EFFECT OF TRITON X-100 ON THE ISOELECTRIC FOCUSING PROFILE OF FIBROBLAST SPHINGOMYELINASE

Guy T. N. BESLEY

Department of Pathology, Royal Hospital for Sick Children, Edinburgh, EH9 1LF, Scotland

Received 5 November 1976

1. Introduction

Sphingomyelinase is the only mammalian enzyme known to hydrolyse sphingomyelin. Hence, the inherited deficiency of this enzyme in Niemann-Pick disease results in the characteristic sphingomyelin lipidosis. The disease is, however, expressed in at least five different phenotypes [1] of which only types A and B lack sphingomyelinase activity. In types C, D and E where the lipidosis is less severe, as also is the course of the disease, normal enzyme activities have been measured. Recently, however, Callahan et al. [2–4] have demonstrated multiple forms of sphingomyelinase following isoelectric focusing of extracts of brain, liver and cultured skin fibroblasts. Of particular interest was their finding that a specific sphingomyelinase component was absent in Niemann-Pick diseases types C and E.

In view of the importance of this finding, the present work is reported in order to extend these observations on the behaviour of fibroblast sphingomyelinase during isoelectric focusing. The results suggest that in the original study the enzyme was probably in a bound state but when solubilized with Triton X-100, it moves to a much higher p*I*.

2. Materials and methods

[(*Methyl*-^{14}C)choline]sphingomyelin was obtained from New England Nuclear Gmbh (Germany), ampholine from LKB-Produkter AB (Sweden), sodium cholate from Sigma Chemical Co. Ltd. (USA) and purified Triton X-100, bovine sphingomyelin and 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside from Koch Light Labs. (UK).

2.1. *Cultured skin fibroblasts*

Skin fibroblasts were grown and harvested as described [5]. The cell pellet was lyophilised and extracted into 1% (w/v) glycine [4] and the 31 500 × *g* 30 min supernatant (1–2 mg protein) used for electrofocusing and enzyme analysis. All cultures were judged to be free of mycoplasma contamination.

2.2. *Enzyme assay*

Sphingomyelinase activity was measured as described [6]. The assay mixture contained, in a final volume of 0.2 ml, enzyme extract (50 μl or 100 μl), 250 μg sodium cholate, 62 nmol [^{14}C]sphingomyelin (50 000 cpm) and 20 μmol acetate buffer, pH 5.0. Incubation (3 h at 37°C) was stopped by the addition of cold water (0.9 ml), 10% (w/v) bovine serum albumen (0.1 ml) and 50% (w/v) trichloroacetic acid (0.2 ml). The precipitate was washed once with 10% (w/v) trichloroacetic acid (1 ml) and the released [^{14}C]phosphorylcholine in the pooled supernatants (1 ml) was counted in Bray's scintillation mixture. In the absence of added enzyme, the blank was 50–60 cpm.

Hexosaminidase activity was determined as described [7] and protein measured by the Lowry method [8].

2.3. *Isoelectric focusing*

Electrofocusing was performed in J-tubes of 10 ml working capacity [7] but in the presence or absence of 0.1% (v/v) Triton X-100, as indicated. On completion of a run (18 h at 400 V and 4°C) fractions (10 drops with Triton and 4 drops without Triton)

were collected and analysed for pH (at 4°C) and enzyme activity.

2.4. *Acid precipitation*

The glycine extracted supernatant was added to an equal volume of 0.4 M acetate buffer, pH 5.0, (assay buffer) in the presence or absence of 0.1% (v/v) Triton X-100. After 30 min at 4°C, each was centrifuged at 1000 × *g* 10 min, the supernatant was retained and the pellet reconstituted in its original volume of appropriate buffered solution. Sphingomyelinase activity was determined in all fractions, and in those not containing Triton X-100, additions were made to maintain a constant concentration in all assays.

