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Abstract

Fumonisin B_1 is a mycotoxin produced by fungi of the genus *Fusarium,* common pathogens of corn and other grain plants. Toxic effects associated with fumonisin B_1 include equine leukoencephalomacia, porcine pulmonary edema, rat renal carcinoma, and murine hepatocellular carcinoma. Increased risk for esophageal cancer in humans has been epidemiologically associated with consumption of corn contaminated with *Fusarium*, suggesting that fumonisin B_1 may be involved. The biological effects of fumonisin B_1 exposure result primarily from disruption of *de novo* sphingolipid biosynthesis via inhibition of ceramide synthase. Exposure of animals or cultured cells to fumonisin B_1 results in the characteristic accumulation of sphinganine, a toxic sphingolipid intermediate, concomitant with depletion of essential complex sphingolipids.

Ceramide synthase has not been purified to homogeniety and characterized. We prepared crude ceramide synthase from detergent-extracted rat liver homogenates using PEG-precipitation and cation exchange chromatography. Ceramide synthase activity was then sequestered, using fumonisin B_1 covalently coupled to POROS-NH$_3$ particles, and eluted selectively. The observed 119-fold enrichment in specific activity demonstrates the utility of fumonisin-POROS affinity chromatography in the purification of ceramide synthase.

Introduction

Fumonisin B_1 (FB1) is a toxin produced by members of the fungal genus *Fusarium* (Gelderblom et al., 1988; Thiel et al., 1991). *Fusarium moniliforme,* a common source of FB1, is a typical fungal contaminant of economically important cereal crops. The infection of corn with *Fusarium,* particularly problematic after periods of drought-induced stress followed by warm, moist weather, produces ear rot and stalk rot diseases that can be devastating to crop productivity (Schaafsma et al., 1993).

FB1 produces diverse pathological effects in animals consuming FB1-contaminated food. FB1 causes equine leukoencephalomacia in horses (Marasas et al., 1988), pulmonary edema in swine (Harrison et al., 1990), immunosuppression in turkey poults (Li et al., 2000), and renal toxicity in rabbits (Gumprecht et al., 1995; Bucci et al., 1998). Two-year FB1 exposure studies in rodents, funded by the National Toxicology Project and performed at the National Center for Toxicological Research, were recently completed. These studies demonstrated that dietary exposure to FB1 produced hepatic carcinoma in female B6C3F$_1$/NCTR mice and renal carcinoma in male Fischer 344/NCTR rats (Howard et al., 1999). Epidemiological studies have associated the consumption of corn contaminated with *Fusarium* with esophageal cancer in humans (Marasas et al., 1988; Chu and Li, 1994).

Disruption of sphingolipid biosynthesis via inhibition of ceramide synthase appears to be the principal biochemical effect of exposure to FB1 (reviewed in Merrill et al., 1995). FB1 is structurally similar to the sphingoid base substrates of the ceramide synthase catalyzed reaction (Fig. 1). Sphinganine, an acyl group acceptor substrate for the ceramide synthase catalyzed reaction, accumulates rapidly in cultured cells or tissues exposed to FB1. Ceramides can be liberated from complex sphingolipids via enzymatic hydrolysis or produced via *de novo* biosynthesis through the ceramide synthase catalyzed reaction. Increased ceramide levels produced by either mechanism have been associated with apoptosis (Mathias et al., 1998). Blocking the ceramide synthase catalyzed N-acylation of sphinganine by FB1 also causes depletion of complex sphingolipid pools, including: dihydroceramides, ceramides, glucosylceramides, sphingomyelin, gangliosides, cerebrosides, and sulfatides. Maintenance of complex sphingolipid pools are important for cell survival and depletion of complex sphingolipids contributes to FB1 toxicity (Yoo et al., 1996; Tolleson et al., 1999).

