Korean J. Chem. Eng., **19**(5), 815-817 (2002)

Chromatographic Analysis of Ceramide III of *Saccharomyces cerevisiae*

Mi Soon Yeo*, Duk Hui Kang*, Se Kyung Kim*,, Hyun Shik Yun*, Yoon Mo Koo*, Youn-Woo Lee*** and Kyung Ho Row*,**†**

*Center for Advanced Bioseparation Technology and **Div. of Chem. Eng.,
Inha University, 253 Yonghyun-Dong, Nam-Ku, Incheon 402-751, Korea
***National Research Lab. for Supercritical Fluid, KIST, Seoul 136-791, Korea
(*Received 22 January 2002 • accepted 10 March 2002*)

Abstract−Ceramide was prepared by the cultivation of *Saccharomyces cerevisiae* from cell extracts by solvent extraction and analyzed by NP-HPLC using a UV detector. The mobile phase was composed of hexane, methanol, and IPA. From the experimental conditions, the composition of mobile phase was 72/5/23 (hexane/IPA/methanol, vol%). Quantitative analysis of ceramide was performed. Based on the analytical conditions, the effect of cultivation temperature for the production of ceramide was investigated and the optimum cultivation temperature was found to be 35 °C.

Key words: Ceramide, *Saccharomyces cerevisiae*, Preparative HPLC

INTRODUCTION

The intercellular lipid of stratum corneum protects the organism against undesirable influences from the environment; and the compositions are ceramide 40%, cholesterol 25%, fatty acid 25%, and cholesterol solvate 10%. Among them ceramide is known as one of the most important sphingolipids that play significant roles as structural and functional components in biomolecular membranes [Rupcic and Maric, 1998]. Ceramides are involved in the barrier function of the skin [Gildenast and Lasch, 1997] and consist of fatty acids bound to the amino groups of sphingoid bases or related long chain bases [Raith and Neubert, 2000]. Ceramides also constitute the hydrophobic backbones of the complex shphingolipids: sphingomyelin, cerebrosides, gangliosides, etc. [Kolesnick et al., 2000]. Ceramides are important not only for the maintenance of the barrier function of the skin but also for the water-binding capacity of the stratum corneum [Gildenast and Lasch, 1997]. Though the effectiveness of ceramide is not understood fully, ceramide has become a widely used ingredient in cosmetic and pharmaceutical industries [Raith and Neubert, 2000; Dickson and Lester, 1999].

Compared to other sources of materials, yeast is more suitable for the production of ceramide because yeasts grow fast and are non-toxic [Rupcic and Maric, 1998]. As the production of ceramide from animal sources possesses possible problems such as bovine spongiform encephalopathy, using yeasts as a source of ceramide is advantageous. However, ceramide production from yeast has not been widely studied and most work was carried out for only two strains: *Saccharomyces cerevisiae* and *Torulopsis*(*Candida*) *utilis* [Rupcic et al., 1998].

The classical approach in ceramide analysis is based on thin layer chromatography (TLC) [Raith et al., 2000; Robson et al., 1994]. However, this method has several disadvantages, such as quantitative separation of individual species is very difficult [Raith et al., 2000] and incorrect. If a better specific analysis is demanded, liquid chromatography or gas chromatography is the method of choice. Since ceramides do not show a sufficient volatility, the polar hydroxyl groups have to be subjected to derivatization [Raith et al., 2000] in case of application of gas chromatography. Since ceramides are classified as their polar hydroxyl groups, normal phased HPLC (NP-HPLC), a powerful tool specially used in the separation of natural plants [Lee and Row, 2001], was selected for the analysis tool.

The purpose of this work was to find the proper condition of chromatographic analysis of ceramide produced by *Saccharomyces cerevisiae* using NP-HPLC with UV detector. The ternary mobile phases of hexane, methanol, and IPA were used as mobile phases.

EXPERIMENTAL

1. Chemicals

Hexane, isopropanol (IPA), and methanol were obtained from Duksan Chemical Co. (Incheon, Korea) and were HPLC grade. Ceramide III standard was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

2. Cell Cultivation

2-1. Yeast Strain and Culture Condition

The strain used in this research is *Saccharomyces cerevisiae* (KCCM 50515) that is known as a sphingolipid producer. Cultivation medium was YEPD (glucose, 20 g/L; bacto peptone, 20 g/L; yeast extract, 10 g/L) [Patton and Lester, 1994]. Cultivation was performed in Erlenmeyer flasks on a rotary shaker [Rupcic and Maric, 1998]. Agitation speed was 200 rpm and cultivation time was 48 hours.

