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DRAMATIC ALTERATION OF SPHINGOLIPID BASES OF HANSENULA CIFERRII BY EXOGENOUS FATTY ACID*

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Summary: Hansenula ciferrii, a yeast which normally excretes large amounts of C_{18}-phytosphingosine in the form of its tetra-acetyl derivative, was grown in basal medium containing added pentadecanoic acid (0.1%) and Brij 58 (1%). Analyses of the sphingolipid bases recovered from the culture medium indicated the presence of significant quantities of C_{17}-phytosphingosine (39%) and C_{19}-phytosphingosine (15%) in addition to the normal C_{18}-phytosphingosine (46%). The sphingolipid bases were characterized by gas-liquid chromatography combined gas-liquid chromatography-mass spectrometry, and chemical degradation.

C_{18}-Phytosphingosine is one of the three major sphingolipid bases. The latter compounds are essential constituents of a variety of complex lipids of eukaryotes including ceramides, sphingomyelins, gangliosides, and other complex glycosphingolipids. The precise nature of the enzymatic reactions leading to the formation of C_{18}-phytosphingosine are unclear. For the most part, studies of the biosynthesis of C_{18}-phytosphingosine have involved Hansenula ciferrii, a yeast which excretes large quantities of C_{18}-phytosphingosine in the form of its tetra-acetyl derivative (1). Greene et al. (2) have demonstrated the incorporation of the label of [9,10-^{3}H]-palmitic acid and of [3-^{14}C]-serine into C_{18}-phytosphingosine in H. ciferrii. Under the same conditions efficient incorporation of the label of [9,10-^{3}H]-α-hydroxypalmitic acid into C_{18}-phytosphingosine was not observed. While the enzymatic formation of C_{18}-phytosphingosine in a cell-free preparation has not been reported, it has been shown that microsomal preparations from H. ciferrii catalyze the pyridoxal phosphate-dependent condensa-

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tion of serine and palmityl coenzyme A to yield 3-ketosphinganine which is then reduced, in an NADPH-dependent reaction, to give dihydrosphingosine (3 and references cited therein). While the formation of sphingosine has been demonstrated to occur in this system when trans-hexadec-2-enoyl coenzyme A was used as a substrate, no formation of C_{18}-phytosphingosine was detected when α-hydroxypalmityl coenzyme A was used as the substrate (3). The combined results of two laboratories (4,5) have indicated that the major, if not exclusive, pathway involved in the incorporation of palmitic acid into C_{18}-phytosphingosine in H. ciferrii occurs with the loss of only one hydrogen atom (from C-2) of palmitic acid.

In two other species of yeast, Candida lipolytica and Saccharomyces cerevisiae, de novo fatty acid biosynthesis has been reported to be suppressed by exogenous fatty acids (6). When these organisms were grown in basal medium supplemented with pentadecanoic acid (0.1%), the fatty acid composition of these yeasts was shown to have been significantly modified and to be comprised largely of fatty acids with either fifteen or seventeen carbon atoms (6). We wished to explore this effect in H. ciferrii. If exogenous pentadecanoic acid suppressed de novo fatty acid synthesis in H. ciferrii and were utilized by the organism in sphingolipid base synthesis, the formation of significant amounts of sphingolipid bases with odd-chain lengths would be anticipated. The presence of such bases in H. ciferrii has not been reported. Small amounts (3-7%) of C_{19}-phytosphingosine have been reported to be present in corn and Torulopsis utilis (7,8).

Materials and Methods

Hansenula ciferrii (mating type F-60-10; NRRL 1031) was a gift from C. P. Kurtzman (Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Illinois). Dextrose, peptone, yeast extract, and malt extract were obtained from Difco Laboratories. Brij 58 and Triton X-100 were purchased from Sigma Chemical Company. Lauric acid and pentadecanoic acid (purity of each in excess of 99% as judged by gas-liquid chromatographic analyses of the methyl esters) were obtained from Eastman Kodak and Aldrich Chemical Company, respectively.