The regulation of ceramide synthase catalytic activity must be critical for cell survival because it affects the concentrations of sphingolipid mediators that influence cell survival and death processes. Furthermore, signal transduction pathways regulating ceramide synthase gene expression and its enzymatic activity may exert an effect on the homeostatic balance of strategic sphingolipids. The first step in understanding the biochemical nature of ceramide biosynthesis is the isolation of the active enzyme. Several groups have reported the partial purification of ceramide synthase, but none have been successful in completing its isolation to homogeniety. We endeavored to test a FB1 affinity chro-

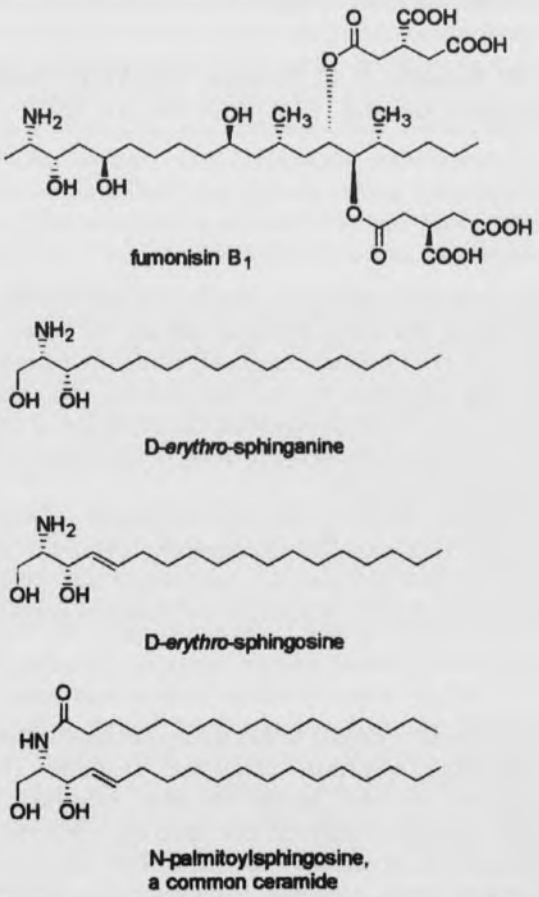

Fig. 1. Structures of fumonisin B_1, sphinganine, sphingosine, and N-palmitoylsphingosine, depicting stereochemical similarities.

matography column for isolating fumonisin-binding proteins expected to be present in rat liver. We previously discovered that our FB1 affinity column sequestered rat liver argininosuccinate synthetase from rat liver extracts and that both rat liver and recombinant human argininosuccinate synthetases are inhibited by FB1 *in vitro* (Ki' = 6 mM and 35 mM for human and rat liver argininosuccinate synthetase, respectively; Jenkins et al., 2000). Although ceramide synthase is believed to represent the most significant target for the biological action of FB1, other enzyme systems are affected by FB1. Fukuda *et al.* (1996) showed that FB1 inhibits recombinant rat protein phosphatase 5 (PP5) with an IC_{50} of 80 µM and PP1γ2, PP2A, PP2B, and PP2C with IC_{50} values 300-1000 µM. PP5 is reportedly expressed in many tissues, including hepatocellular carcinoma cell lines, but it is poorly expressed in normal liver samples and would not be expected in the sample used in this study (Shirato et al., 2000). We predicted that ceramide synthase or other FB1-binding proteins could be recovered from rat liver extracts using FB1 affinity chromatography.

Materials and methods

Sample Preparation.--All sample processing and chromatographic procedures were performed at 4°C except where indicated. Rat liver extract was prepared by mechanical distruption (Polytron, Brinkmann Instruments Company, Westbury, NY) of frozen rat livers in 50 mM sodium phosphate buffer, pH 6.5 containing 10 mM EDTA, 150 mM sodium chloride, and 2% Triton X-100™ followed by 30 min incubation at 0°C. Cellular debris and other insoluble matter was removed by centrifugation for 15 min at 21,000g. Polyethylene glycol (PEG) was added to 10% (w/v) to the supernatant and contaminating proteins were removed from the rat liver detergent extract by precipitation and centrifugation. Ceramide synthase activity was precipitated by increasing the PEG concentration to 20%. In some cases rat liver cytosol was prepared by disrupting rat liver samples in the absence of detergents, then removing cellular debris and organelles by high speed centrifugation, adding Triton X100 to 2% and preparing cytosolic 10-20% PEG fractions. The 10-20% PEG precipitates were collected by centrifugation and redissolved in 25 mM sodium phosphate buffer, pH 6.5 containing 1 mM EDTA and 2% Triton X-100, and applied to a 5x20 cm SP-Sepharose strong cation exchange column. Ceramide synthase activity was eluted from the cation exchange column using an increasing pH and salt gradient (linear gradient from 0-40% Buffer B over 120 min; flow rate 3.0 mL/min; Buffer A: 25 mM sodium phosphate, 1 mM EDTA, 0.2% Brij-35, pH 6.5; Buffer B: 25 mM sodium phosphate, I mM EDTA, 0.2% Brij-35, 2.0 M sodium chloride, pH 7.5). Fractions containing ceramide synthase activity were pooled and dialyzed twice against 50 volumes of 50 mM sodium phosphate, 1 mM EDTA, 0.2% Brij-35, pH 6.8.

