ISOENZYMES OF SPHINGOMYELINASE AND THE GENETIC DEFECT IN NIEMANN-PICK DISEASE, TYPE C

J.W. Callahan, M. Khalil and J. Gerrie. Research Institute, The Hospital for Sick Children, Toronto, Canada

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SUMMARY

Isoenzymes of sphingomyelinase have been resolved by isoelectric focusing. The two major species (I and II) in human liver have distinct isoelectric points, pH optima and Km values. Liver from Niemann-Pick disease Type C contained isoenzyme I (pI 4.6) while isoenzyme II (pI 5.2) was absent. The absence of isoenzyme II likely constitutes the genetic defect in this disease.

Sphingomyelinase is a lysosomal hydrolase whose only known substrate is sphingomyelin. It has been partially purified from a variety of mammalian sources including human liver and spleen (1-5). In their work on the spleen enzyme Schneider and Kennedy reported the presence of enzyme activity at pH 7.4 which was stimulated by magnesium ions (4). The enzyme acting under these conditions may be different from that localized primarily in the lysosomes. There is however little evidence to date for the existence of sphingomyelinase isoenzymes in mammalian tissues.

Multiple forms of this enzyme may be inferred from biochemical studies on Niemann-Pick disease. This is a group of disorders, designated Types A-E, all of which are characterized by an abnormally high content of sphingomyelin especially in the major visceral organs (6). In two forms of this disease (Types A and B) sphingomyelinase activity is markedly reduced (4, 6, 7). Types C and D however are characterized by a near normal activity when measured in crude extracts. The primary genetic defects in Types C and D, Niemann-Pick disease have not been recognized to date. In this communication, we present evidence for the existence of isoenzymes of sphingomyelinase in human liver and the absence of one of these species from liver of Type C Niemann-Pick disease.

MATERIALS AND METHODS

Tissue

Autopsy specimens of human liver were obtained within 24 hours of death and stored at -20°C. The Niemann-Pick, Type C liver examined was obtained from the brother of a previously reported case (8).

Preparation of Tissue Extracts

Tissues were minced with scissors and the enzyme was extracted according to Sandhoff et al (9). A large scale preparation is as follows: 4.5 g of normal human liver was homogenized in 10 vols of cold 0.05 M citrate-phosphate buffer pH 4.5 containing 0.25% (v/v) Triton X-100. Homogenization was performed in a Sorvall Omnimixer at setting 8 for 30 seconds followed by cooling for 5 minutes. This extraction was then repeated. The extract was centrifuged in a Beckman J-21 centrifuge with a JA-20 rotor at 2°C for 30 min at 31,500 g avg. The supernatant fluid was dialyzed 17-20 hours against 2 liters of 1% glycine at 2°C and re-centrifuged as above. This final fluid was then subjected to isoelectric focusing. For more routine analysis, 1 gram of liver tissue was treated as above.

Isoelectric Focusing of Tissue Extracts

Isoelectric focusing was carried out in a Uniphor 7900 column electrophoresis system (LKB Products) and the general procedure followed is outlined in their manual. The enzyme extract was exposed to current for 43 hours in 3% carrier ampholines, pH range 4-7, in a sucrose gradient. Temperature was thermostatically controlled with a Haake FK 10 at 2.8-3.6°C and the maximum voltage was 500 V. The column was then eluted at a flow rate of 1 ml/min and fractions of about 0.8 ml were collected. The pH of the fractions was measured with a combination micro-electrode in an ice bath.

Sphingomyelinase Assay

Tritium-labelled bovine brain sphingomyelin (New England Nuclear Corp.) was purified by chromatography on Silica gel G layers, 250 μ with $CHCl_3/CH_3OH/H_2O$ (70:30:4) as solvent. Pure sphingomyelin was eluted from the gel with 2/1 C/M containing 7% H_2O and diluted with cold carrier to give a final specific activity of 2×10^6 CPM/mg. The pure substrate was stored in 2/1 C/M in the cold.

Sphingomyelinase activity was measured in the eluates in the presence of ampholines and sucrose according to Schneider and Kennedy (4). The assay contained in a final volume of 200 μl: 125 mM acetate buffer pH 5.0, 0.167 mM sphingomyelin (50,000 CPM), 0.2 mg Triton X-100, water and 10 μl eluate. Incubation was for 1 hour at 37°C. The [^{3}H]-ceramide released was counted in 10 ml of a toluene scintillant with a Beckman LS-255 liquid scintillation spectrometer. The blank value was 100-150 CPM and counting efficiency was 40%.

A unit of activity refers to the hydrolysis of one nanomole of sphingomyelin per hour at 37°C. Protein was measured according to Lowry et al (10).

