Mobile Phase Compositions for Ceramide III by Normal Phase High Performance Liquid Chromatography

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Abstract Ceramide III was prepared by the cultivation of *Saccharomyces cerevisiae*. Ceramide III was partitioned from the cell extracts by solvent extraction and analyzed by Normal Phase High Performance Liquid Chromatography (NP-HPLC) using Evaporative Light Scattering Detector (ELSD). We experimentally determined the mobile phase composition to separate ceramide III with NP-HPLC. Three binary mobile phases of n-hexane/ethanol, *n*-hexane/Isoprophyl Alcohol(IPA) and *n*-hexane/*n*-butanol and one ternary mobile phase of *n*-hexane/IPA/methanol were demonstrated. For the binary mobile phase of *n*-hexane/ethanol, the first mobile phase composition, 95/5 (v/v), was step-increased to 72/23 (v/v) at 3 min. In the binary mobile phase, the retention time of ceramide III was 7.87 min, while it was 4.11 min respectively in the ternary system, where the mobile phase composition of *n*-hexane/IPA/methanol, 85/7/8 (v/v/v), was step-increased to 75/10/15 (v/v/v) at 3 min. However, in the ternary mobile phase, the more peak area of ceramide III was observed.

Keywords: *Saccharomyces cerevisiae*, cermaide III, mobile phase, NP-HPLC, ELSD

INTRODUCTION

The main functions of the skin are to act as a barrier to substances and protect organism against undesirable influences for the environment. The main barrier of the skin resides in the upper layer of the skin, the stratum corneum, which consists of corneocytes embedded in lipid lamellar regions [1]. The stratum corneum is an effective barrier to the loss of water and on the other hand to physical, chemical and biological influences from the environment [2]. The compositions of the intercellular lipid of stratum corneum 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 establish a barrier for cell permeability and form a matrix for the association of membrane proteins [3,4]. 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 [3]. It is difficult to isolate free ceramide quantitatively and incompletely pure form since its content in biological materials is quite low. As ceramides are key compounds in the metabolism of sphingolipids and are thought to be important second messengers, exact analysis of their molecular species and concentrations would seem to be crucial for elucidating their function and metabolism [4].

Compared to other materials, yeast is relatively suitable source for production of ceramide because it has advantages such as fast growth, non-toxicity and ability to be genetically manipulated. Nevertheless, there has been no systematic analysis of ceramide occurrence and their level in yeast cells. Their presence has, until now, been detected in only two yeasts, *Saccharomyces cerevisiae* and *Torulopsis (Candida) utilis* [5].

As ceramides are classified as their polar hydroxy groups and long chain based fatty acid, normal phase high performance liquid chromatography (NP-HPLC) was determined to analyze the polar hydroxy group for the analysis tools. There has been a need to develop simple, quantitative analytical methods to assay complex lipids. More recently, evaporative light scattering detector (ELSD) has been reported as the appropriate detection method for analysis of ceramides [6-8]. ELSD is a semi-universal mass detector which is ideal for analysis of lipid classes. This detector has come into wide use recently for the detection of both nonpolar and polar lipids. Wells *et al.*[9] developed a normal-phase HPLC method for use with an ELSD to quantify ceramides in crude yeast lipid extracts [10].

The purpose of this work is to find the optimum condition of mobile phase composition to analyze ceramide III from *Saccharomyces cerevisiae* based on the peak area of ceramide III using NP-HPLC with ELSD.

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MATERIALS AND METHODS

Chemicals

The HPLC grade solvent, *n*-hexane, ethanol, IPA and methanol were obtained from Duksan Chemical Co. (Incheon, Korea). *n*-Butanol was purchased from Dong Yang Chemical Co.(Seoul, Korea) and it was filtered by decompressing pump (Division of Millipore, Waters) through 0.5 μm of filter film. Ceramide III standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Yeast Strain and Culture Condition

Used strain in this research was *Saccharomyces cerevisiae* (KCCM 50515) that is known as sphingolipid producer. Cultivation medium was YEPD (glucose, 20 g/L; Bacto peptone, 20 g/L; yeast extract, 10 g/L) [5]. Cultivation was performed in Erlenmeyer flasks on the rotary shaker [11]. Cultivation condition was 200 rpm and cultivation time was 48 h.

Recovery of Lipid

Centrifugation and ultrasonification method were used for recovery of lipid. *Saccharomyces cerevisiae* was harvested by centrifugation at 4,000 rpm and washed twice with distilled water [12].