POROS-FB1 Affinity Chromatography.--The POROS-FB1 affinity matrix was prepared by coupling FB1 tricarbyllate groups to free primary amine groups on 20 µm POROS-NH™ particles (Perceptive Biosystems, Foster City, CA), as previously described (Newkirk etal., 1998; Jenkins et al., 2000). A suspension of POROS-FB1 matrix was slurry packed into a 0.46 cm x 10.0 cm polymer column jacket fitted with 2 µm end frits and installed into a Varian ProStar biocompatible HPLC system equipped with a Varian ProStar variable wavelength UV/Vis detector, a BioRad Econo Gradient monitor, and a BioRad Model 2128 fraction collector. Data were collected and processed using MacIntegrator II Software (Varian) and a Macintosh Quadra personal computer, which also provided control for the pump units.

The POROS-FB1 affinity column was equilibrated in dialysis buffer and loaded at 4 mL/min. Ceramide synthase activity was eluted from the POROS-FB1 column using a two-step gradient of increasing ionic strength (linear gradient from 0-25% Buffer B from 0-10 minutes, 25-100% Buffer

B rom 10-15 min; flow rate 4 mL/min; Buffer A: 50 mM sc ium phosphate, 1 mM EDTA, 0.2% Brij-35, pH 6.8; B fer B: 2.0 M sodium chloride dissolved in Buffer A).

Analytical Procedures.--Protein concentrations were d ermined using the Pierce BCA Protein Assay Kit with b vine serum albumin standards (Pierce Endogen, R ckford, IL) according to the manufacturer's specifica- ti ns. Denaturing polyacrylamide gel electrophoresis was p formed using 4-20% gradient Ready Gels (BioRad, H rcules, CA) with BioSafe Coomassie Stain. Ceramide sy thase activity was measured using the method described b Hirshberg et al. (1993) and adapted slightly for our use (J nkins et al., 2000). Assay reactions were performed in 50 mM sodium phosphate buffer, pH 6.9. Samples were desalted prior to activity measurements by three sequential dilutions (1:10) with cold assay buffer and centrifugal ultrafiltration at 4°C using Ultrafree 0.5 microconcentrator devices (10,000 MWCO, Millipore, Bedford, MA). Desalted samples were incubated with 0.2 µCi [^{14}C]-palmitoyl coenzyme A (60 mCi/mmole, NEN, Boston, MA) and 40 µM D-*erythro*-sphingosine (Sigma Chemical Co., St. Louis, MO) in 0.25 mL reactions for 20 min at 37°C. The reactions were quenched by placing the tubes in an ice/water bath for 1 min prior to extraction. Samples containing polyethylene glycol were extracted twice with 0.5 mL diethyl ether. All other samples were extracted once with 0.6 mL 2:1 chloroform/methanol and once with 0.4 mL chloroform. The organic extracts were dried *in vacuo*, dissolved in chloroform, and applied to LK5DF silica thin layer plates (Whatman Inc., Clifton, NJ). Thin layer chromatography was performed using 90:7:3 chloroform/methanol/concentrated ammonium hydroxide. Authentic N-palmitoylsphingosine was included as a reference standard and visualized by post-run exposure to iodine vapor. [^{14}C]-N-palmitoylsphingosine reaction products were quantified by phosphorimagining (Molecular Dynamics Corporation, Sunnydale, CA) with ImageQuant software. Background reaction rates for protein-free reactions were subtracted from all measurements. Specific activity values were determined by dividing the net amount of ceramide product formed, expressed as volume integration counts, by the amount of protein added to the reaction.

