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A mass spectrometry-based method for the assay of ceramide synthase substrate specificity

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ABSTRACT

The acyl composition of sphingolipids is determined by the specificity of the enzyme ceramide synthase (EC 2.3.1.24). Ceramide contains a long-chain base (LCB) linked to a variety of fatty acids to produce a lipid class with potentially hundreds of structural variants. An optimized procedure for the assay of ceramide synthase in yeast microsomes is reported that uses mass spectrometry to detect any possible LCB and fatty acid combination synthesized from unlabeled substrates provided in the reaction. The assay requires the delivery of substrates with bovine serum albumin for maximum activity within defined limits of substrate concentration and specific methods to stop the reaction and extract the lipid that avoid the non-enzymatic synthesis of ceramide. The activity of ceramide synthase in yeast microsomes is demonstrated with the four natural LCBs found in yeast along with six saturated and two unsaturated fatty acyl-coenzyme As from 16 to 26 carbons in length. The procedure allows for the determination of substrate specificity and kinetic parameters toward natural substrates for ceramide synthase from potentially any organism.

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Sphingolipids are bioactive molecules that can enact profound outcomes on cell fate in the form of cell division or cell death [1–4]. Ceramides are synthesized de novo from fatty acyl-coenzyme A (CoA)[1] and long-chain base (LCB) that when phosphorylated may also affect cell fate decisions. Indeed, the ratio between long-chain base phosphate and ceramide is proposed to function as a rheostat that regulates cell fate [5–7]. The synthesis of ceramide, therefore, is a critical reaction in sphingolipid metabolism that has the potential to coordinate LCB and ceramide levels [8–10]. In addition, the LCB and fatty acid combinations of the ceramide, and thus the final complex sphingolipid, are important components in determining the ultimate role of the individual sphingolipids in the cell [11–14]. Ceramide is synthesized by the enzyme sphingosine *N*-acyl transferase (EC 2.3.1.24), commonly referred to as ceramide synthase. In many organisms, ceramide synthase has multiple isoforms with different specificities for LCB and fatty acyl-CoA substrates that contribute significantly to the variation found in sphingolipid structure [15]. For example, in plant ceramides up to 10 different LCBs are found combined with 14 or more different fatty acids to produce hundreds of theoretical species of ceramide [16]. Given the importance of the ceramide synthase reaction to the overall composition of the cell's sphingolipid profile, the enzymatic and regulatory properties of the individual isoforms have been the subject of significant investigation [17].

A thorough enzymatic characterization of ceramide synthase is challenging due to the fact that ceramide synthase is an integral membrane protein and its substrates and products are not readily soluble in aqueous solutions [18]. Although ceramide synthase can be solubilized from the membrane using detergents such as octylglucoside [19] and digitonin [20], most of the activity is lost; hence, the majority of reports have characterized the enzyme in isolated membranes [21]. The most common method to assay ceramide synthase in vitro is through the use of radiolabeled 3,4-^{3}H dihydrosphingosine ([3,4-^{3}H]DHS) prepared by reduction of sphingosine. Subsequent to the reaction, radiolabeled substrates and reaction products are separated by normal-phase thin-layer chromatography and quantified. Using this approach, previous reports have detailed the substrate specificities for the mammalian ceramide synthases: CerS1 (C18 CoA) [22], CerS2 (C22–C26 CoAs) [23], CerS3 (C18–C20 CoAs) [24], CerS4 (C18–C20 CoAs) [25], CerS5 (C16 CoA) [25], and CerS6 (C14–C16 CoAs) [26]. This methodology is eminently suitable for investigation of mammalian sphingolipid metabolism where the predominant LCB is DHS.

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[1] *Abbreviations used:* CoA, coenzyme A; LCB, long-chain base; DHS, dihydrosphingosine; LC–MS, liquid chromatography coupled to mass spectrometry; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; MTBE, methyl-*tert*-butyl-ether; MeOH, methanol; PC, phosphatidylcholine.

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However, in plants and certain fungi, the predominant LCB found in sphingolipids is 4-hydroxy-DHS or phytosphingosine along with a number of different unsaturated LCBs. None of these is readily available as a radiolabeled substrate, meaning that if it is to be used to measure ceramide synthase activity, a different method for detecting the products of the ceramide synthase assay must be employed. Other methodologies to assay ceramide synthase activity are conducted using radiolabeled fatty acyl CoAs [27], fluorescent LCB analogues [28], in vivo feeding experiments with radiolabeled substrate [24,29], and mass spectrometry [28,30] along with synthetic odd-chain substrates [31,32]. Although these methods work in detecting produced ceramide, they all have distinct limitations. In vivo assays are of only limited use in characterizing enzyme activity, whereas not all acyl-CoAs/fatty acids or LCBs are available as radiolabeled substrates and the cost of purchasing multiple radiolabeled CoA substrates is significant. To circumvent the difficulties with obtaining radiolabeled substrates, a method to assay ceramide synthase activity in vitro was developed that uses non-radiolabeled phytosphingosine (t18:0) or dihydrosphingosine (d18:0) and detection of the products of the ceramide synthase reaction by liquid chromatography coupled to mass spectrometry (LC–MS). This was applied to the assay of ceramide synthase in *Saccharomyces cerevisiae* using purified natural LCB and fatty acid substrates.

Materials and methods

All chemicals, unless otherwise indicated, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acyl-CoAs and lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA). LCBs were purchased from Matreya (Pleasant Gap, PA, USA). Solvents were OmniSolv grade from EMD Millipore (Billerica, MA, USA) unless otherwise noted. Chloroform (ethanol stabilized) was obtained from Thermo Fisher Scientific (Rockford, IL, USA).

