J. Inher. Metab. Dis. 19 (1996) 278–285

A fluorimetric enzyme assay for the diagnosis of Sanfilippo disease type A (MPS IIIA)

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MS received 12.10.95 Accepted 5.12.95

Summary: 4-Methylumbelliferyl-α-D-*N*-sulphoglucosaminide (MU-α-GlcNS) was synthesized and shown to be a substrate for the lysosomal heparin sulphamidase. Sanfilippo A patients' fibroblasts ($n=42$) and lymphocytes ($n=1$) showed 0–3% of mean normal heparin sulphamidase activity; in total leukocytes from patients ($n=8$) sulphamidase activity was clearly deficient. In fibroblasts from obligate heterozygotes for Sanfilippo A, the sulphamidase activity was reduced in 9 out of 10 cases. Heparin sulphamidase desulphates MU-αGlcNS to MU-αGlcNH$_2$ and further hydrolysis during a second incubation is required to liberate 4-methylumbelliferone, which can be measured. Yeast α-glucosidase, which has low but sufficient α-glucosaminidase activity, was used to hydrolyse the reaction intermediate MU-αGlcNH$_2$ to release 4-methylumbelliferone and free glucosamine.

Sanfilippo disease (mucopolysaccharidosis type III, MPS III) comprises four different autosomal recessive subtypes (for review see Neufeld and Muenzer 1995) 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, patients store excessive amounts of this mucopolysaccharide in various tissues.

MPS III type A is caused by a deficiency of heparin sulphamidase (EC 3.10.1.1; Kresse et al 1973). Although the mucopolysaccharidoses are rare disorders, MPS IIIA is relatively common. Heparin sulphamidase is routinely assayed using [*N*-^{35}S-sulphonate]heparin (Kresse et al 1973). This assay has many pitfalls related to the instability and short half-life of the substrate and the poor relationship between amount of enzyme and activity. The use of [^{3}H]heparin-derived oligosaccharides has overcome most of these difficulties (Hopwood and Elliot 1981a,b, 1982).

In this paper we describe a novel, simple fluorimetric assay for heparin sulphamidase, using 4-methylumbelliferyl-α-D-*N*-sulphoglucosaminide, and show its usefulness in the diagnosis of MPS IIIA.

MATERIALS AND METHODS

4-Methylumbelliferyl-α-D-*N*-sulphoglucosaminide (MU-αGlcNS; 2-sulphamino-2-deoxy-D-glucopyranosyl α1-[4-methylumbelliferone]) has been synthesized by selective *N*-sulphation of the free amino group in 4-methylumbelliferyl-α-D-glucosaminide by an excess of triethylamine–sulphur trioxide complex in dimethylformamide–methanol solution. After purification of the resulting mixture, by passing through a cation exchange resin and substitution of triethylamino group to sodium, the product was lyophilized. The synthesis of starting material with free amino group was described earlier (Voznyi et al 1991).

Total leukocytes were isolated from heparinized blood as described previously (van Diggelen et al 1990), and lymphocytes were prepared using LymphoSep (ICN Flow). The white blood cells were frozen before use. Skin fibroblasts were cultured according to routine procedures in Ham's F10 medium supplemented with 15% fetal bovine serum and antibiotics. The cells were harvested with trypsin 7 days after the last subculture and stored at −80°C until use. Fibroblasts from patients with Sanfilippo types A, B, C and D or multiple sulphatase deficiency were obtained from the European Human Cell Bank, Rotterdam, The Netherlands (Dr W. J. Kleijer).