Lipid Extraction

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 sonicated three times in sonicator (IKA U200S) for 5 min. The disrupted cell suspension was filtered through 0.2 μm RC filter (Sartorius). The residual cell debris was suspended in 50 mL of chloroform-methanol mixture (2:1, v/v) and stirred by magnetic stirrer for 30 min at room temperature and filtered again. This process was repeated four times. Ceramide was produced at 20°C, pH 4. Extraction method was the same as above process.

Preparation for HPLC

The cell extracts were 150 mL before any pretreatments. The cell extracts were concentrated into 3 mL using rotary evaporator. The concentrations were adjusted with chloroform. As the extracts contained small amount of water, the phase separation was occurred. The chloroform phase was used the separation.

NP-HPLC

HPLC was performed with Waters 600S solvent delivery system (Waters, Milford, MA, USA). Data acquisition system was Autochro 2.0 (Youngin Instrument, Anyang, Korea) installed on an HP Vectra 500 PC. The detection system was ELSD from Alltech (Deerfield, USA).

RESULTS AND DISCUSSION

Optimum cell cultivation conditions for the production of ceramide from *Saccharomyces cerevisiae* were studied using NP-HPLC. As mentioned earlier, ceramide is characterized by its polar hydroxyl groups, long-chain sphingoid base, and fatty acid. As the results, normal phase chromatography was used for the analysis of ceramide.
In case of using UV for detection of ceramide III, chloroform could not be used for mobile phase because of its relative high UV cutoff. Therefore, hexane was the second choice for mobile phase. It has been reported that the detection of ceramide with UV detector was insensitive because of its weak UV functionality [11]. Thus, the better detection device for ceramide III such as ELSD was needed for the accurate analysis and quantification.

The mobile phase to separate ceramide III from *Saccharomyces cerevisiae* was investigated with hexane, ethanol, *n*-butanol, IPA, methanol and their polarity indexes were 0, 5.2, 3.9, 3.9 and 6.6, respectively. The combination of the mobile phase was *n*-hexane/ethanol, *n*-hexane/*n*-butanol, *n*-hexane/IPA and *n*-hexane/IPA/methanol. The injection amount was fixed as 5 μL. This experimental was carried out at ambient temperature. The mobile phases were degassed with helium. The flow rate of mobile phase was fixed at 1.0 mL/min. The column used in this work was a generous gift from RStech (Daejeon, Korea). The dimension of column was 250 × 4.6 mm. The packing material was OP Sil-51002546 (Daejeon, Korea).

Binary Mobile Phases

The calibration curves of ceramide III were estimated to confirm the amount of ceramide in cell extracts. Ceramide III ranging from 2.5 to 20 μg was injected with same mobile phase composition mentioned in the experimental section. The calibration curve equations and regression coefficients (r^2) were $Y = 419581X$ and 0.9796 under *n*-hexane/ethanol, $Y = 408814X$ and 0.9695 under *n*-hexane/*n*-butanol and $Y = 414020X$ and 0.8629 under *n*-hexane/ IPA, where Y is peak area(Vsec) and X is the amount (μg) of injected ceramide III. Tables 1, 2 and 3 showed the effect of the composition of binary mobile phase on the retention factor and the peak area of ceramide III to find the optimum mobile phase condition for the largest peak area of ceramide III with ELSD.

Three binary mobile phase conditions were investigated. First, the initial mobile phase composition was n-hexane/ethanol = 95/5 (v/v) and then step-increased to 77/23 (v/v) after 3 min. This condition showed highest sensitivity with ELSD. The chromatogram of the separated *Saccharomyces cerevisiae* with the mobile phase of *n*-hexane/ethanol is given in Fig. 1. Retention time of ceramide III was 7.87 min. As the modifier is gradually increased, retention factor is decreased. The drift temperature of ELSD was set at 65°C and flow rate of the nebulizer gas (nitrogen) was 1.6 L/min.

Second, the mobile phase is changed to *n*-hexane and *n*-butanol. The initial mobile phase composition, *n*-hexane/

Table 1. Effect of the mobile phase composition (*n*-hexane/ethanol) on retention factor, response factor and peak area of ceramide III

mobile phase condition (*n*-hexane/ethanol)	*k* (retention factor)	*response factor	peak area (V sec)
97/3 → 77/23	1.31	0.809	1484
96/4 → 77/23	1.28	0.909	1668
95/5 → 77/23	1.22	1.000	1835
94/6 → 77/23	1.02	0.193	354
95/5 → 81/19	1.28	0.132	242
95/5 → 80/20	1.24	0.922	1692
95/5 → 79/21	1.23	0.987	1811
95/5 → 78/22	1.22	0.947	1738
95/5 → 77/23	1.22	1.000	1835
95/5 → 76/24	0.96	0.869	1594
95/5 → 78/22	0.81	0.726	1331

