Structure and Phase Behavior of Synthetic Ceramide 2 ((2*S*,3*R*)-2-Octadecanoylamino-octadecane-1,3-diol) and Its Interaction with Limonene

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In this study, we studied the structure and phase behavior of synthetic ceramide 2 ((2*S*,3*R*)-2-Octadecanoylamino-octadecane-1,3-diol) (S-CER2) using differential scanning calorimetry (DSC) and X-ray diffraction. In addition, we also investigated the effects of limonene on S-CER2. It was found that the phase behavior of S-CER2 depends on its thermal history and that the DSC thermograms depend apparently on the scan rates. The present study suggested a relatively strong affinity between limonene and S-CER2.

Key words: ceramide, phase behavior, metastable phase, skin penetration enhancer

1. INTRODUCTION

The main function of skin is to prevent entry of foreign matter and the loss of water. The barrier function has been shown to be associated with intercellular lipids in the stratum corneum that is the outmost layer of the skin [1,2]. The detailed molecular mechanism is however not clearly understood. Stratum corneum lipids mainly consist of ceramides (~50%), cholesterol (~25%) and free fatty acids (~10%) [3]. These lipids are formed a multilamellar structure in the stratum corneum [1,4]

Judging from the correlation between dysfunction of the skin barrier and a significant decrease of the amount of ceramides found in atopic dermatitis [5,6], ceramides are believed to play a central role in the skin barrier function. In order to clarify the detailed mechanism of the barrier function, the phase behaviors and structures of ceramides and lipid mixtures containing ceramides have been investigated using various methods. In many of these studies, synthetic ceramides have been used, because there are only small amounts of ceramides in living tissues and extracted ceramides that contain various molecular species with subtle different chemical structures. Different nine subclasses of ceramide have been found in human stratum corneum [7]. Isolation and separation of native ceramides are significantly difficult and high-cost task. To overcome this problem, various

ceramide analogues have been synthesized.

Recently, Ishida [8] has succeeded in synthesizing an optically active ceramide 2 analogue, ((2*S*,3*R*)-2-Octadecanoylamino-octadecane-1,3-diol) (S-CER2) (Fig.1). Ceramide 2 is the most abundant ceramide in keratinized tissue [9, 10]. Naturally occurring ceramide 2 has a sphingosine chain, while S-CER2 has a sphinganine chain. Hence, the difference between them is only the presence or absence of one double bond in the hydrocarbon chain.

On the basis of their differential scanning calorimetry (DSC) results obtained by using a scan rate of 5 °C/min, Watanabe et al. [11] have reported that the phase diagram of S-CER2-cholesterol binary mixtures was a eutectic type in which the eutectic point was located at 50:50 ratio of S-CER2 to Cholesterol. A study performed using the same DSC method but with a different scan rate (1.0 °C/min), however, showed that the phase diagram of the mixtures was the same eutectic type but the composition of the eutectic point was different, i.e., 60:40 ratio of S-CER2 to Cholesterol (H. Kasahara, Unpublished). This suggests that DSC thermograms of S-CER2-cholesterol mixtures depend on the scan rate.

Here we report the scan rate dependent phase behavior of pure S-CER2 system together with its structural properties and thermal history dependent phase behavior, based on the results obtained by DSC and X-ray diffraction measurements. In addition, we also investigated the effects of limonene on the structure and phase behavior of S-CER2. Limonene has been well known to be a skin penetration enhancer of drugs [12].