Purification of LCB substrate from S. cerevisiae

LCBs were hydrolyzed from 1 g of lyophilized yeast as described previously [33]. After hydrolysis, total LCBs were separated from fatty acids by weak cation exchange solid-phase extraction (Supelclean LC-WCX SPE, Sigma–Aldrich). The cartridge was equilibrated with 4 ml of 0.5 N acetic acid in methanol followed by 7 ml of methanol, and the LCB sample was applied in 4 ml of diethyl ether/acetic acid (98:5, v/v). The cartridge was washed with 10 ml of chloroform/methanol (3:1) to remove all traces of fatty acids, and the bound LCBs were eluted with 4 ml of 1 N acetic acid in methanol. Individual LCBs were purified by semi-preparative, reverse-phase high-performance liquid chromatography (HPLC) on a Zorbax XDB C18 column (9.4×250 mm; Agilent Technologies, Santa Clara, CA, USA) using a Shimadzu Prominence HPLC device and an FRC-10A fraction collector. LCBs were separated by a binary gradient of buffer A (10 mM ammonium acetate and 20% methanol, pH 7.0) and buffer B (methanol) at a flow rate of 1.5 ml/min and a column temperature of 30 °C with a gradient as described previously [16]. Fractions containing the relevant LCBs were identified by mass spectrometry, pooled, dried under nitrogen, and quantified by *o*-phthalaldehyde derivatization as described previously [33].

Microsome isolation

S. cerevisiae strain BY4741 was maintained on YPD Broth (RPI Y20090) agar plates. A liquid batch culture was grown to OD_{600} of 2, and the cells were harvested by centrifugation at 8000*g* for 10 min. The resulting pellet was washed once with 40 ml of sterile water and harvested by centrifugation as before. The washed cells were resuspended to a final concentration of OD_{600} = 200/ml in TNE buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, and 1 mM phenylmethylsulfonyl fluoride) containing Protease Inhibitor Cocktail and 1 μl/ml TNE buffer. Cells were lysed at 4 °C by vortexing with 0.5 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA) for 1 min followed by 1 min on ice, repeated 10 times. Cell debris was removed by centrifugation at 4 °C for 10 min at 8000*g*. The supernatant was removed and centrifuged a second time as before. The supernatant was removed and centrifuged at 100,000*g* for 1 h at 4 °C, and the pellet was resuspended by gentle pipetting in reaction buffer (20 mM potassium phosphate [pH 7.5] and 250 mM sorbitol). The microsomes were harvested again by centrifugation at 100,000*g* for 1 h at 4 °C, followed by final resuspension in reaction buffer using a Dounce homogenizer. Microsomes were snap-frozen in liquid nitrogen and stored at −80 °C. Protein concentration was measured using the Pierce BCA (bicinchoninic acid) Protein Assay (Thermo Fisher Scientific) with bovine serum albumin (BSA) used as a standard curve.

BSA/LCB complex formation

Fatty acid-free BSA (Sigma–Aldrich A7030) and LCB were used to create BSA/LCB complexes. Stock solutions of BSA were made in reaction buffer (w/v), and the LCB was dissolved in 2:1 (v/v) ethanol/dimethyl sulfoxide (DMSO) to a final concentration of 2 mM. The complexes were made with a final BSA concentration of 100 μM and varying amounts of LCB not exceeding 150 μM. An additional 2:1 (v/v) ethanol/DMSO was added as necessary to standardize all solutions at 10% by volume 2:1 (v/v) ethanol/DMSO. The final concentration of the BSA/LCB complex was a 10× solution for direct addition to the ceramide synthase assay.

LCB equilibration into microsomes

First, 10× LCB/BSA complexes were made as described above. In addition, a BSA-free 10× LCB solution was made exactly as described above but omitting the BSA. Then, 10 μl of 15 μM LCB solution was added to an 8-ml glass tube with a Teflon-lined screw cap containing 20 mM potassium phosphate (pH 7.5), 250 mM sorbitol, and microsomes containing 10 μg of protein in a final volume of 100 μl and incubated in a digital heating block at 30 °C for 10 min. Reactions were then moved to an ultracentrifuge tube and spun at 100,000*g* for 1 h. The supernatant was removed, and LCBs were extracted by the addition of 750 μl 1:1 (v/v) methyl-*tert*-butyl-ether (MTBE)/methanol (MeOH) followed by the addition of 5 nmol of d17:1 LCB as an internal standard, 850 μl of MTBE, and 312 μl of 100 mM ammonium hydroxide. The MTBE layer was removed and dried under a stream of air at 60 °C. LCBs were resuspended in 100 μl of tetrahydrofuran/methanol/water (2:1:2, v/v/v) containing 0.1% formic acid and analyzed by LC–MS.

Ceramide synthase in vitro assay

The assay was performed in an 8-ml glass tube with a Teflon-lined screw cap and a final volume of 100 μl. The reaction mix contained a final concentration of 20 mM potassium phosphate (pH 7.5), 250 mM sorbitol, 50 μM acyl-CoA, 10 μM BSA, up to 15 μM LCB, and up to 10 μg microsomal protein. All components for the assay, except the microsomal protein, were mixed with a pipet tip and equilibrated for 10 min at 30 °C in a digital heating block. The reaction was started by the addition of the microsomal protein with gentle mixing using a pipet tip and incubated for 30 min. To stop the reaction, 750 μl of 1:1 (v/v) MTBE/MeOH was added and mixed with a vortex mixer. Then, 50 pmol of C12 ceramide was added as an

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