Homogenates were prepared by sonication of cell material in water. The standard heparin sulphamidase reaction mixtures for fibroblasts and lymphocytes consisted of 10 μl homogenate (10 or 15 μg protein, respectively) and 20 μl MU-αGlcNS (5 or 10 mmol/L, respectively) in Michaelis' barbital sodium acetate buffer, pH 6.5 (29 mmol/L sodium barbital, 29 mmol/L sodium acetate, 0.68% (w/v) NaCl, 0.02% (w/v) sodium azide; adjusted to pH 6.5 with HCl) and the reaction mixtures were incubated for 17 h at 37°C or 47°C, respectively. MU-αGlcNS is available from Moscerdam Substrates (for inquiries: O.P. van Diggelen, Rotterdam). The standard assay for leukocytes was different: 10 μl homogenate (60 μg protein) plus 20 μl 10 mmol/L MU-αGlcNS in barbital/sodium acetate buffer, pH 6.5 (see above), containing 0.225 mg/ml Pefabloc (a protease inhibitor from Boehringer; not stable in solution) was incubated for 17 h at 47°C. For all assays, after the first incubation at either 37°C or 47°C, 6 μl twice-concentrated McIlvain's phosphate/citrate buffer, pH 6.7, containing 0.02% sodium azide and 10 μl (0.1 U) yeast α-glucosidase (Sigma) in water were added and a second incubation of 24 h at 37°C was carried out. Long incubations at 37°C (17–24 h) were carried out in 96-well titreplates which were sealed airtight with broad sticky tape, limiting evaporation to < 15%. Incubations at 47°C were carried out, in Eppendorf tubes, under oil, to prevent evaporation; after the first incubation, the mixtures were transferred quantitatively to the wells of a titreplate and the second incubation was carried out as described above.

Next, 200 μl 0.5 mol/L Na_2CO_3/$NaHCO_3$, pH 10.7, was added and the fluorescence of the released 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-α-D-*N*-sulphoglucosaminide (MU-αGlcNS) as substrate for the heparin sulphamidase was investigated. The liberation of MU from MU-αGlcNS cannot be accomplished by the sole action of heparin sulphamidase, since this enzyme only cleaves the *N*-sulphate, yielding MU-αGlcNH$_2$. In previous studies we had

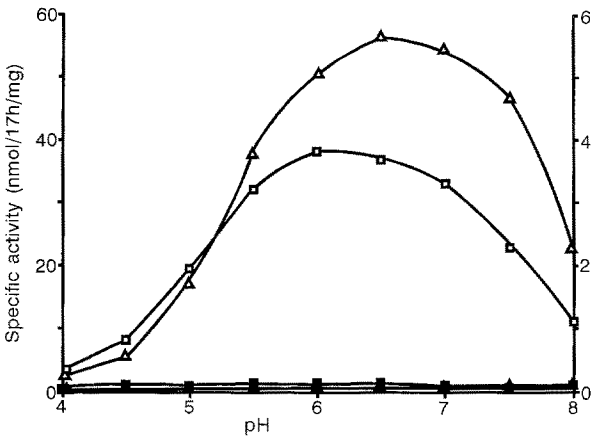

Figure 1 pH-dependence of heparin sulphamidase activity. Fibroblasts from a control (△) and a Sanfilippo A patient (▲); leukocytes from a control (□) and a Sanfilippo patient (■). Triangles, left scale; squares, right scale. Reaction conditions as in standard assay, except for pH

found that α-glucosidase from various sources has low, but significant, α-glucosaminidase activity towards MU-α-$GlcNH_2$, releasing free glucosamine (Wang He et al, unpublished observation). We applied this observation to our sulphamidase assay by subjecting the reaction mixtures to a second incubation (24h at 37°C) in the presence of 0.1 U yeast α-glucosidase, which caused complete hydrolysis of the reaction intermediate MU-α-$GlcNH_2$ between 10 and 1000 pmol.

In the experiments described below, heparin sulphamidase was assayed under standard conditions in barbital/sodium acetate buffer as described in the Materials and Methods section (except for the condition which was being investigated). The pH optimum of heparin sulphamidase from fibroblasts and leukocytes was pH 6.5, whereas fibroblasts and leukocytes from a Sanfilippo A patient had practically no activity in the pH range tested (Figure 1). The apparent K_m was estimated to be 4 mmol/L for fibroblasts and 8 mmol/L for leukocytes (Figure 2). The sulphamidase activity increased nearly linearly with protein up to 20 μg for fibroblasts and up to 60 μg for leukocytes (Figure 3). A nearly linear rate of reaction was observed over a period of up to 17 h incubation (Figure 4). Michaelis' barbital/sodium acetate buffer was the only buffer found useful at pH 6.5, since the more commonly used buffers were inhibitory (Table 2). Phosphates and sulphates are also powerful inhibitors in our standard sulphamidase assay (Table 2).