2-2. Recovery of Lipid

Centrifugation and ultrasonification were applied for recovery of lipid. Cells were harvested in the stationary growth phase by centrifugation at 4,000 rpm and washed twice with distilled water [Lewis et al., 2000].

2-3. Extraction of Lipid

Five grams (wet weight) of yeast cells were suspended in 20 ml of a chloroform-methanol mixture (1 : 2, v/v) and the mixture was

†To whom correspondence should be addressed.
E-mail: rowkho@inha.ac.kr
‡This paper is dedicated to Dr. Youn Yong Lee on the occasion of his retirement from Korea Institute of Science and Technology.

sonicated three times in a U200S sonicator (IKA) for 5 min. The disrupted cell suspension was filtered through 0.2 μm RC filter (Sartorius). The residual cell debris was resuspended in 50 ml of chloroform-methanol mixture (2 : 1, v/v) and mixed by magnetic stirrer for 30 min at room temperature and then filtered. This process was repeated four times [Rupcic and Maric, 1998]. Production of ceramide was examined at various temperatures: 25 °C, 30 °C, 35 °C, and 40 °C.

3. NP-HPLC

HPLC was performed with Waters 600S solvent delivery system and 2487 UV dual channel detector (Waters, Milford, MA, U.S.A.). Data acquisition system was Millennium 3.2 installed in an HP Vectra 500 PC. Dual UV wavelengths were set at 208 nm and 210 nm. The mobile phases were degassed with helium. The flow rate of mobile phase was fixed at 1.0 ml/min. The HPLC pressure drop ranged from 400 to 600 psi. The mobile phases were hexane, IPA and methanol, respectively. For the packing material, 15 μm diameter of Si60 Lichrosphere was purchased from Merck (Darmstadt, Germany). It was in-house packed with an aspirator (Tokyo RIKAKIKAI, Tokyo, Japan). The column size was fixed as 0.39×15 cm for the analysis. Mobile phase composition used was : hexane/IPA/Methanol=72/5/23, vol%. This experiment was performed at ambient temperature.

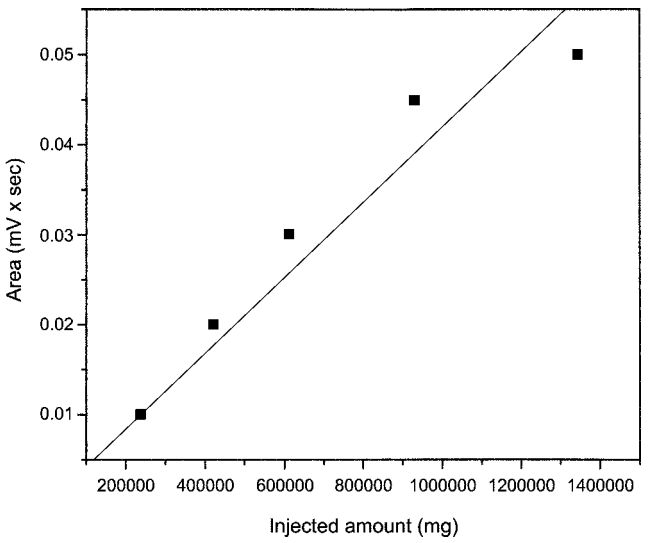

Fig. 1. Calibration curve of ceramide III for normal phased HPLC with UV detector.

To estimate the calibration curve for ceramide III, a standard of ceramide was loaded in ranging from 0.01 to 0.05 mg.

