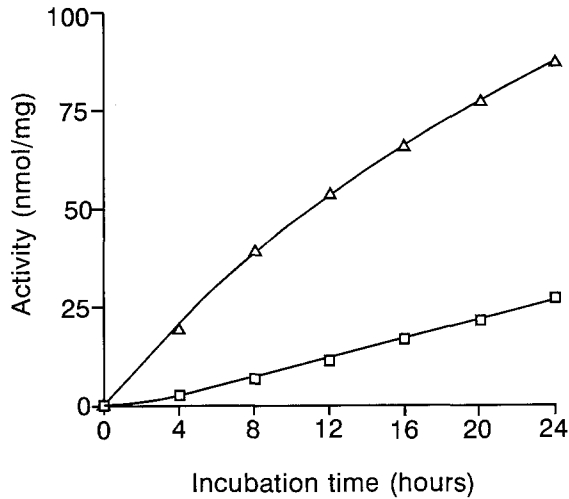
**Figure 2** pH dependence of GlcNAc-6S sulphatase activity in normal leukocytes ( $\square$ ; right scale) and fibroblasts ( $\Delta$ ; left scale). Reaction conditions as in standard assay, except for pH



**Figure 3** Lineweaver-Burk plot for GlcNAc-6S sulphatase activity in normal leukocytes (a) and fibroblasts (b). Reaction conditions as in standard assay, except for MU- $\alpha$ GlcNAc-6S concentration

investigated were  $< 1\%$  of the mean control activity, and the sulphatase activity in cells from a patient with multiple sulphatase deficiency was 3%. The conventional GlcNAc-6S sulphatase assay uses radiolabelled oligosaccharides, derived from heparan sulphate (Kresse et al 1980; Freeman and Hopwood 1992); this is still the most widely used assay. Nawakowski et al (1989) have described a GlcNAc-6S sulphatase assay with the monosaccharide GlcNAc-6S. This method requires separation of substrate and free GlcNAc and is based on the colorimetric determination of GlcNAc. Our fluorogenic assay is sensitive and specific and obviously more convenient than the existing assays.

In undialysed homogenates, the GlcNAc-6S sulphatase activity was barely detectable. This suggested the presence of inhibitors, possibly inorganic sulphates and phosphates, which are known powerful inhibitors of GlcNAc-6S sulphatase (Basner et al 1979). It was surprising that the activity in dialysed homogenates was still extremely low and reached a plateau with only 3–5  $\mu\text{g}$  protein, suggesting that inhibitors were still present in the reaction mixtures. Addition of lead acetate to the



**Figure 4** Time course of GlcNAc-6S sulphatase activity in normal leukocytes ( $\square$ ) and fibroblasts ( $\triangle$ ). Reaction conditions as in standard assay, except for incubation time

**Table 1** GlcNAc-6S sulphatase activity in leukocytes and fibroblasts

	<i>GlcNAc-6S sulphatase activity</i>	
	<i>Leukocytes</i> (nmol/24 h per mg)	<i>Fibroblasts</i> (nmol/17 h per mg)
Normal range (mean $\pm$ SD)	14–22 ( $n = 11$ ) (18 $\pm$ 3)	41–86 ( $n = 12$ ) (57 $\pm$ 14)
Sanfilippo D patient(s)	0.0 ( $n = 1$ )	0.0 ( $n = 3$ )
Multiple sulphatase deficiency		1.8
Sanfilippo A ( $n = 1$ )		60
Sanfilippo B ( $n = 1$ )		42
Sanfilippo C ( $n = 1$ )		52

reaction mixtures, which will precipitate traces of free sulphates and phosphates, increased the activities  $> 10$ -fold and also resulted in a nearly linear relationship between enzyme activity and amount of protein. We concluded that inorganic phosphates and sulphates are formed during the incubation and that these trace amounts must be removed.

Assays with MU-substrates requiring more than one enzyme to yield the fluorochrome are uncommon. This principle was successfully applied for the first time for galactose-6-sulphate sulphatase to allow the diagnosis of Morquio disease type A (van Diggelen et al 1990) and recently for acetyl-CoA: glucosaminide-*N*-acetyltransferase, which facilitated the diagnosis of Sanfilippo disease type C (Voznyi et al 1993). This approach could also result in replacement of other cumbersome radiochemical assays. Potential MU substrates for the diagnosis of MPS IIIA and MPS II are currently being synthesized.

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