Under the standard conditions of fibroblast assay, heparin sulphamidase activity was readily detectable in lymphocytes but barely detectable in total leukocytes. Compared to fibroblasts and lymphocytes, the activity in leukocytes was unexpectedly low, and we considered that sulphamidase was being degraded by granulocyte proteases during the long incubation at a neutral pH (6.5). Several protease inhibitors were tested and a mixture prepared by Boehringer (Pefabloc) greatly increased sulphamidase activity. Heparin sulphamidase is a very heat-stable enzyme, and this has been exploited to increase sulphamidase activity by incubation at elevated temperatures (Whiteman and Young 1977; Hopwood and Elliott 1981a,b, 1982). In our assay, raising the incubation temperature from

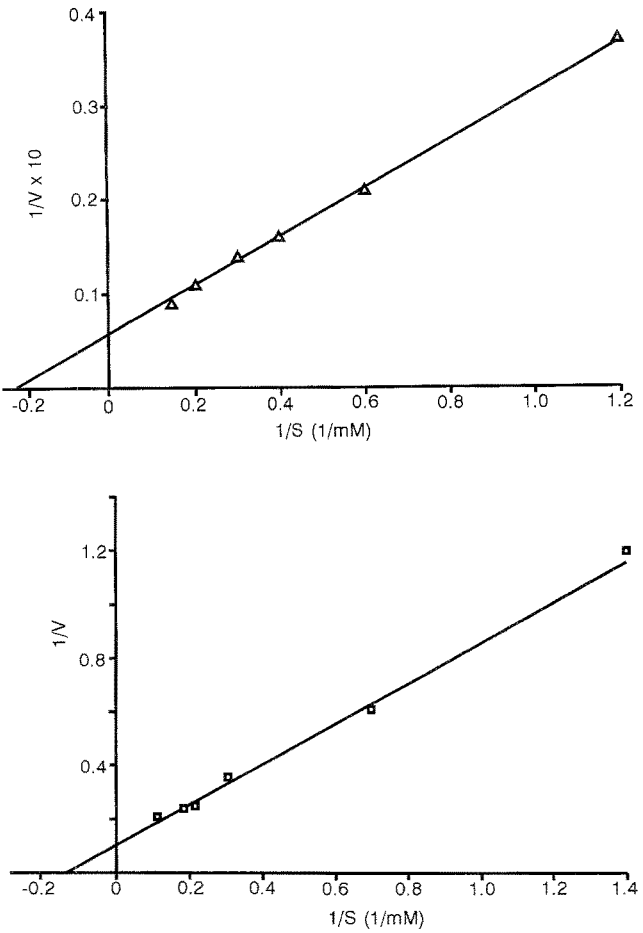

Figure 2 Lineweaver–Burk plot for heparin sulphamidase activity with MU-αGlcNS. Normal fibroblasts (upper panel) and leukocytes (lower panel). *V* is in units of nmol/17h per mg protein. Reaction conditions as in standard assay, except for MU-αGlcNS concentration

37°C to 47°C resulted in 2- to 3-fold increase. The addition of Pefabloc in the 47°C assay led to a 3-fold increase of sulphamidase activity in leukocytes, with an optimum effect obtained between 0.1 and 0.3 mg/ml. At higher concentrations it was inhibitory. Using the protease inhibitor Pefabloc, heparin sulphamidase activity could be measured reliably in total leukocytes. (The method is described as the standard leukocyte assay under Materials and Methods.) Dialysis of leukocyte homogenate did not increase the sulphamidase activity appreciably. Increasing the incubation temperature from 37°C to 47°C caused a 1.5- to 2-fold increase in sulphamidase activity of lymphocytes and fibroblasts. Since the activity in fibroblasts at 37°C is high, this standard incubation temperature is to be preferred for diagnostic laboratories.

The heparin sulphamidase activity was determined in material from patients and controls under standard assay conditions. Sulphamidase activity in fibroblasts and lympho-