RESULTS

Isoelectric Focusing of Normal Liver Sphingomyelinase

Two major species of sphingomyelinase were resolved by isoelectric

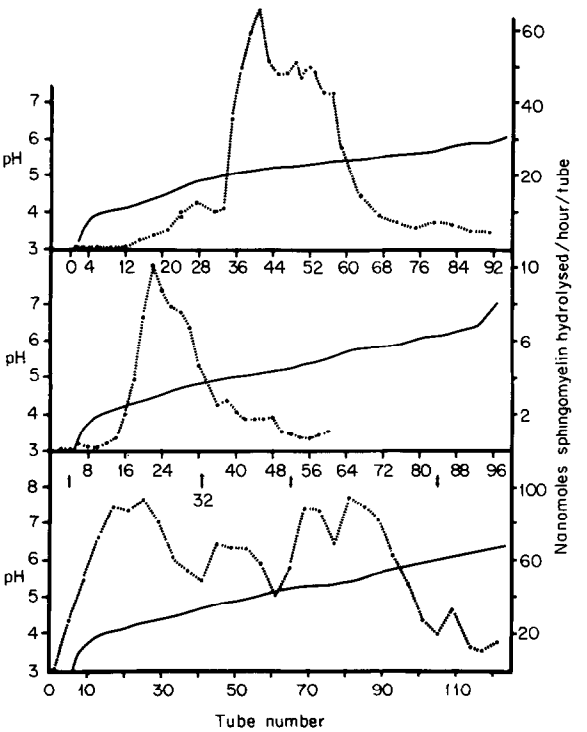

Figure 1: Isoelectric focusing of normal human liver. Details of the extraction and other experimental conditions are presented in Methods. In the lower panel, the crude liver extract (283 mg protein, 7500 units of sphingomyelinase) was focused for 43 hours at 500 V max. Tubes 5-41 were pooled and when re-focused (475 units) gave a pI of 4.5. (Isoenzyme I, Fig. 1 Middle panel). Tubes 65-105 (1783 units) were pooled and re-focused as shown in Fig. 1 Upper. Isoenzyme IIa had a pI 5.15 and Isoenzyme IIb a pI 5.2-5.3. The pH gradient is the solid line. Recovery of enzyme was 69-80%.

focusing of human liver extracts (Fig. 1). The first (Isoenzyme I) when re-focused under the same conditions had a pI of 4.5 at 3°C whereas the second species (Isoenzyme II) showed a peak at pI 5.15 (IIa) and a lower plateau of activity in the range 5.2-5.4 (IIb).

Enzymes IIa and IIb were not resolved in this experiment. Recovery of enzyme activity was generally 69-80 per cent for the first fractionation. When isoenzyme I was dialyzed prior to re-focusing about 63 per cent of the enzyme was recovered in the protein pellet after centrifugation. The resulting supernatant fluid was used as enzyme source (475 units). In

this instance alone recovery was low (34%), presumably due to enzyme instability in dilute solution. Two minor isoenzymes of sphingomyelinase were also identified in crude extracts but they have not been studied to date.

Fractions of re-focused enzyme were pooled, centrifuged and studied further. Isoenzyme I differs from IIa and IIb in pH optimum and Km values. Species I showed a pH optimum of 4.8 in acetate buffers and this was not altered after dialysis for 48 hours against 0.05 M sucrose. The pH optimum for species IIa and IIb were more acidic (Figure 2). IIa

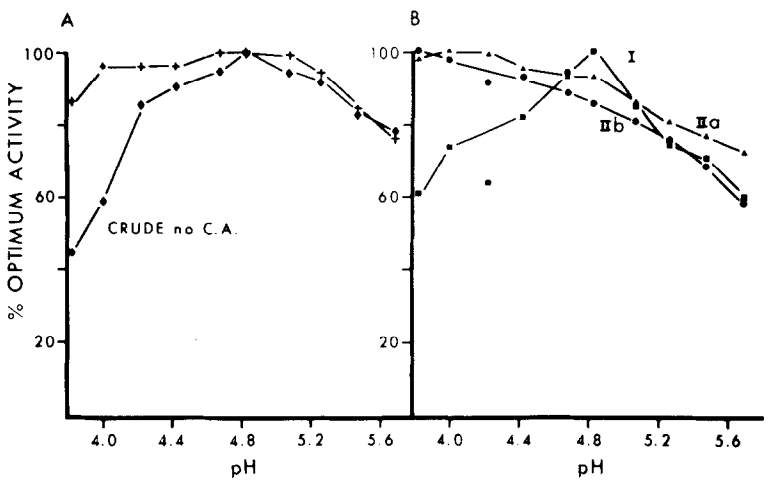

Figure 2: Sphingomyelinase activity as a function of pH. Eluates from the re-focused enzymes were pooled and assayed in duplicate. In A, the crude extract was examined with (✚ —— ✚) and without (◆ —— ◆) carrier ampholines. In B, Isoenzyme I (■ —— ■) has a pH optimum different from either isoenzyme IIa (▲ —— ▲) or IIb (● —— ●). Acetate buffers were used and the units of activity were expressed as a function of the optimum value.