The basal medium for the growth of the yeast contained yeast extract (0.3%), malt extract (0.3%), peptone (0.5%), dextrose (6%), Brij 58 (1%), and either pentadecanoic acid (0.1%) or lauric acid (0.1%). The medium (200 ml) was trans-

ferred to an Erlenmeyer flask (1,000 ml), autoclaved, innoculated with 5 ml of a 26 hour culture, and incubated for 74 hours on a rotary shaker at 24-26°. The cells were collected by centrifugation, washed three times with Triton X-100 (4%) and twice with water, and lyophilized.

Analysis of Fatty Acids

The lyophilized yeast was heated under reflux with 15% KOH in 90% ethanol (20 ml) for 3 hours. After cooling to room temperature, water (10 ml), 6N HCl (10 ml), and water (20 ml) were successively added. The resulting mixture was extracted 3 times with ether (50 ml portions) and the combined ether extracts were washed 3 times with water (20 ml portions) and dried over anhydrous magnesium sulfate. After evaporation of the solvent, the resulting fatty acids were esterified with diazomethane (9). The fatty acid methyl esters were analyzed by gas-liquid chromatography on a column of SP-2330 (10%) on Chromosorb W AW (Supelco, Inc.) at 180°C. Peak areas were determined using an electronic integrator (Hewlett-Packard Model 3370B). The fatty acid methyl esters were further characterized by combined gas-liquid chromatography-mass spectrometry using an LKB-9000S spectrometer equipped with a column packed with the same support as noted above. The temperature of the column was programmed from 170 to 250°C at 2° per minute. Operating conditions for the mass spectrometer were as described previously (10).

Analysis of Sphingolipid Bases

The initial supernatant obtained upon centrifugation of the fermentation medium was extracted 3 times with hexane (200 ml portions). The combined extracts were evaporated to dryness under reduced pressure. The resulting residue was dissolved in methanol (20 ml). 1N KOH (2 ml) was added and the resulting mixture was allowed to stand overnight at room temperature. After removal of most of the methanol under reduced pressure, water (20 ml) was added and the resulting mixture was extracted once with hexane (100 ml) and twice with ether (100 ml portions). The combined ether extracts were washed with water and evaporated to dryness under reduced pressure. The resulting residue, in chloroform, was subjected to chromatography on an activated silicic acid (Unisil; 100-200 mesh; Clarkson Chemical Company) column (18 cm x 0.75 cm). After the passage of chloroform (50 ml) and 97:3 chloroform-methanol (50 ml) through the column, the N-acetylated bases were eluted with 2:1 chloroform-methanol (20 ml). Portions of the N-acetylated bases were silylated according to the procedure of Carter and Gaver (11) and the resulting trimethylsilyl derivatives of the N-acetylated bases were examined by gas-liquid chromatography on a 3% OV-17 on Gas Chrom Q (Applied Science Laboratories) column at 240° and by combined gas-liquid chromatography-mass spectrometry using the same column packing and column temperature. Periodate oxidation of the N-acetylated bases was performed by the following modifications of the procedure described by Sweeley and Moscatelli (12). The sample (0.3-10 mg) was dissolved in a mixture of chloroform and methanol (1:1; 8.3 ml) and 0.2 M $NaIO_4$ (0.7 ml) was added. After standing 2.5 hours in the dark at room temperature, a mixture of methanol and water (1:1; 8.3 ml) and chloroform (10 ml) were successively added. After thorough shaking, the separated lower phase was removed and washed once with a mixture of methanol and water (1:1; 5 ml). After removal of the solvent under a stream of nitrogen, the resulting aldehydes were extracted from the residue with two 1.5 ml portions of hexane and examined by gas-liquid chromatography on a 3% OV-17 on Gas-Chrom Q column at 155°C.

Results

The growth of H. ciferrii did not appear to be affected by the presence of the pentadecanoic acid or the detergent, Brij 58, since the yield of yeast (dry

weight) from the medium containing pentadecanoic acid and the detergent (1.2 g) was comparable to that (1.1 g) from the medium containing lauric acid and the detergent, and with that found in separate experiments using basal medium alone and basal medium plus Brij 58.

