Enzymatic Hydrolysis of Sphingomyelins: Use in Structure Analysis

KARL-ANDERS KARLSSON

Institute of Medical Biochemistry, University of Göteborg, Göteborg, Sweden

Sphingomyelins, the major mammalian sphingolipids, are usually characterized by analysis of their lipid soluble hydrolysis or methanolysis products (fatty acids, long-chain bases). Methods in common use [1–3] for long-chain bases involve degradation of sphingomyelins in hydrochloric acid-containing water-methanol mixtures. However, besides incomplete [4–6] dephosphorylation of bases (50 %) and by-product formation (*e.g.* from allylic bases), this type of analysis does not reveal different fatty acid-base combinations. The recent finding in sphingomyelins of trihydroxy bases [7] (phytosphingosine and related bases) in addition to dihydroxy bases (sphingosine and related bases), and 2-hydroxy fatty acids [8] in addition to normal fatty acids, makes many amide combinations possible, which may have structural importance in relation to, *e.g.*, membrane functions. Concerning phosphoglycerides Renkonen [9] and others [10] have used an enzymatic conversion to less polar compounds (diglycerides) for the characterization of molecular species. In a similar way Renkonen [9,10] outlined the separation of blood plasma sphingomyelins, in the form of ceramide (fatty acid-base amide) diacetates, into groups according to unsaturation, using silver nitrate impregnated adsorbents. The present method is an extension of this principle and takes advantage of a commercially available enzyme preparation capable of converting sphingomyelins to ceramides, which are better separable [7,9] and more easily cleaved [6] by alkali (no by-product formation) than sphingomyelins. After the suggestion was made [11] of this procedure for long-chain base analysis, earlier unknown sphingomyelin ceramides [7,8] and long-chain bases [8,12] have been isolated and characterized, and the method is now in use in other laboratories.[5]

Crude bacterial preparations of lecithinase C (phosphatidylcholine: choline phosphohydrolase, EC 3.1.4.3) have long been known [13] to degrade, in addition to phosphatidylcholines to diglycerides, sphingomyelins to ceramides. This has been considered to be caused by a single enzyme, but recent experiments [14] demonstrate phosphatidylcholine and sphingomyelin hydrolysis in separate fractions. The enzyme preparation of the present work (α-toxin from *Clostridium welchii*, Sigma Chemical Company, St. Louis, U.S.A.) has been used without further purification and given reproducible results with different batches during a three year period of use. With a substrate-toxin ratio of 100 human brain sphingomyelins are degraded quantitatively within 30 min, using the conditions described below. Ceramides and no free fatty acids or long-chain bases are found in the lipid phase, and phosphorylcholine and no choline detected in the water phase. The influence of ceramide structure variation on hydrolysis is summarized in Table

Table 1. Behavior of different sphingomyelin species towards α-toxin treatment.

	Composition of ceramide part		Hydrolysis
	Fatty acid	Long-chain base	
1.	Normal acid	Monohydroxy base (Sphingine)	+
2.	Normal acid	Dihydroxy base	+
3.	Normal acid	Trihydroxy base	+
4.	Normal acid	Dihydroxy base acetylated in position 3	−
5.	2-Hydroxy acid (D,L) [a]	Dihydroxy base	+
6.	2-Acetoxy acid (D,L) [a]	Dihydroxy base	−

[a] Synthetic compounds (K.-A. Karlsson, I. Pascher, unpublished).

1. For analytical and microscale preparative purposes the following procedure has been adopted.

A solution containing 2—20 mg of sphingomyelins is evaporated in a 15 ml test tube with a teflon-faced screw cap (Kimble Products, Toledo, Ohio, U.S.A.). 1 mg of toxin, 5 ml of Tris-HCl buffer [9] pH 7.3 (6.1 g tris (hydroxymethyl) aminomethane, 2.2 g $CaCl_2 \cdot 6H_2O$, 3.5 ml hydrochloric acid, water ad 1000 ml) and 5 ml of diethyl ether are added. The tube is shaken in a horisontal position overnight at room temperature (the solubilization of more polar sphingomyelins may proceed slowly). Ceramides are extracted with 5 times 5 ml of diethyl ether.