(gradient time, 3min.)
* response factor = a peak area / the largest peak area

Table 2. Effect of the mobile phase composition (*n*-hexane/*n*-butanol) on retention factor, response factor and peak area of ceramide III

mobile phase condition (*n*-hexane/*n*-butanol)	*k* (retention factor)	* response factor	peak area (V sec)
98/2 → 54/46	1.19	0.810	1333
97/3 → 54/46	1.18	0.874	1438
96/4 → 54/46	1.18	1.000	1646
94/5 → 54/46	1.18	0.931	1532
96/4 → 80/20	1.35	0.354	582
96/4 → 70/30	1.24	0.851	1400
96/4 → 57/43	1.20	0.881	1450
96/4 → 56/44	1.19	0.973	1601
96/4 → 54/46	1.18	1.000	1646
96/4 → 55/45	1.18	0.874	1438
96/4 → 53/47	1.18	0.868	1428

(gradient time, 3min.)
* response factor = a peak area / the largest peak area

n-butanol = 96/4 (v/v), was step-increased to 54/46 (v/v) at 3 min. The drift temperature of ELSD was set as 70°C and flow rate of the nebulizer gas (nitrogen) was 1.6 L/min. The maximum concentration of ceramide was found at 7.73 min. With *n*-hexane/ethanol, retention factor is gradually decreased when the modifier is gradually increased. Fig. 2 shows the separated *Saccharomyces cerevisiae* as the mobile phase was *n*-hexane and *n*-butanol.

Third, mobile phase is fixed to *n*-hexane/IPA. The initial mobile phase composition, *n*-hexane/IPA = 95/5 (v/v), was step-increased to 65/35 (v/v) at 3 min. The drift temperature of ELSD was set as 55°C and flow rate of

Table 3. Effect of the mobile phase composition (*n*-hexane/IPA) on retention factor, response factor and peak area of ceramide III

mobile phase conditions (*n*-hexane/IPA)	*k* (retention factor)	* response factor	peak area (V sec)
90/10 65/35	0.76	0.083	141
91/9 65/35	0.86	0.220	372
93/7 65/35	1.14	0.918	1552
94/6 65/35	1.16	0.979	1655
95/5 65/35	1.18	1.000	1690
96/4 65/35	1.19	0.279	471
97/3 65/35	1.25	0.214	362
95/5 66/34	1.18	0.288	486
95/5 65/35	1.18	1.000	1690
95/5 64/36	1.17	0.951	1606
95/5 63/37	1.16	0.946	1599
95/5 62/38	1.16	0.938	1586

(gradient time, 3min.)
* response factor = a peak area / the largest peak area

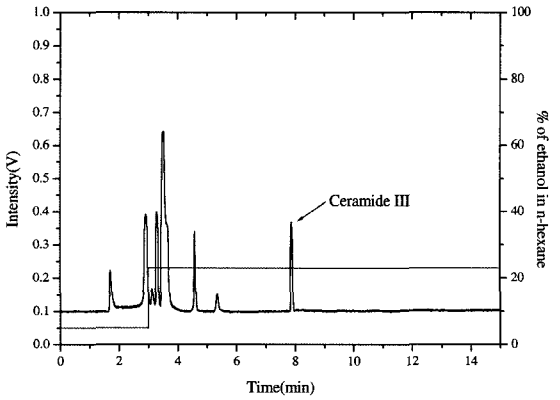

Fig. 1. Chromatogram of ceramide III obtained from *Saccharomyces cerevisiae*, using *n*-hexane/ethanol as the mobile phase.

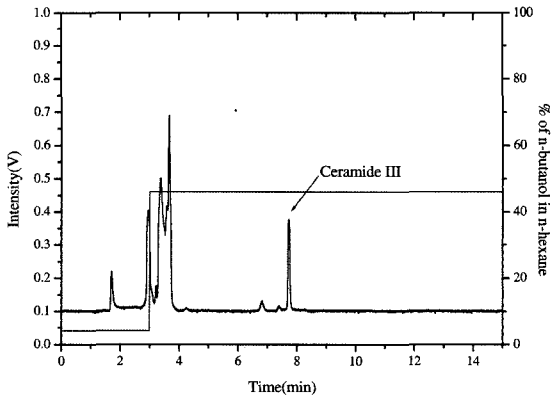

Fig. 2. Chromatogram of ceramide III obtained from *Saccharomyces cerevisiae*, using *n*-hexane/*n*-butanol as the mobile phase.