Analysis of the fatty acid methyl esters indicated that the yeast grown in the medium containing pentadecanoic acid had a much higher percentage of fatty acids of odd chain length (69%; the major odd-chain fatty acids were pentadecanoic acid (28.3%) and a C_{17} monounsaturated acid (31.7%)) than did yeast grown in medium containing lauric acid (2%). The total fatty acid content (calculated as the methyl esters) of the yeast grown in the medium containing the pentadecanoic acid (11.8 mg per gm dry cells) was slightly higher than that of the yeast grown in the medium containing the lauric acid (7.8 mg per gm dry cells).

Analysis of the N-acetylated bases (in the form of their trimethylsilyl derivatives) isolated from the yeast grown in the medium containing lauric acid indicated the presence of only one (>99%) component which had the identical retention time and mass spectrum as that of the corresponding derivative of authentic N-acetyl-C_{18}-phytosphingosine. A similar analysis of the N-acetylated bases isolated from the yeast grown in the medium containing pentadecanoic acid indicated the presence of three major bases (Figure 1). The second peak had the same retention time as that of the trimethylsilyl derivative of authentic N-acetyl-C_{18}-phytosphingosine. The relative retention times of the three components (relative to that of the trimethylsilyl derivative of authentic N-acetyl-C_{18}-phytosphingosine) were 0.75, 1.00, and 1.31. There was a linear relationship between the logarithm of the retention time and the chain length (vide infra) in the three compounds.

The mass spectrum of the component corresponding to the first peak (39% of total area) in Figure 1 indicated that this material represented the trimethylsilyl derivative of N-acetyl C_{17}-phytosphingosine. Major ions in the spectrum (with their relative intensities and tentative assignments) were as follows: 546 (26%; M-CH_3), 387 (33%; C2-C3 cleavage with charge retention by the C3 fragment), 368 (22%), 297 (12%), 285 (80%; C3-C4 cleavage with charge retention by

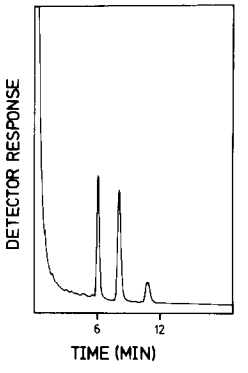

Figure 1. Gas-liquid chromatographic analysis of trimethylsilyl derivatives of N-acetyl sphingolipid bases recovered from culture medium of Hansenula ciferrii grown in the presence of basal medium supplemented with pentadecanoic acid.

the C4 fragment), 276 (78%; C3-C4 cleavage with charge retention by the C3 fragment), 247 (27%; an ion arising from a cyclic rearrangement described by Hammarstrom et al. (13)), 246 (34%), 218 (100%), 204 (18%), 191 (17%), 187 (73%), 186 (72%), 174 (40%; C2-C3 cleavage with charge retention by the C2 fragment), 157 (45%; loss of trimethylsilanol from the ion at 247 (14)), 147 (48%; $(CH_3)_2Si{=}O{-}Si{-}CH_3)_3$), 132 (91%; 174 - ketene), 116 (42%), 103 (32%; C1-C2 cleavage with charge retention on the C1 fragment), 96 (21%), 83 (16%), 75 (47%; $(CH_3)_2Si{=}OH$), and 73 (80%; $(CH_3)_3Si$).