3. Results and discussion

When run in the absence of Triton X-100, the isoelectric focusing profile of fibroblast sphingomyelinase was (fig.1a) closely similar to that reported by Callahan and Khalil [4]. The major enzyme peak was at about pH 5.0, with a p*I* between 4.95 and 5.20 on two separate occasions, there was a shoulder of activity at p*I* 8.1. There was evidence of two sphingomyelinase components in the major peak, the second form (p*I* 5.2) possibly being the component whose deficiency has been associated [3,4] with Niemann-Pick diseases types C and E. Shown also in fig.1a, as a reference enzyme, is the profile of fibroblast hexosaminidase activity. The two characteristic peaks of hexosaminidase A (p*I* 5.2) and hexosaminidase B (p*I* 8.5) are seen. The p*I* of hexosaminidase B is somewhat higher than that reported earlier [9] and would appear to be due to the presence of glycine, also some loss of hexosaminidase B activity is evident following its sedimentation at 31 500 × *g* for 30 min.

The sphingomyelinase assay unfortunately necessitated 1–2 mg protein to be loaded on the column which resulted in a considerable band of precipitated protein in the region of the major sphingomyelinase peak. In order to alleviate this problem, Triton X-100 was included in subsequent focusing experiments. Under these conditions it was found (fig.1c) that the major band of sphingomyelinase then focused at p*I* 9.0. (between p*I* 8.75 and 9.10, on two occasions). On the acid side of this peak a number of other components were evident, at p*I* values of 8.3, 7.8, a broad band between p*I* 7.0–6.3 and one at p*I* 5.1. The enzyme peak could be demonstrated using either [^{14}C]sphingomyelin or [^{3}H]dihydrosphingomyelin [4] as substrate

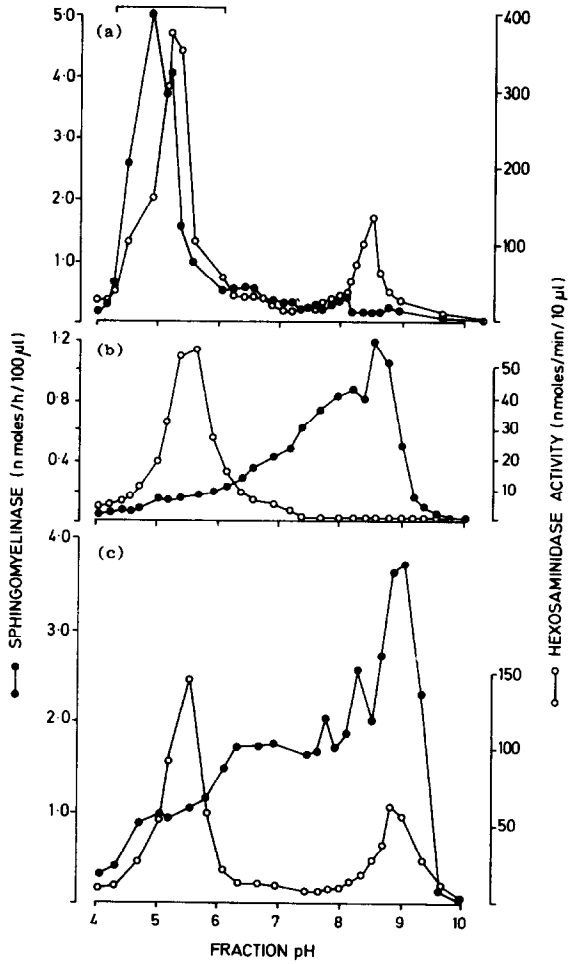

Fig.1. Isoelectric focusing profiles of fibroblast sphingomyelinase (•) and hexosaminidase (○) activities. (a) Focusing performed in the absence of Triton X-100 as described in the text. Supernatant contained 1.50 mg protein, sphingomyelinase activity 71.5 nmol/h/mg protein (representing 75% extraction from the total homogenate). (b) Fractions between p*I* 4.3 and 6.1 on fig.1 (a) refocused but in the presence of 0.1% (v/v) Triton X-100. (c) Focusing performed in the presence of 0.1% (v/v) Triton X-100. Supernatant contained 1.96 mg protein, sphingomyelinase activity 46.7 nmol/h/mg protein (60% enzyme extraction).

and enzyme recovery from the column was estimated to be about 80% of that applied. Under these conditions, however, the hexosaminidase profile was unchanged.