For thin-layer chromatography 0.15 mm thick layers of Silica gel G (Fluka) are dried for 30 min at 120°C immediately before sample application (about 0.1 mg) and development (chloroform-methanol 95:5, v/v). Spots are made visible by iodine vapor. Different groups of ceramides are shown in Fig. 1. Identification was made by infrared

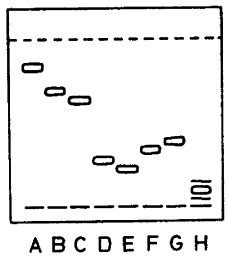

Fig. 1. Thin-layer chromatographic properties of some ceramide species. Ceramides with the following fatty acid and long-chain base composition are shown. *A*: Normal fatty acid and monohydroxy base (sphingine). *B*: Normal, C_{24}, fatty acid and monounsaturated dihydroxy base. *C*: Normal, C_{18}, fatty acid and monounsaturated dihydroxy base. *D*: 2-D-hydroxy, C_{24} fatty acid and monounsaturated dihydroxy base. *E*: 2-D-hydroxy, C_{18}, fatty acid and monounsaturated dihydroxy base. *F*: 2-L-hydroxy, C_{18}, fatty acid and saturated dihydroxy base. *G*: Normal, mostly C_{24}, fatty acids and saturated trihydroxy base.[7] *H*: 2-hydroxy, mostly C_{26}, fatty acids and saturated trihydroxy base. Combination *H* is cerebrin from *Torulopsis utilis* [22] and has not yet been found in sphingomyelins. All bases are of natural configuration, that is *erythro* for dihydroxy and *ribo* for trihydroxy bases.

spectroscopy, analysis of hydrolysis products and by comparison with synthetic compounds (K.-A. Karlsson, I. Pascher, unpublished). For microscale preparative purposes about 5 mg of ceramides are separated on each plate. After development and indication with iodine, bands are scraped off and transferred to a column of 9 mm i.d. packed with 1 g of silicic acid. Ceramides are eluted with 20 ml of chloroform-methanol 2:1, v/v. For complete removal of fine adsorbent particles a re-chromatography on an identical column may be performed, using 10 ml of chloroform-methanol 95:5, v/v, as eluent. For the separation of the indicated groups of ceramides (Fig. 1) according to unsaturation (fatty acids and bases may have up to two double bonds) an earlier described procedure [9] may be used. Similarly to the analysis of glycerides [15] isolated groups (homologs) of ceramides may finally be separated according to carbon numbers by gas chromatography of their trimethylsilyl ethers.[16]

For component analysis ceramides are degraded [17] for 10 h by reflux in 1 M KOH in methanol-water 9:1, v/v (less than 10 mg lipid per ml). After acidification to pH 1 with hydrochloric acid and partition (chloroform-methanol-water 8:4:3, v/v/v), the lower phase is evaporated and fatty acids and long-chain base hydrochlorides eluted from silicic acid (column as above) with 10 ml of chloroform-methanol 98:2, v/v, and 1:3, v/v, respectively. Fatty acids are esterified overnight at 60°C in 2 ml of 1 M HCl in methanol in a test tube with a teflon-faced screw cap. After partition by addition of 4 ml of chloroform and 1.5 ml of water, the lower phase is evaporated and analyzed by gas chromatography.[8,18] About 0.5 mg of long-chain base hydrochlorides may be oxidized for 1 h at 50°C with about 10 mg of lead tetraacetate in 0.1 ml benzene. After addition of 1 ml of water the derived aldehydes are extracted with 2 ml of heptane and analyzed by gas chromatography.[19] Alternatively, trimethylsilyl derivatives of free bases [3,11] or *N*-acetyl bases [3] may be analyzed, preferably in combination with mass spectrometry.[3,11] However, for a closer characterization (*e.g.* configuration) or isolation of individual long-chain bases *N*-dinitrophenyl derivatives are preferred.[4,7,12,19–21] Using these procedures natural configurations of bases are retained.[21]

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