Fig. 1. Chemical structure of synthetic ceramide 2, ((2*S*,3*R*)-2-Octadecanoylamino-octadecane-1,3-diol) (S-CER2)

2. MATERIALS AND METHODS

Powder sample of S-CER2 was supplied by Takasago International (Tokyo, Japan). D-limonene was purchased

Fig. 2. DSC thermograms of S-CER2 recorded at various heating scan rates for (a) first heating scan, and (b) second heating scan after rapid cooling ($>$ about $10\text{ }^{\circ}\text{C/min}$) from a melted state above $120\text{ }^{\circ}\text{C}$.

from Nacalai Tesque, Inc. (Kyoto, Japan) and other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals were of analytical grade and used as received without further purification. X-ray diffraction measurements were carried out at beamlines 9C and 6A [13] at Photon Factory of KEK (Tsukuba, Japan). DSC measurements were performed using a DSC6100-Exstar6000 system (Seiko Inst., Japan). Crystallized S-CER2 solid samples from organic solution were prepared as follows. First, a powder sample of S-CER2 was completely dissolved into various organic solvents and then the solvents were evaporated by stream of nitrogen gas. Further, in order to remove the residual solvents, the samples were kept under reduced pressure (about 100 Pa) for 10 hours. The remaining solvents did not detected by human eyes.

3. RESULTS AND DISCUSSION

Figure 2 represents DSC thermograms recorded with various heating scan rates. The data obtained in the 1st heating scan and the 2nd heating scan after rapid cooling from $120\text{ }^{\circ}\text{C}$ to a room temperature with a cooling rate of $\sim 10\text{ }^{\circ}\text{C/min}$ are displayed in Figs 2 (a) and (b), respectively. As increasing scan rates, the temperatures of the largest peak temperatures shift to higher side (Fig.2). Linear regression analysis gave the intercept values of $104.6\text{ }^{\circ}\text{C}$ and $105.4\text{ }^{\circ}\text{C}$, and the slope values of 0.44 min and 0.14 min , for 1st and 2nd heating scans, respectively (Fig.3).

This apparent scan-rate dependence DSC thermograms of S-CER2 is likely to be a reason why two different compositions of the eutectic point of S-CER2/Cholesterol mixture has been estimated by DSC studies by using different scan rates. The detailed study on the scan-rate dependence phase behavior of S-CER2/Cholesterol mixture is now under the progress. As increasing scan rates, the endothermic peaks indicated by arrows become smaller (Fig.2). As described below, by X-ray diffraction study, the peak was confirmed to correspond to a melting process after recrystallization. Since at faster scan rates temperature reaches quickly above the main melting temperature before completing recrystallization, the highest

Fig. 3. Plots of peak temperature of the largest DSC peak in thermograms of S-CER2 for 1st scan (closed circles), and 2nd scan (closed squares) as a function of heating scan rates.

Fig. 4. DSC thermograms of (a) non-heat-treated powder S-CER2 sample as the state when it was supplied and (b) S-CER sample after keeping at $106\text{ }^{\circ}\text{C}$ for 1 hours. The scan rate was $1.0\text{ }^{\circ}\text{C/min}$.

temperature endothermic peaks become smaller.

It can be seen from these thermograms that the phase behavior of S-CER2 depends on its thermal history as well as the heating scan rate. Mainly endothermic peaks were observed at around $105\text{--}110\text{ }^{\circ}\text{C}$ in the first heating DSC thermogram of untreated powder S-CER2 sample as the state when it was supplied. On the other hand, at least two exothermic peaks and four endothermic peaks were observed in second immediately heating DSC scan

Fig. 5. (a) Small-angle and (b) wide-angle X-ray diffraction patterns of S-CER2 with different thermal history.

after cooling from a melted state above 120 °C. For both first and second scan, the total enthalpy values were independent on the scan rate. The values were ~150 J/g and ~67 J/g for the first scan and second scan, respectively. This suggests that S-CER2 adopts a metastable state after rapid cooling.

DSC thermogram recorded after keeping at 106 °C for one hour was completely different from that of no-heat-treated sample (Fig.4). After heat-treatment, only single endothermic peak was observed at 110.5 °C with the transition enthalpy value of ~90 J/g. The enthalpy value is smaller than that of no-heat-treated sample (~150 J/g), indicating that the state is less stable than that of initial state. The enthalpy value did not change for the sample kept at 106 °C more than three hours.