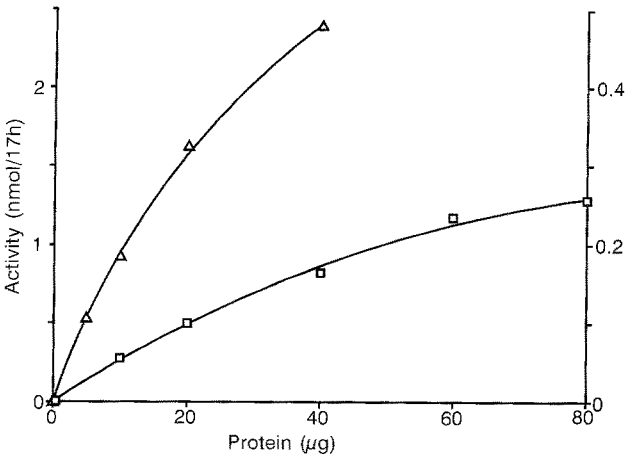

Figure 3 Protein dependence of heparin sulphamidase activity. Normal fibroblasts (△; left scale) and leukocytes (□; right scale). Reaction conditions as in standard assay, except for amount of protein

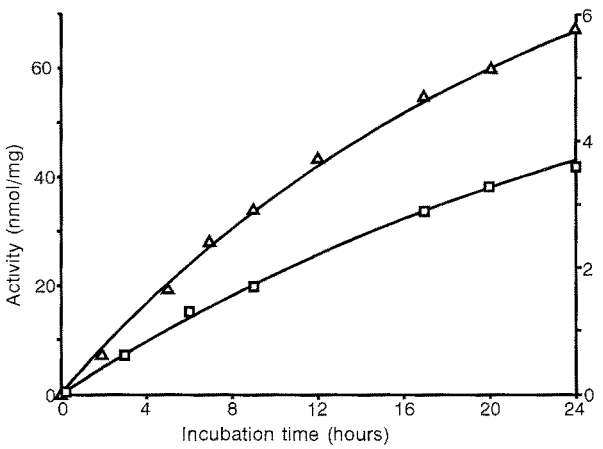

Figure 4 Time course of heparin sulphamidase activity. Normal fibroblasts (△; left scale) and leukocytes (□; right scale). Reaction conditions as in standard assay, except for incubation time

cytes from MPS IIIA patients was 0–3% of mean control activity (Table 1). The residual activity in total leukocytes from MPS IIIA patients was 0–10%. Fibroblasts from patients with multiple sulphatase deficiency had 0.7–3.4% activity, whereas patients with Sanfilippo disease types B and C had normal sulphamidase activity. Surprisingly, all three patients with Sanfilippo D had reduced sulphamidase activity in the range for heterozygotes of MPS IIIA (Table 1).

DISCUSSION

We have defined conditions for the fluorimetric determination of heparin sulphamidase in a two-step assay, using the substrate 4-methylumbelliferyl-α-D-*N*-sulphoglucosaminide

Table 1 Heparin sulphamidase activity in fibroblasts and leukocytes

	Heparin sulphamidase activity (nmol/17 h per mg protein)		
	Fibroblasts	*Leukocytes*	*Lymphocytes*
Normal range	42–152 (n=28)	1.8–6.8 (n=11)	11–20 (n=7)
(mean ± SD)	(68 ± 24)	(4.1 ± 1.4)	(13.7 ± 3.3)
MPS IIIA patients, range	0.0–2.2 (n=42)	0.0–0.4 (n=8)	0.3 (n=1)
MPS IIIA carriers, range	15–43 (n=10)		
(mean ± SD)	(31 ± 7)		
Multiple sulphatase deficiency	0.5; 2.3 (n=2)		
MPS IIIB	66 (n=1)		
MPS IIIC	73 (n=1)		
MPS IIID	26; 32; 38 (n=3)		

Table 2 Inhibitors of heparin sulphamidase activity

	Relative sulphamidase activity (%)
Various buffers at pH 5.8	
Sodium acetate	100
Michaelis' barbital/sodium acetate	100
Gomori's Tris/maleate	<5
Sodium citrate/NaOH	10
Sodium citrate/citric acid	10
Imidazole/HCl	50
Anions in barbital/sodium acetate	
Sulphate (0.1 mol/L)	1.5
Phosphate (0.1 mol/L)	2

(MU-αGlcNS). Desulphation is accomplished by heparin sulphamidase and the fluorochrome MU is released from the reaction intermediate (MU-αGlcNH$_2$) during a second incubation with α-glucosidase, which also has α-glucosaminidase activity (Wang He et al, unpublished observation).