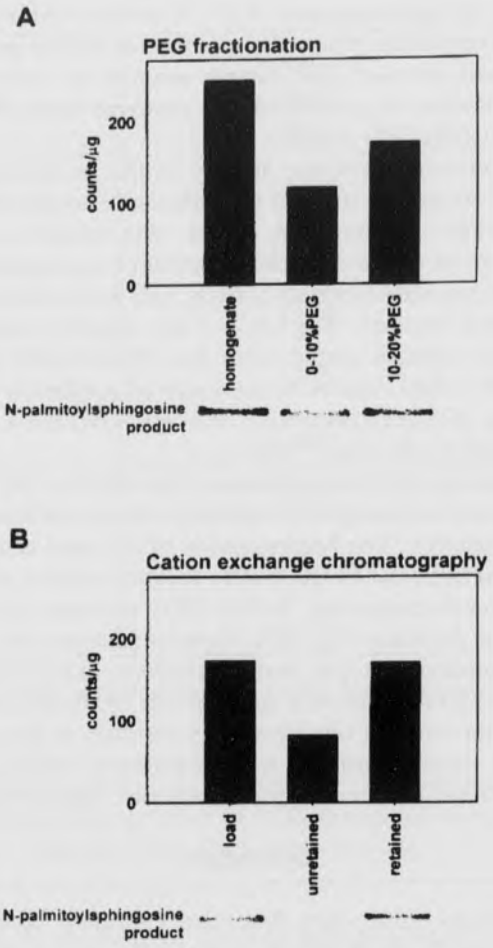

Fig. 2. Extraction of ceramide synthase activity from rat liver. (A) Ceramide synthase activity in rat liver fractions (100 µg protein/reaction) was determined as given in Materials and Methods. (B) Ceramide synthase activity in cation exchange chromatography fractions. Aliquots (100 µg/reaction) from 10-20% PEG fraction (load), unretained fraction, and retained fractions were subjected to ceramide synthase assay as described in Materials and Methods. Insets displayed under plots: [^{14}C]-N-palmitoylsphingosine reaction products quantified by tlc and phosphorimaging.

Results

Ceramide synthase activity was detected in rat liver detergent extracts and concentrated using polyethylene glycol precipitation (Fig. 2A). Prior work in our laboratory determined that ceramide synthase activity could be detected in the absence of detergents. However, the addition of detergents prevented excessive losses of ceramide synthase through nonspecific association with heterogenous protein precipitates (data not shown). A large amount of extraneous protein was removed by differential precipitation with PEG, and the sample volume was reduced (Table 1). Although the apparent specific activity appeared to be relatively unchanged following PEG precipitation, the presence of PEG in these samples may have interfered with enzyme catalyzed reaction. We observed that the specific activity recovered somewhat after cation exchange chromatography in the subsequent step (Fig. 2B). Ceramide synthase activity

was retained by the SP-Sepharose column and eluted at 135-140 min in approximately 0.35 M sodium chloride. UV spectroscopy of the retained and nonretained fractions (data not shown) revealed that a large amount of nonproteinaceous UV-absorbing material was removed from the redissolved 10-20% PEG fraction.

The ceramide synthase activity in the pooled and dialyzed SP-Sepharose fraction was efficiently adsorbed by the POROS-FB1 column and eluted with sodium chloride whereas most of the extraneous protein was excluded (Fig. 3A-C). Ceramide synthase activity was not detected in the unretained sample (Fig. 3C). The specific activity of ceramide synthase eluted from the POROS-FB1 column increased 17-fold relative to the material applied to the column (Fig. 3C), and the protein recovery indicated a 119-fold enrichment by this step (Table 1).

Denaturing gel electrophoresis showed that the heterogeneity of active samples correlated inversely with increased specific activity. The heterogeneity of the most active sample eluted from the POROS-FB1 column was less than that of rat liver homogenate, 10-20% PEG fractions, or the SP-Sepharose fractions (Fig. 3D). Several proteins are particularly prominent in the active fractions eluted from the POROS-FB1 column with apparent Mr of 29, 38, 51, and 74 kDa. Prior work in our laboratory resulted in the purification of a small quantity of a homogeneous 38 kDa protein that exhibited ceramide synthase activity (data not shown).

