



Short Communication

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

Lauren S. Whyte, John J. Hopwood, Kim M. Hemsley, Adeline A. Lau *

Lysosomal Diseases Research Unit, South Australian Health and Medical Research Institute, PO Box 11060, Adelaide, SA 5001, Australia

ARTICLE INFO

Article history:

Received 20 July 2015

Received in revised form 13 October 2015

Accepted 13 October 2015

Available online 22 October 2015

Keywords:

N-sulfoglucosamine sulfohydrolase

Enzyme activity

Fluorometry

Sanfilippo

Mucopolysaccharidosis

ABSTRACT

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.

© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Sanfilippo syndrome (mucopolysaccharidosis (MPS¹) 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 (SGSH²; 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-UOA) 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 radio-labeled substrate and product peaks by high performance liquid

chromatography. A second method, using a 4-methylumbelliferyl- α -D-*N*-sulfoglucosaminide (4MU- α GlcNS³ substrate), has been described [6]. Here, SGSH cleaves the *N*-sulfate of the 2-sulfamino-2-deoxy-D-glucopyranosyl residue to generate 4MU- α GlcNH₂. 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.

Congenic 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

* Corresponding author at: Lysosomal Diseases Research Unit, South Australian Health and Medical Research Institute (SAHMRI), PO Box 11060, Adelaide, SA 5001, Australia.

E-mail addresses: lauren.whyte@sahmri.com (L.S. Whyte),

john.hopwood@sahmri.com (J.J. Hopwood), kim.hemsley@sahmri.com (K.M. Hemsley),

adeline.lau@sahmri.com (A.A. Lau).

¹ MPS, mucopolysaccharidosis.

² SGSH, *N*-sulfoglucosamine sulfohydrolase.

³ 4MU- α GlcNS, 4-methylumbelliferyl- α -D-*N*-sulfoglucosaminide.

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. $\alpha\text{-Glucosidase}$ 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