In order to demonstrate that this apparent change in sphingomyelinase p*I* was brought about by the presence of Triton X-100, fractions between pH 4.3 and 6.1 from the first column (fig.1a) were pooled and refocused in the presence of Triton X-100. The enzyme now focused with its major peak at p*I* 8.6–8.8 (fig.1b) with a shoulder of other components on its acid side, closely resembling that profile seen in fig.1c.

Since the p*I* of bound sphingomyelinase corresponds to that of its activity pH optimum [4,10] it would seem likely that during enzyme assay, in the absence of Triton X-100, considerable precipitation might occur, resulting in apparent loss of sphingomyelinase activity. For example, when assayed in the presence of 0.025% (v/v) Triton X-100, sphingomyelinase activities of the supernatant fraction and its pellet were 432 and 232 nmol/h/ml, whereas in the absence of the detergent, activities were reduced by some 85% to 62 and 40 nmol/h/ml. It can be seen that sphingomyelinase extraction into glycine was generally poor (60–75%), however, extraction into sodium cholate [10] gave recoveries of 93–96%. Addition of Triton X-100 to this extract improved little (10%) its sphingomyelinase activity, and its focusing profile was similar to that shown in fig.1c. Shown in table 1 is the effect of Triton X-100 in preventing enzyme precipitation by acetate buffer, pH 5.0. Virtually no enzyme was precipitated in the presence of Triton X-100 whereas in its absence 30% of the sphingomyelinase was lost to the sediment.

The foregoing results indicate that sphingomyelinase normally exists in a bound state but that it can be freed by the presence of Triton X-100. However, the exact nature of the material to which it is bound is not known. Gatt and Gottesdiner [11] have speculated that in rat brain the enzyme exists as a multienzyme aggregate of about 300 000 daltons and that in the presence of Triton X-100 this was reduced to 200 000 daltons. Furthermore, cellular distribution studies [12] have indicated that sphingomyelinase activity may not be found exclusively in the lysosomes. Whether such a situation is responsible for the existence of its various component forms must remain to be seen.

Acknowledgements

I am most grateful to Mrs Eleanor Cochrane for the cell culture. This work was supported, in part, by a grant from the Scottish Home and Health Department.

References

[1] Fredrickson, D. S. and Sloan, H. R. (1972) in: The Metabolic Basis of Inherited Disease (Stanbury, J. B., Wyngaarden, J. B. and Fredrickson, D. S. eds) 3rd edn. pp. 783–807, McGraw-Hill, USA.
[2] Callahan, J. W., Khalil, M. and Gerrie, J. (1974) Biochem. Biophys. Res. Commun. 58, 384–390.
[3] Callahan, J. W., Khalil, M. and Phillippart, M. (1975) Pediat. Res. 9, 908–913.
[4] Callahan, J. W. and Khalil, M. (1975) Pediat. Res. 9, 914–918.
[5] Besley, G. T. N. and Bain, A. D. (1976) J. Med. Genet. 13, 195–199.
[6] Kanfer, J. N., Young, O. M., Shapiro, D. and Brady, R. O. (1966) J. Biol. Chem. 241, 1081–1084.

Table 1
Effect of Triton X-100 on acid precipitation of sphingomyelinase

	Sphingomyelinase activity (nmoles/h/ml)	
	Plus Triton X-100	Minus Triton X-100
Extract in 0.2 M Acetate, pH 5.0	225	225
Supernatant	230	152
Precipitate	2	66

[7] Besley, G. T. N. and Broadhead, D. M. (1976) Biochem. J. 155, 205–208.
[8] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[9] Broadhead, D. M. and Besley, G. T. N. (1975) Biochem. Soc. Trans. 3, 247–250.
[10] Besley, G. T. N. (1977) Ann. Clin. Biochem. (in press).
[11] Gatt, S. and Gottesdiner, T. (1976) J. Neurochem. 26, 421–422.
[12] Weinreb, N. J., Brady, R. O. and Tappel, A. L. (1968) Biochim. Biophys. Acta 159, 141–146.