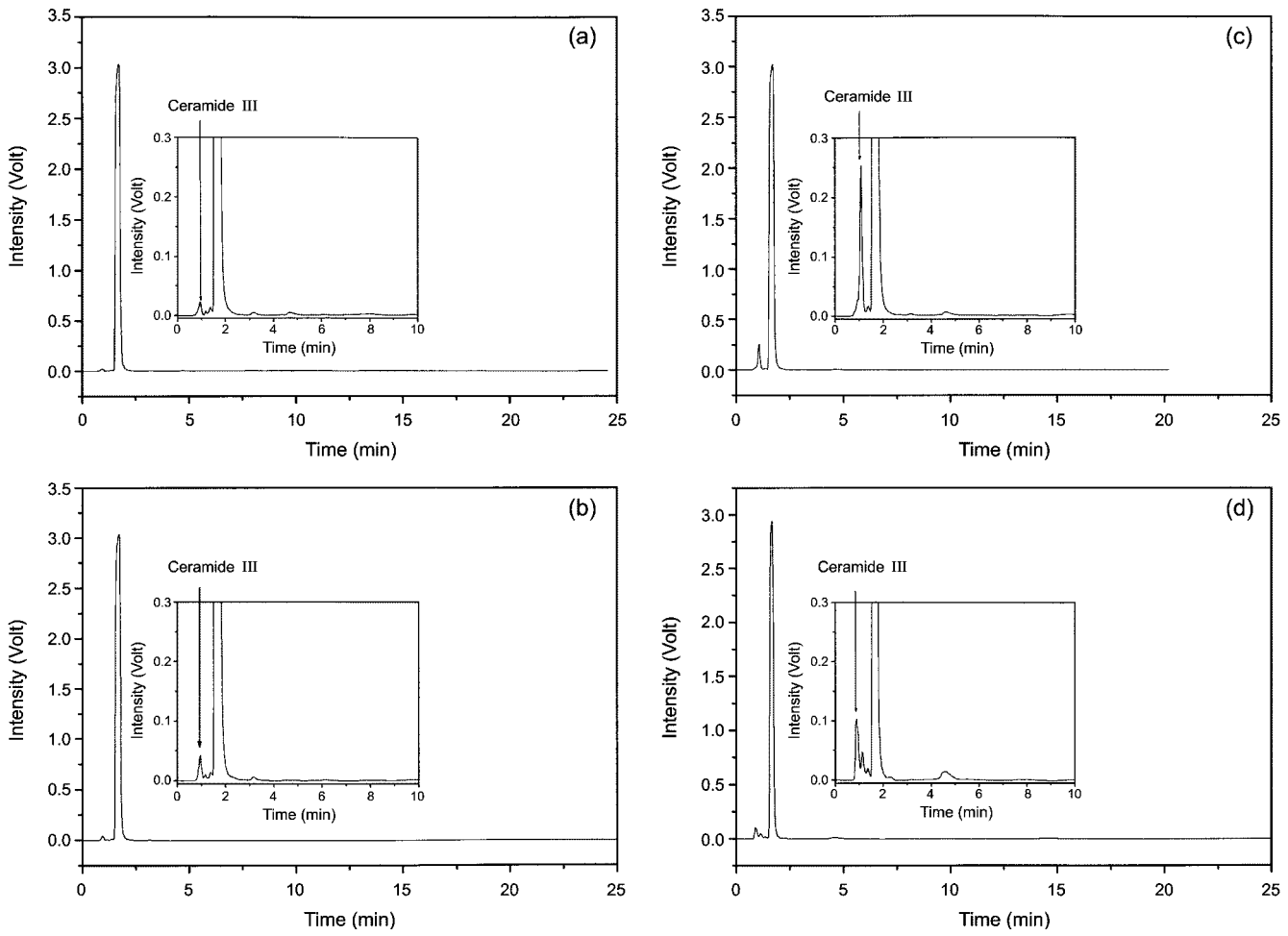

Fig. 2. Chromatogram of ceramide III obtained from *Saccharomyces cerevisiae*.
a: 25 °C; b: 30 °C; c: 35 °C; d: 40 °C

Table 1. Ceramide quantification analysis by normal phased HPLC with UV detector

Temperatures	Peak area	Amount of ceramide (mg/100 ml)
25 °C	37682	16.17
30 °C	39540	16.97
35 °C	567186	242.34
40 °C	375177	161.00

RESULTS AND DISCUSSION

The calibration curve of ceramide III was estimated to confirm the amount of ceramide in cell extracts. The response of UV for ceramide was linear as shown in Fig. 1. Ceramide III ranging from 0.01 to 0.05 mg was injected with the same mobile phase composition mentioned in the experimental section. The calibration curve equation was $y=4.218\times10^{-8}x$, where y is the amount (mg) of injected ceramide and x is area (mV*sec). The value of regression coefficient was 0.966. Ceramide III contains several components. From the standard sample of ceramide III, three different peaks were found by UV detector. The largest peak was considered to represent ceramide. Optimum cell cultivation conditions for the production of ceramide from *Saccharomyces cerevisiae* were studied at various temperatures by using NP-HPLC. The optimum cell cultivation temperature was found by comparing the amount of produced ceramide in the cell extracts. As mentioned earlier, ceramide is characterized by its polar hydroxyl groups, long-chain sphingoid base, and fatty acid. As a result, normal phase chromatography was selected for the analysis of ceramide. The results of NP-HPLC are shown Fig. 2. Ceramide III was identified by comparing retention time of the samples with that of ceramide standard. As shown in Table 1, cells cultured at 35 °C were found to contain the largest amount of ceramide. Subsequently, this tendency might imply that the best activation temperature for the production of ceramide from *Saccharomyces cerevisiae* was 35 °C.