The mass spectrum of the component corresponding to the second peak (46% of total area) in Figure 1 indicated that this material represented the trimethylsilyl derivative of N-acetyl C_{18}-phytosphingosine. Major ions in the spectrum (with their relative intensities and tentative assignments) were as follows: 560 (11%; M-CH_3), 401 (11%; C2-C3 cleavage with charge retention by the C3 fragment), 382 (8%), 311 (5%), 299 (30%; C3-C4 cleavage with charge retention on the C4 fragment), 276 (28%; C3-C4 cleavage with charge retention by the C3 fragment), 247 (11%; an ion arising from cyclic rearrangement proposed by Hammarstrom et al. (13)), 246 (12%), 218 (56%), 204 (7%), 187 (30%), 186 (29%), 174 (15%; C2-C3 cleavage with charge retention by the C2 fragment), 157 (16%; loss of trimethyl-

silanol from the ion at 247 (14)), 147 (20%; $(CH_3)_2Si{=}O{-}Si(CH_3)_3$), 132 (92%; 174 - ketene), 129 (16%), 116 (21%), 103 (13%; C1-C2 cleavage with charge retention by the C1 fragment), 96 (8%), 83 (7%), 75 (23%; $(CH_3)_2Si{=}OH$), and 73 (100%; $(CH_3)_3Si$). The mass spectrum was identical to that obtained from the trimethylsilyl derivative of authentic N-acetyl-C_{18}-phytosphingosine isolated from yeast grown in basal medium.

The mass spectrum of the component corresponding to the third peak (15% of total area) in Figure 1 indicated that this material represented the trimethyl silyl derivative of N-acetyl C_{19}-phytosphingosine. Major ions (with their relative intensities and tentative assignments) were as follows: 574 (8%; $M{-}CH_3$), 415 (15%; C2-C3 cleavage with charge retention on the C3 fragment), 396 (9%), 313 (34%; C3-C4 cleavage with charge retention on the C4 fragment), 299 (9%), 276 (40%; C3-C4 cleavage with charge retention on the C3 fragment), 247 (14%; an ion arising from a cyclic rearrangement proposed by Hammarstrom et al. (13)), 246 (15%), 218 (72%), 204 (9%), 187 (39%), 186 (35%), 174 (20%: C2-C3 cleavage with charge retention on the C2 fragment), 157 (24%; loss of trimethylsilanol from 247 (14)), 147 (27%; $(CH_3)_2Si{=}O{-}Si(CH_3)_3$), 132 (100%; 174 - ketene), 116 (21%), 103 (20%; C1-C2 cleavage with charge retention on the C1 fragment), 96 (9%), 83 (8%), 75 (37%; $(CH_3)_2Si{=}OH$), and 73 (85%; $(CH_3)_3Si$).

The mixture of the N-acetylated bases isolated from the yeast grown in the presence of pentadecanoic acid was subjected to periodate oxidation. The resulting aldehydes were examined by gas-liquid chromatography. Three components were observed, with retention times of 4.0, 6.4, and 10.2 minutes. The ratio of the peak areas was 31:51:18, a finding which is in reasonable agreement with the corresponding ratio (39:46:15) observed in the case of the trimethylsilyl derivatives of the N-acetylated bases (Figure 1). The component eluting at 6.4 minutes cochromatographed with an authentic sample of pentadecanal. There was a linear relationship between the logarithm of the retention time and the chain length for the three aldehydes. This latter finding, coupled with a similar observation for the trimethylsilyl derivatives of the N-acetylated bases, indicates

that the C_{17} and C_{19} sphingolipid bases were straight-chain in nature.

Discussion

The results presented herein demonstrate that exogenous pentadecanoic acid causes a marked alteration of the cellular fatty acid composition of H. ciferrii, a finding similar to those reported previously in C. lipolytica and S. cerevisiae (6). Of particular importance is our finding that exogenous pentadecanoic acid also effects a remarkable modification of the composition of the sphingolipid bases excreted by H. ciferrii. Analyses of the sphingolipid bases recovered from the culture medium indicated the presence of significant quantities of C_{17}-phytosphingosine (39%) and C_{19}-phytosphingosine (15%) in addition to the normal C_{18}-phytosphingosine (46%). The sphingolipid bases were characterized by gas-liquid chromatography, combined gas-liquid chromatography-mass spectrometry, and chemical degradation. This approach offers significant promise of utility in studies of sphingolipid base formation and for the preparation of sphingolipid bases of unusual structure. Moreover, the findings presented herein have obvious implications with respect to factors operative in the regulation of the biosynthesis of sphingolipid bases.

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