Discussion

Scribney (1966) first described ceramide biosynthesis (i.e., ceramide synthase) in brain and liver microsomes. Morell and Radin (1970) determined the acyl group specificity specificity of ceramide synthase in rat brain extracts. Later, the presence of ceramide synthase activity in liver microsomal fractions was confirmed by Narimatsu et al. (1986) and extended by Mandon et al. (1992) and Hirshberg et al. (1993) who detected ceramide synthase activity present on the cytosolic surface of the endoplasmic reticulum. Shimeno et al. (1995, 1998) also observed ceramide synthase activity in mitochondrial fractions. Studies performed in our laboratory confirmed that ceramide synthase activity could be detected in both fractions and that conditions that partially disrupt organelle integrity released ceramide synthase activity into the soluble, microsome-free cytosolic fraction. We utilized Triton X-100 as a nondenaturing detergent to extract ceramide synthase from whole liver homogenates.

The purification of ceramide synthase has been a daunting problem for several research groups. Shimeno et al. (1998) employed anion exchange chromatography followed by sphingosine affinity chromatography in the partial purification of ceramide synthase. Sphingosine was coupled to the column matrix via its primary amine group, resulting in

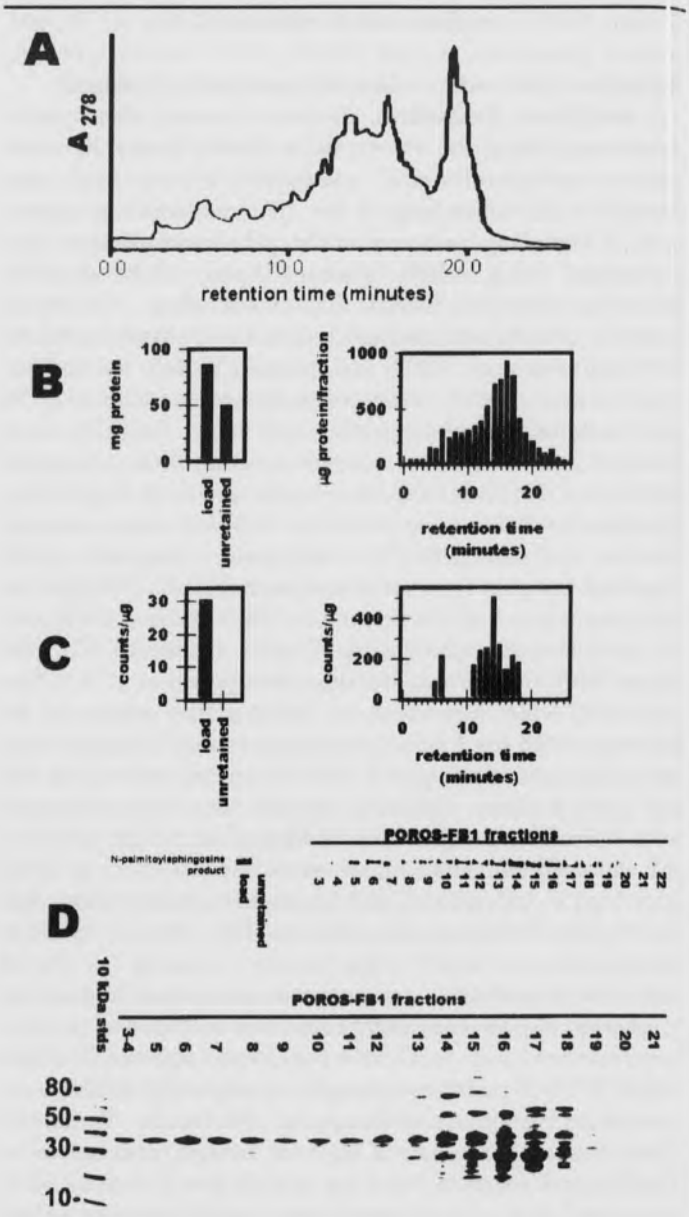

Fig. 3. POROS-FB1 affinity chromatography. (A) POROS-FB1 chromatogram. Dialyzed fraction from strong cation exchange chromatography containing crude ceramide synthase activity was applied to 0.46x10 cm POROS-FB1 column and eluted with sodium chloride. (B) Protein levels in POROS-FB1 chromatographic samples. (C) Ceramide synthase activity in POROS-FB1 chromatographic samples. Insets under plots: [^{14}C]-N-palmitoylsphingosine reaction products quantified by tlc and phosphorimaging. (D) Denaturing polyacrylamide gel electrophoresis of POROS-FB1 chromatographic samples.

