Variables influencing fluorimetric N-sulfoglucosamine sulfohydrolase (SGSH) activity measurement in brain homogenates

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Deficient N-sulfoglucosamine sulfohydrolase (SGSH) enzyme activity causes mucopolysaccharidosis (MPS) type IIIA. A fluorimetric SGSH activity assay is commonly used to examine patient cells. Here, we modified this method for brain homogenates and define the parameters for assay linearity. SGSH activity was suppressed outside of these parameters. This method will enable the accurate measurement of SGSH activity in MPS IIIA tissues to examine disease pathogenesis and evaluate therapies.

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1. Introduction

Sanfilippo syndrome (mucopolysaccharidosis (MPS) type III) is caused by the absence of specific lysosomal enzymes involved in the catabolism of heparan sulfate glycosaminoglycans. In the case of MPS IIIA (OMIM 252900), insufficient N-sulfoglucosamine sulfohydrolase (SGSH; EC 3.10.1.1) activity prevents hydrolysis of the terminal N-sulfated bond from the non-reducing terminal glucosamine residue of heparan sulfate oligosaccharides. Consequently, partially-degraded heparan sulfate accumulates within the lysosome causing progressive neurological deterioration [1]. Although there are several therapies being assessed in clinical trials [2–4], treatment of MPS IIIA patients is currently directed at supportive care. Studies in MPS IIIA animal models are required to evaluate the efficacy and optimize the application of therapies and therefore a method to assess SGSH activity in brain tissue is needed.

Two methods for measuring SGSH activity have been described. A tritiated tetrasaccharide substrate derived from heparin (GlcNS-UA-GlcNS-UA-GlcNS-UA) has been used diagnostically and can separate MPS IIIA patients from normal or obligate carriers, and from other MPS subtypes [5]. However, this substrate is not available commercially, is time-consuming to prepare and requires separation of the radiolabeled substrate and product peaks by high performance liquid chromatography. A second method, using a 4-methylumbelliferyl-α-D-N-sulfoglucosaminide (4MU-αGlcNS substrate), has been described [6]. Here, SGSH cleaves the N-sulfate of the 2-sulfamino-2-deoxy-α-D-glucopyranosyl residue to generate 4MU-αGlcNH2. The 4MU moiety can then be liberated by digestion with α-glycosidase, due to α-glucosaminidase activity on the glucosamine residue. This substrate is available for purchase and the assay requires a fluorometer equipped with the correct filters for measuring the umbelliferone fluorescence.

Karpova et al. [6] described the assay conditions influencing SGSH activity in skin fibroblasts and leukocytes. Whilst SGSH activity in MPS IIIA tissue homogenates has been reported (e.g. [7–9]), the parameters influencing the accuracy of the method in this sample type have not been delineated. In this study, we investigate the effect of total protein content and assay incubation time on the rate of brain homogenate SGSH activity.

2. Materials and methods

2.1. Animals and tissue collection.

Mongrel C57BL/6 MPS IIIA mice [10] and wild-type (+/+) and heterozygote (+/−) controls were used in this study (n = 2–7 mice/group). Genotyping was performed as previously described [11]. The mice were housed in the Women’s and Children’s Health Network Animal Care Facility and all breeding and experimental procedures were performed in accordance with the Declaration of Helsinki and the guidelines of the South Australian Health and Medical Research Institute’s Animal Care and Use Committee.

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undertaken with the approval of the Women's and Children's Health Network Animal Ethics Committee (Adelaide, Australia) in accordance with the Australian Code For The Care And Use Of Animals For Scientific Purposes 8th edition (2013). Mice were killed via carbon dioxide asphyxiation and trans-cardially perfused with ice-cold PBS to remove blood. The brain was removed, divided along the midline, some samples further dissected into 2-mm slices, and snap frozen in liquid nitrogen. Samples were stored at −80 °C until required.

2.2. SGSH activity assay

Half-brains (Fig. 1A, B) or 2-mm hemi-coronal brain slices (Fig. 1C-E) were homogenized with an Ultra-Turrax T10 mechanical homogenizer (IKA Works, Selangor, Malaysia) in 500 μL of 0.02 M Tris, 0.5 M NaCl, pH 7.4, and sonicated with a VibraCell ultrasonic processor (Sonics and Materials, Newtown, CT, USA) for 2 cycles of 30 s on ice, prior to being dialyzed into 0.2 M sodium acetate, pH 6.5, overnight at 4 °C. Total protein was determined using a Micro-BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA; cat. no. 23235).