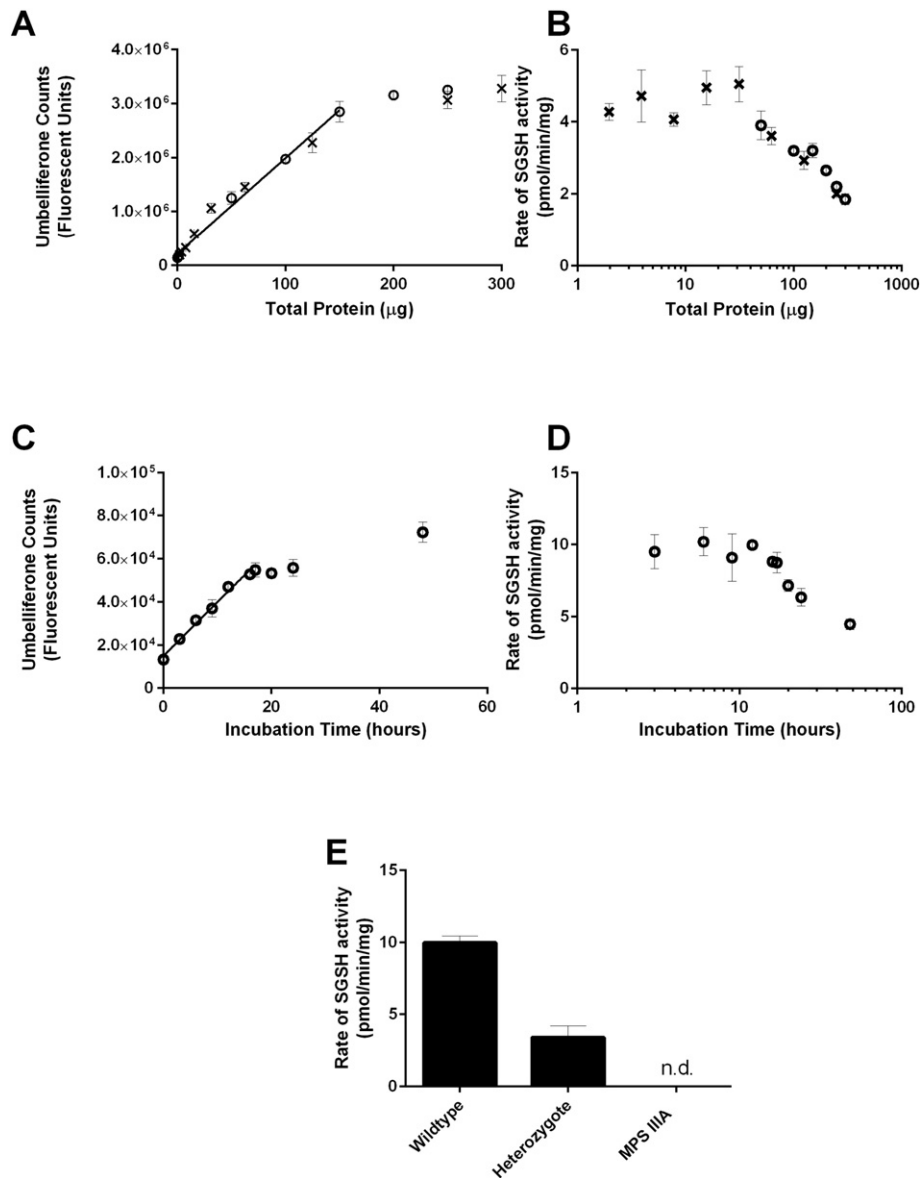


Fig. 1. (A, B) Effect of total protein concentration on umbelliferone counts and SGSH activity in wild-type mouse brain homogenate incubated at 47°C for 17 h. X and O represent different experiments. (C, D) Effect of incubation time on umbelliferone counts and SGSH activity on 15 μg of wild-type mouse 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 \pm SEM (n = 2–7 per group).

Na₂CO₃/NaHCO₃, 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 linearity with increasing protein concentrations up to 150 µg total protein (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 using the natural tetrasaccharide substrate in fibroblasts and leukocytes, where activity was reduced at higher protein concentrations [5]. Karpova et al. [6] demonstrated that SGSH activity showed a dose-dependent response with protein concentration up to 20 µg and 60 µg for fibroblasts and leukocytes, respectively. Brain tissues were not examined. Selection of a protein range in different tissues/cell types where the activity measured is independent of protein concentration is desirable to allow comparison of SGSH activity rates between experiments.

The effect of incubation time on SGSH activity was then determined using wild-type brain homogenate. Umbelliferone counts strongly correlated with incubation length up to 17 h at 47 °C, with an *R*-squared value of 0.99 for a linear fit trendline (Fig. 1C). Although the umbelliferone counts continued to rise with increasing incubation length, there was no linearity beyond this time-point. The calculated rate of SGSH activity was also consistent up to 17 h of incubation, after which a reduction in the calculated rate of SGSH activity was found (Fig. 1D). The linearity of SGSH activity in brain homogenates over this time-frame is consistent with that published in fibroblasts and leukocytes, where a near linear rate of reaction was demonstrated up to 17 h of incubation [6].

SGSH activity was then measured in 5-month old mouse brain homogenates using 15 µg total protein and a 16 h incubation. SGSH activity was readily detectable in wild-type and heterozygote mice (9.98 ± 0.46 pmol/min/mg and 3.39 ± 0.80, respectively; Fig. 1E). However, under these conditions, SGSH activity was unable to be detected in MPS IIIA mouse brains. The SGSH activity measured in brain homogenates using the artificial 4MU-αGlcNS substrate shows reduced sensitivity compared to the 3.2% and 3.9% wild-type SGSH activity levels measured in C57BL/6 MPS IIIA mouse liver homogenates or mixed strain MPS IIIA mouse brain homogenates assayed with the natural tritiated tetrasaccharide substrate derived from heparin [10]. This is significant given that some enzymes, such as hexosaminidase A, show differential activities towards natural and artificial 4MU-conjugated substrates,

increasing the complexity of differentiating affected patients from normal and carrier controls [12].

Between 0.8% and ~6% of wild-type SGSH activity have been reported for MPS IIIA C57BL/6 mouse brain using the 4MU-αGlcNS substrate; however the amount of protein and reaction length were not reported [7,8]. Likewise, 3% of wild-type SGSH activity in MPS IIIA mouse brain was reported when 60 µg total protein was used [9]. When we performed this assay outside of the parameters required for linearity (250 µg, 17 h of incubation), we could also detect counts above the assay blank in MPS IIIA brain homogenates (0.12 ± 0.02 pmol/min/mg; 2.2% of wild-type SGSH activity; data not shown). In these latter samples, SGSH activity was no longer detectable above the assay blank in MPS IIIA homogenates when the assay was repeated with samples that had been freeze/thawed once (data not shown).