MU-αGlcNS was shown to be useful in the diagnosis of Sanfilippo type A disease. The residual heparin sulphamidase activity in fibroblasts, leukocytes or lymphocytes from the 48 MPS IIIA patients investigated was 0–3% of the mean control activity. ^{35}S-Radiolabelled heparin is by far the most widely used substrate for assay of heparin sulphamidase. This method, originally described by Kresse et al (1973), is inconvenient because the substrate has a short half-life and it needs to be repurified regularly. Furthermore, separation techniques are required to isolate the reaction product; the relationship between amount of protein and enzyme activity is poor; and reproducibility of the assay occasionally causes problems, e.g. high residual activity in MPS IIIA patients. Our fluorogenic assay is sensitive (Table 1), requiring only 10 μg protein from a fibroblast homogenate, and specific with low residual activity in patients (<3%). It is obviously more convenient than the conventional assay. The assay, which was adequate for fibroblast and lymphocytes, was

too insensitive for total leukocytes. Since leukocytes are the most widely used material for enzyme analysis, we have modified the assay to increase the activity in control leukocytes. These modifications, raising the incubation temperature to 47°C, increasing the substrate concentration, and adding the protease inhibitor Pefabloc (Boehringer, unpublished composition), allowed determination of heparin sulphamidase deficiency in MPS IIIA patients. Occasionally the residual activity was rather high (10%, Table 1).

Carrier detection in fibroblasts with this assay was informative in 9 out of 10 of the obligate heterozygotes having reduced sulphamidase activity.

The natural substrate for heparin sulphamidase is polymeric heparin or heparan sulphate; thus heparin sulphamidase is always exposed to the terminal oligomeric stretch of heparin. Hopwood and co-workers (Hopwood and Elliott 1981a,b; Freeman and Hopwood 1986) have prepared a series of di-, tri- and tetrasaccharides derived from heparin, and investigated the importance of the saccharides adjacent to the terminal *N*-sulphated glucosamine. Using a series of structurally different disaccharides, it was shown that the presence of a C-6 carboxyl group and a C-2 *O*-sulphate on the adjacent uronic acid moiety increased sulphamidase activity 100–800 times. Increasing the substrate length by two residues led to a 10-fold increased sulphamidase activity, relative to the best disaccharide substrate (Hopwood and Elliott 1982); free GlcNS was not de-*N*-sulphated under these conditions (Hopwood and Elliott 1981b). These results implied an essential role for the structure of the adjacent residues in the mechanism of action or binding of sulphamidase towards oligosaccharides with terminal *N*-sulphated glucosamine. The V_{max} for MU-αGlcNS is essentially the same as the V_{max} for the best oligosaccharide, a tetrasaccharide (Hopwood and Elliott 1982). This demonstrates that heparin sulphamidase has no absolute requirement for adjacent heparin-specific saccharides for its enzymatic action. Our results suggest that the presence of any group linked α-glycosidically to *N*-sulphoglucosamine is sufficient to create a substrate for heparin sulphamidase. However, the K_m of our derivatized monosaccharide substrate (4 mmol/L) is much greater than those for the oligosaccharides: GlcNS-IdOA, 35–220 μmol/L (Hopwood and Elliott 1981a,b; Freeman and Hopwood 1986); GlcNS-UA-GlcNS-UOA, 30 μmol/L (Hopwood and Elliott 1982): GlcNS-IdoA-GlcNS-IdOA, 10 μmol/L (Freeman and Hopwood 1986). This suggests that the adjacent saccharides contribute to the binding of the substrates, especially at low substrate concentrations.

Assays with MU substrates requiring more than one enzyme to yield the fluorochrome are uncommon. This principle was successfully applied for the first time to a new assay of galactose-6-sulphate sulphatase for 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 iduronate- and glucuronate-2-sulphatase are currently being synthesized.

ACKNOWLEDGEMENTS

We thank Professor Hans Galjaard for his continuing support. This work was supported by INTAS (International Association for the Promotion of Co-operation with Scientists from the former Soviet Union; project 94-3625). We thank Drs Ries Duran and Frans Scholte for making available blood from an MPS IIIA patient for lymphocyte isolation. Doctors

Ron Wevers and Jan Bakkeren are kindly acknowledged for making available fibroblasts from patients with Sanfilippo type D. We also thank Dr D.J. van Rhenen and the staff of the 'Bloedbank, Rotterdam' for providing indispensable blood samples from control individuals.

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