SGSH activity was determined according to the method described by Karpova et al. [6]. 4MU-αGlcNS substrate from Carbosynth Limited (Berkshire, UK; cat. no. EM06602) was dissolved in Michaelis' barbital sodium acetate buffer (29 mM sodium barbital, 29 mM sodium acetate, 0.6% (w/v) sodium chloride, 0.02% (w/v) sodium azide) at a concentration of 5 mM. Twenty microliters of 4MU-αGlcNS substrate was mixed with samples of 1–300 μg total protein (made up to a total volume of 10 μL with 0.2 M sodium acetate pH 6.5) and incubated at 47 °C for up to 48 h. All incubations were carried out in the dark and in screw-cap tubes to prevent evaporation. Twice-concentrated McIlvaine’s phosphate/citrate buffer, pH 6.7, containing 0.02% (w/v) sodium azide (6 μL) was used to terminate the reaction. α-Glucosidase from Bacillus stearothermophilus (0.1 U in 10 μL; Sigma-Aldrich, Castle Hill, Australia; cat. no. G3651) was then added to the samples and incubated for 24 h at 37 °C to liberate the 4MU from the glucosamine residue. This second reaction was terminated by adding 100 μL of 0.5 M

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**Fig. 1.** (A, B) Effect of total protein concentration on umbelliferone counts and SGSH activity in wild-type mouse brain homogenate incubated at 47 °C for 17 h. X and O represent different experiments. (C, D) Effect of incubation time on umbelliferone counts and SGSH activity on 15 μg of wild-type mouse brain homogenate incubated at 47 °C. (E) Measurement of SGSH activity in 15 μg of wild-type (+/+), heterozygote (+/−) and MPS IIIA (−/−) mouse brain homogenates after incubation at 47 °C for 16 h. n.d., not detected. All data are mean ± SEM (n = 2–7 per group).
\[ \text{Na}_2\text{CO}_3/\text{NH}_4\text{HCO}_3, \text{pH} 10.7. \] Samples were then transferred to a non-binding, flat-bottom, 96-well microtiter plate (Interpath, Heidelberg West, Australia; cat. no. 655101). 4-Methylumbelliferone (Sigma-Aldrich; cat. no. M1381) standard was included to convert sample counts to SGSH activity rates in units of pmol/min/mg total protein. Fluorescence of the released 4MU was measured on a VICTOR3 plate reader (Perkin Elmer, MA, USA) at excitation 355 nm and emission 460 nm.

3. Results and discussion

First, we determined the effect of brain homogenate protein concentration on SGSH activity. Between 1 and 300 μg of wild-type mouse brain homogenate was assayed at 47 °C with the incubation time set to 17 h as per Karpova et al. [6]. The umbelliferone counts approached a plateau at higher protein concentrations (>200 μg). SGSH activity rates were consistent only up to 31.25 μg of total protein, with an average SGSH activity of 4.6 pmol/min/mg in wild-type mouse brain homogenate for the two-fold dilution series, ranging from 31.25 to 0.98 μg per assay (8.7% coefficient of variation). However, for total protein values above 31.25 μg, the calculated rate of SGSH activity reduced with increasing protein concentrations (Fig. 1A). A plateau was noted at higher protein concentrations (≥200 μg). SGSH activity rates were consistent only up to 31.25 μg of total protein, with an average SGSH activity of 4.6 pmol/min/mg in wild-type mouse brain homogenate for the two-fold dilution series, ranging from 31.25 to 0.98 μg per assay (8.7% coefficient of variation). However, for total protein values above 31.25 μg, the calculated rate of SGSH activity reduced with increasing protein concentrations (Fig. 1B). Similar observations have been made in SGSH enzyme assays of SGSH activity reduced with increasing protein concentrations up to 17 h at 47 °C, with an umbelliferone count strongly correlating with incubation length. For reliable brain SGSH activity measurement, the assay should be conducted on freshly prepared homogenates using less than 31 μg of total protein per assay, with incubations undertaken for up to 17 h at 47 °C.

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