In conclusion, the 4MU-αGlcNS substrate provides a simple and convenient two-step assay for SGSH activity measurement in tissue homogenates. 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.

Acknowledgments

We thank Shire Human Genetic Therapies for kindly providing a comparative and alternate sample of MU-αGlcNS, Ms. Meghan Setford and Ms. Sarah Tamang for breeding and genotyping the mice in this study and Mrs. Barbara King for preparing the substrate buffer.

References

- [1] M.J. Valstar, S. Neijs, H.T. Bruggenwirth, R. Olmer, G.J. Ruijter, et al., Mucopolysaccharidosis type IIIA: clinical spectrum and genotype-phenotype correlations, *Ann. Neurol.* 68 (2010) 876–887.
- [2] M. Tardieu, M. Zerah, B. Husson, S. de Bournonville, K. Deiva, et al., Intracerebral administration of adeno-associated viral vector serotype rh.10 carrying human SGSH and SUMF1 cDNAs in children with mucopolysaccharidosis type IIIA disease: results of a phase I/II trial, *Hum. Gene Ther.* 25 (2014) 506–516.
- [3] K.M. Hemsley, B. King, J.J. Hopwood, Injection of recombinant human sulfamidase into the CSF via the cerebellomedullary cistern in MPS IIIA mice, *Mol. Genet. Metab.* 90 (2007) 313–328.
- [4] K.H. Kim, C. Dodsworth, A. Paras, B.K. Burton, High dose genistein aglycone therapy is safe in patients with mucopolysaccharidoses involving the central nervous system, *Mol. Genet. Metab.* 109 (2013) 382–385.
- [5] J.J. Hopwood, H. Elliott, Diagnosis of Sanfilippo A syndrome by estimation of sulphamidase activity using a radiolabelled tetrasaccharide substrate, *Clin. Chim. Acta* 123 (1982) 241–250.
- [6] E.A. Karpova, V. Voznyi Ya, J.L. Keulemans, A.T. Hoogveen, B. Winchester, et al., A fluorimetric enzyme assay for the diagnosis of Sanfilippo disease type A (MPS IIIA), *J. Inher. Metab. Dis.* 19 (1996) 278–285.
- [7] A. Ruzo, S. Marco, M. Garcia, P. Villacampa, A. Ribera, et al., Correction of pathological accumulation of glycosaminoglycans in central nervous system and peripheral tissues of MPSIIIA mice through systemic AAV9 gene transfer, *Hum. Gene Ther.* 23 (2012) 1237–1246.
- [8] A. Fraldi, K. Hemsley, A. Crawley, A. Lombardi, A. Lau, et al., Functional correction of CNS lesions in a MPS-IIIa mouse model by intracerebral AAV-mediated delivery of sulfamidase and SUMF1 genes, *Hum. Mol. Genet.* 16 (2007) 2693–2702.
- [9] A. Langford-Smith, F.L. Wilkinson, K.J. Langford-Smith, R.J. Holley, A. Sergijenko, et al., Hematopoietic stem cell and gene therapy corrects primary neuropathology and behavior in mucopolysaccharidosis IIIA mice, *Mol. Ther.* 20 (2012) 1610–1621.
- [10] A.C. Crawley, B.L. Gliddon, D. Auclair, S.L. Brodie, C. Hirte, et al., Characterization of a C57BL/6 congenic mouse strain of mucopolysaccharidosis type IIIA, *Brain Res.* 1104 (2006) 1–17.
- [11] A.A. Lau, N.J. Shamsani, L.K. Winner, S. Hassiotis, B.M. King, et al., Neonatal bone marrow transplantation in MPS IIIA mice, *JIMD Rep.* 8 (2013) 121–132.
- [12] J.F. Tallman, R.O. Brady, R. Navon, B. Padeh, Ganglioside catabolism in hexosaminidase A-deficient adults, *Nature* 252 (1974) 254–255.