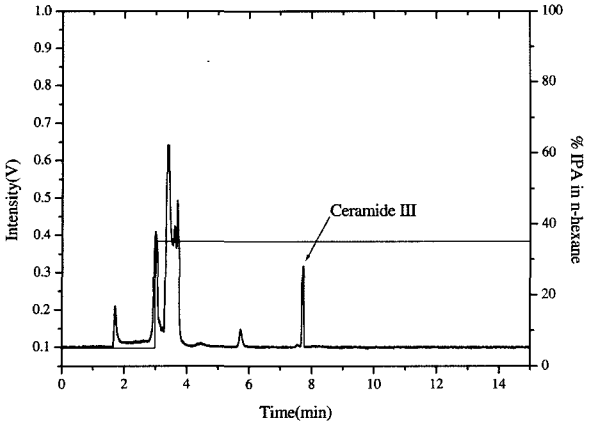

Fig. 3. Chromatogram of ceramide III obtained from *Saccharomyces cerevisiae* using *n*-hexane/IPA as the mobile phase.

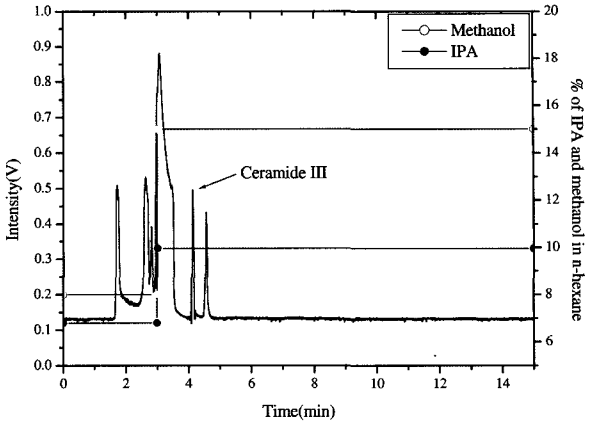

Fig. 4. Chromatogram of ceramide III obtained from *Saccharomyces cerevisiae* using *n*-hexane/IPA/methanol as the mobile phase.

Table 4. Effect of the mobile phase composition (n-hexane/IPA/methanol) on retention factor, response factor and peak area of ceramide III

mobile phase conditions (*n*-hexane/IPA/methanol)		*k* (retention factor)	* response factor	peak area (V sec)
85/7/8	75/12/13	0.14	0.838	2948
85/7/8	75/11/14	0.14	0.974	3428
85/7/8	75/10/15	0.15	1.000	3518
85/7/8	75/9/16	0.16	0.827	2909
85/7/8	75/8/17	0.17	0.811	2854
85/6/9	75/10/15	0.16	0.742	2609
85/7/8	75/10/15	0.15	1.000	3518
85/8/9	75/10/15	0.14	0.809	2847
85/9/8	75/10/15	0.13	0.806	2834

(gradient time, 3 min.)
* response factor = a peak area / the largest peak area

the nebulizer gas (nitrogen) was 1.6 L/min. The retention time of ceramide III was 7.71 min. Ceramide III was separated well from the *Saccharomyces cerevisiae* as shown in Fig. 3. In these binary phases used in this work, ceramide III was well separated from the *Saccharomyces cerevisiae*.

Ternary Mobile Phase

In order to calculate amount of the ceramide III in the *Saccharomyces cerevisiae*, calibration curve is calculated in ternary system. The sample range of ceramide III was 2.5 to 20 μg. The calibration curve and regression coefficient (r^2) was $Y = 452604X$ and 0.9757, where Y is peak area ($V \times$ sec) and X is the amount (μg) of injected ceramide III. Table 4 shows the effect of the composition of ternary mobile phase on the retention factor and peak area. As the same cases of the three binary mobile phases were used, the initial mobile phase composition, *n*-hexane/IPA/methanol = 85/7/8 (v/v/v), was step-increased to 75/10/15 (v/v/v) at 3 min. Retention time of ceramide III was 4.06 min. The drift temperature of ELSD was set at 55°C and flow rate of the nebulizer gas (nitrogen) was 1.6 L/min. Ceramide III was separated from the *Saccharomyces cerevisiae* using the *n*-hexane, IPA and methanol as shown in Fig. 4. Ternary mobile phase condition showed the best result in terms of sensitivity and separation time.

CONCLUSION

Ceramide III was prepared by the cultivation of *Saccharomyces cerevisiae*. In case of the references applied reverse phase high performance liquid chromatography (RP-HPLC) and UV detector, the sensitivity of detection is lower than that of ELSD. The mobile phases considered in this work were binary system such as *n*-hexane/ethanol, *n*-hexane/*n*-butanol and *n*-hexane/IPA, and ternary system of *n*-hexane/IPA/methanol. The ternary mobile phase system showed the best condition for ceramide III in terms of sensitivity and separation time.

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

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[Received September 19, 2003; accepted November 18, 2003]