gave an optimum at pH 4.0-4.2 while IIb had a pH optimum of 3.8. As in the case of I, the pH optima of IIa and IIb did not change after dialysis. The crude unfractionated liver extract showed a pH optimum of 4.8 both in the presence and absence of carrier ampholines. At pH 5.0, isoenzyme I had a Km value of 26 μM while the Km values of IIa and IIb were the same at pH 4.0 (14 μM).

Isoelectric Focusing of Niemann-Pick C Liver Sphingomyelinase

The pattern obtained for the normal liver indicates the presence of two major species of sphingomyelinase in approximately equal amounts (I and II). It is not clear whether species IIa and IIb are distinct isoenzymes since they share similar properties. When liver from a case of Niemann-Pick disease Type C was analyzed only one major isoenzyme could be discerned (Figure 3). This isoenzyme had a pI range of 4.5-4.7 and

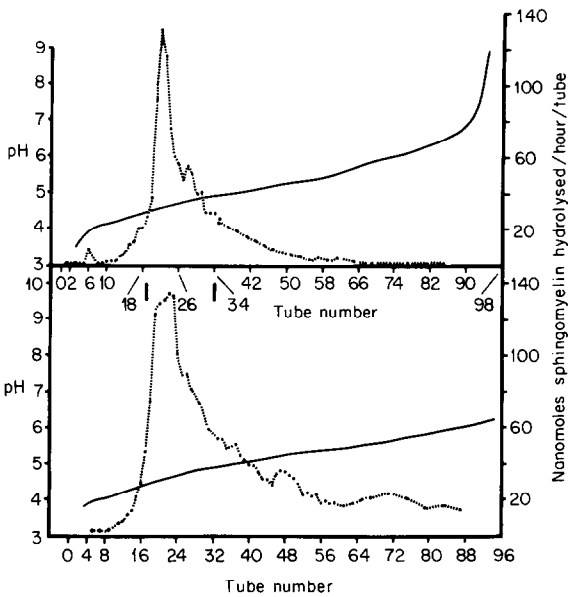

Figure 3. Isoelectric focusing of Niemann-Pick Type C liver. The extract prepared from one gram of tissue contained 62 mg protein and 3,500 units of sphingomyelinase (Lower). The pH gradient measured at 4°C is shown as the solid line. Recovery of enzyme was 79%. The enzyme in tubes 17-32 was pooled and re-focused with a pI of 4.6 (Upper).

when re-focused under the same conditions, the pI was 4.6. There was a small peak of activity (pI 5.2) in the region corresponding to isoenzyme II in normal liver. The sphingomyelinase isoenzyme remaining in the diseased liver was found to have a pH optimum of 4.8-5.0 and the same Km as isoenzyme I of normal liver. Liver from a case of G_{M2}-gangliosidosis contained the two major isoenzymes seen in normal tissue.

DISCUSSION

Two major isoenzymes of sphingomyelinase have been identified in normal human liver. Both have different isoelectric points, pH optima and Km values at their pH optimum. In a liver from a known case of Niemann-Pick disease Type C, isoenzyme I was the only species present. The absence of isoenzyme II likely represents the genetic defect in this form of the disease. The presence of multiple species of sphingomyelinase in human liver raises the question of their roles in sphingomyelin metabolism and their subcellular localization. It is possible that one enzyme species may act in concert with the other or as a modifier of the action of the other. It is also possible that each species is localized in a different region in the lysosomal ultrastructure or in different cell types. Any one of the above hypotheses could explain the storage of sphingomyelin in the presence of the enzyme in Type C liver. Experiments are in progress to provide answers to these questions.

In preliminary experiments on the brain, we have recognized only one major species of sphingomyelinase. It has a pI of 4.6 which is similar to isoenzyme I of liver. The presence of this enzyme in normal brain coupled with the persistence of the same enzyme in Niemann-Pick Type C liver may explain the lack of sphingomyelin storage in the brain in patients with this disease. Similarly, deficiencies in either isoenzyme I or II or both likely underline the genetic defects in the other forms of Niemann-Pick disease.

ACKNOWLEDGEMENT

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