Table 1 – Analysis of ceramide synthase isolation scheme

sample	volume (mL)	protein concentration (mg/mL)	protein yield (mg)
mogenate[a]	596	37.0	22,040
10% PEG fraction[a]	174	70.5	12,300
-20% PEG fraction[a]	87	31.9	2,780
SP-Sepharose (unretained)[a]	331	3.04	1,010
SP-Sepharose (retained)[a]	30	16.3	490
POROS-FB1 load[b]	28	3.10	86.7
POROS-FB1 (unretained)[b]	83.5	0.60	50.1
POROS-FB1 (retained)[b]	4	0.18	0.73

[a]These samples and those presented in Fig. 2 are related to processing 141 g rat liver as described in the Materials and Methods section.

[b]These samples and those presented in Fig. 3 are related to processing 250 g rat liver in the absence of detergent and performing a high speed centrifugation to remove nuclei, mitochondria, and microsomes prior to PEG precipitation in the presence of detergent and SP-Sepharose chromatography. Similar results were obtained with whole liver extracts.

essentially a saturated hydrocarbon, hydrophobic column matrix. Although some enrichment in specific activity was reported using this affinity matrix, it was probably due to hydrophobic interaction chromatography instead of specific ligand binding because the substrate amine subject to enzyme recognition by ceramide synthase was acylated to provide immobilization to the column matrix.

Our approach differs in that we first select for ceramide synthase using solubility in PEG. This eliminated many proteins that did not exhibit ceramide synthase activity. PEG fractionation was followed by cation exchange chromatography (SP-Sepharose) then affinity chromatography using the POROS-FB1 column. The latter chromatographic technique has been used to isolate anti-fumonisin antibodies (Newkirk et al., 1998). That study demonstrated that unique FB1 epitopes available for specific protein binding were present in the POROS-FB1 matrix. Additionally, argininosuccinate synthase was isolated as a protein that bound to POROS-FB1, and inclusion of FB1 inhibited its catalytic activity (Jenkins et al., 2000). POROS-FB1 affinity chromatography has proven to be useful and versatile in the purification of FB1 binding proteins.

The complete purification of ceramide synthase remains an elusive goal. Its hydrophobicity and tendency to associate nonspecifically with other proteins present obstacles for its further purification. The possibility that ceramide synthase exists as a component of a multi-protein complex cannot be excluded by our results. Size exclusion chromatography using Sephacryl S-200 indicated that ceramide synthase segregates with higher molecular weight protein-containing complexes (data not shown). The stability of ceramide synthase catalytic activity present in partially purified ceramide synthase seems to be lower than that of crude extracts, suggesting that the presence of additional proteins may contribute to its stability. These characteristics indicate that native ceramide synthase may be associated in complexes with other proteins.

The nature of the substrates of the ceramide synthase-catalyzed reaction creates difficulties in the accurate determination of its catalytic activity. The sphingoid base substrates for ceramide synthase are considerably hydrophobic. This presents specific biochemical problems in kinetic assays that require saturating substrate concentrations to achieve zero-order rate conditions. The hydrophobicity of sphinganine and sphingosine allows them to associate nonspecifically with many proteins via hydrophobic

interactions. The effective concentrations of these substrates can be lowered through nonspecific adsorption to extraneous proteins in a concentration-dependent manner. Some investigators presaturate carrier proteins with excess sphinganine (or sphingosine) for inclusion in ceramide synthase assays in order to prevent removal of free sphinganine by nonspecific binding. However, this results in an indeterminate amount of free and bound substrate and does not ensure adequate concentrations of free sphingoid base substrate. Thus, reaction rates observed in ceramide synthase activity measurements are generally dependent both on the concentration of the enzyme and the substrate and are only informative in a qualitative way regarding relative catalytic activity. The purification of ceramide synthase will eventually allow accurate determinations of the rate constants of this enzyme for its substrates and determination of inhibition constants for FB1 and related molecules.

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