X-ray diffraction clearly gave an evidence for the metastable state (Fig.5). Only broad diffraction peaks were observed in both small- and wide-angle regions for S-CER2 sample after rapid cooling from a melted state. The center positions of broad peaks were $S = 0.34 \text{ nm}^{-1}$ ($d = 2.94 \text{ nm}$) and $S = 2.46 \text{ nm}^{-1}$ ($d = 0.41 \text{ nm}$) for small- and wide-angle region, respectively. The state can be interpreted as a sort of amorphous (or glass) state. In addition, X-ray diffraction also showed that S-CER2 sample was transformed to an ordered phase by keeping at 106 °C more than one hour (Fig.4). This result

Fig. 6. DSC thermograms of S-CER2 crystallized from various organic solution recorded in (a) first heating scan and (b) successive second heating scan after rapid cooling ($> \text{about } 10 \text{ }^{\circ}\text{C/min}$). (1) Limonene, (2) methyl acetate, (3) chloroform and (4) mixture of Chloroform and ethanol. The scan rate was $1.0 \text{ }^{\circ}\text{C/min}$.

indicates that the exothermic DSC peak at around 105 °C observed at slower scan rates corresponds to a recrystallized process to a stable phase. The ordered phase exhibited d -spacing of 4.62 nm. From the molecular dimension of S-CER2, this spacing is interpreted as the periodic repeat distance of S-CER2 bilayer structure.

Taking account of both DSC and X-ray diffraction results, we concluded that the metastable amorphous phase transforms into an ordered phase by keeping the metastable sample at 106 °C, but the ordered phase is not the most stable phase, judging from the enthalpy values.

For initial sample, two different lamellar peaks appeared in the small-angle region. The one is d -spacing of 7.87 nm and the other is d -spacing of 4.00 nm. The long spacing peaks (7.87 nm) disappeared at about 50 °C with increasing temperature. This change corresponds to a small endothermic peak ($\Delta H = \sim 5 \text{ J/g}$) observed at 50 °C in DSC thermogram (Fig.4 (a)). Since the molecular length of the long axis of S-CER2 is about 2.5 nm, the long spacing of 7.87 nm suggests that S-CER2 adopts so-called V-shape conformation [14]. The fact that d -spacing of non-heat treatment sample (4.41 nm) is shorter than that of sample kept at 106 °C more than one hour (4.62 nm) indicates that tilt angle of the

hydrocarbon chains of former sample is bigger than latter one.

Next we studied the effect of limonene on the phase behavior of S-CER2. X-ray diffraction revealed that the crystal structure of S-CER2 crystallized from limonene solution was almost identical with those of the samples crystallized from other organic solutions (methyl acetate, chloroform, and a mixture of ethanol and chloroform) (data not shown). This is good agreement with the DSC results that, at first scan, S-CER2 solid sample crystallized from S-CER2-limonene solution exhibited almost same phase behavior as compared with the sample obtained from other organic solution (Fig. 6 (a)). In the second heating scan, however, only sample crystallized from limonene solution exhibited different thermal behavior (Fig. 6 (b)). The other sample showed the same DSC thermograms as observed for an initial sample in second heating scan (Fig. 2 (b)). This suggests that small amount of limonene still remains even after keeping the sample under reduced pressure (~100 Pa) more than eight hours. From this DSC study, we concluded that there is a relatively strong interaction between limonene and S-CER2. This nature may be related to the action of limonene as a skin penetration enhancer, although further study is necessary to ascertain whether the conclusion obtained with S-CER2 applies to other types of ceramide found in skin. A recent FTIR study has revealed limonene affects strongly on the alkyl chains of naturally occurring ceramides in comparison with other organic solvents such as ethanol and dimethyl sulfoxide [15].

4. CONCLUSIONS

We have shown that the phase behavior of S-CER2 depends on its thermal history and that observed DSC thermograms of S-CER2 depend apparently on the scan rates. When the systems containing S-CER2 are studied, therefore, it should be paid attention to such effects. We have also revealed that the affinity between limonene and S-CER2 is relatively strong. This would be related to the mechanism of the skin penetration action of limonene.

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