It has been reported that the detection of ceramide with UV detector was insensitive because of its weak chromatographic functionality [Zhou et al., 1999]. The better detection device for ceramide III such as ELSD (Evaporated Light Scanning Detector) might be needed for the accurate analysis and quantification. On the basis of the presented data and other research [Rupcic and Maric, 1998; Zhou et al., 1999; Bouwstra et al., 1996], it can be concluded that yeast ceramides display quite heterogeneous structure depending on the yeast strain and growth conditions. For example, it was reported that ceramides of *Saccharomyces cerevisiae* and *Candia utilis* were characterized by a high abundance of saturated fatty acid, primarily C_{26} hydroxylated fatty acids [Rupcic and Maric, 1998].

CONCLUSION

Ceramide was separated from *Saccharomyces cerevisiae* and analyzed by NP-HPLC with UV detector. The mobile phase composition was 72/5/23 (hexane/IPA/methanol, vol%). Optimum cell cultivation condition for the production of ceramide from *S. cerevisiae* was also found in terms of ceramide content in cell extracts. Consequently, the best activation temperature for production of ceramide from *Saccharomyces cerevisiae* was 35 °C. However, the insensibility of ceramide with ultraviolet (UV) detection is well known because of weak chromatographic functionality in the 200-210 nm region. Thus, better detection for ceramide might be needed for more accurate quantitative analysis.

ACKNOWLEDGEMENTS

The authors were gratefully appreciated for the financial support by KRF (2001-042-E00072)

REFERENCES

Bouwstra, J. A., Cheng, K., Gooris, G. S., Weerheim, A. and Ponec, M., "The Role of Ceramides 1 and 2 in Stratum Corneum Lipid Organization," *Biochimica et Biophysica Acta*, **1300**, 177 (1996).

Dickson, R. C. and Lester, R. L., "Yeast Sphingolipids," *Biochimica et Biophysica Acta*, **1426**, 347 (1999).

Gildenast, T. and Lasch, J., "Isotation of Ceramide Fractions from Human Stratum Corneum Lipid Extracts by High-performance Liquid Chromatography," *Biochimica et Biophysica Acta*, **1346**, 69 (1997).

Kolesnick, R. N., Goni, F. M. and Alonso, A., "Compartmentalization of Ceramide Signaling: Physical Foundations and Biological Effects," *J. Cellular Physiology*, **184**, 285 (2000).

Lee, J. W. and Row, K. H., "New Retention Mechanism of Mononucleotides with Buffer Concentrations in Ion-Suppressing RP-HPLC," *Biotech and Bioprocess Engineering*, **6**(1), 371 (2001).

Lewis, T., Nichols, P. D. and Mcmeekin, T. A., "Evaluation of Extraction Methods for Recovery of Fatty Acid from Lipid-producing Microheterotrophs," *J. Microbiol. Methods*, **43**, 107 (2000).

Long, A. S., Wertz, P. W., Strauss, J. S. and Downing, D. T., "Human Stratum Corneum Polar Lipids and Desquamation," *Arch. Derm. Res*., **277**, 284 (1985).

Patton, J. L. and Lester, R. L., "The Phosphoinositol Sphingolipids of *Saccharomyces cerevisiae* Are Highly Localized in the Plasma Membrane," *J. Bacteriology*, **173**, 3101 (1991).

Raith, K. and Neubert, R. H. H., "Liquid Chromatography-electrospray Mass Spectrometry and Tandem Mass Spectrometry of Ceramides," *Analytica Chimica Acta*, **403**, 295 (2000).

Raith, K., Darius, J. and Neubert, R. H. H., "Ceramide Analysis Utilizing Gas Chromatography-mass Spectrometry," *J. Chrom. A*, **876**, 229 (2000).

Robson, K. J., Stewart, M. E., Michelsen, S., Lazo, N. D. and Downing, D. T., "6-Hydroxy-4-sphingenine in Human Epidermal Ceramides," *J. Lipid Res*., **35**, 2060 (1994).

Rupcic, J. and Maric, V., "Isolation and Chemical Composition of the Ceramide of the *Candida lipolytica* Yeast," *Chemistry and Physics of Lipids*, **91**, 153 (1998).

Rupcic, J., Mesaric, M. and Maric, V., "The Influence of Carbon Source on the Level and Composition of Ceramides of the *Candida lipolytica* Yeast," *Appl. Microbiol. Biotechnol*., **50**, 583 (1998).

Zhou, J. Y., Chaminade, P., Gaudin, K., Prognon, P., Baillet, A. and Ferrier, D., "Postcolumn Fluorescence as an Alternative with Gradient Elution in Non-aqueous Reversed-phase Liquid Chromatography," *J. Chrom. A*, **859**, 99 (1999).