Amino Acids and Their N-Acetylated Derivatives Maintain the Skin’s Barrier Function

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The hydrocarbon-chain packing structure of intercellular lipids in the stratum corneum (SC) is critical to the skin’s barrier function. We previously found that formation of V-shaped ceramide reduces the barrier function of skin. There are few agents, apart from ceramides and fatty acids that can improve the orthorhombic packing (Orth) ratio of the intercellular lipid packing structure. In this study, we investigated agents that directly increase the Orth ratio. We selected an intercellular lipid model consisting of ceramide, cholesterol, and palmitic acid and performed differential scanning calorimetry. We focused on natural moisturizing factor components in the SC, and therefore investigated amino acids and their derivatives. The results of our intercellular lipid model-based study indicate that N-acetyl L-hydroxyproline (AHYP), remarkably, maintains the lamellar structure. We verified the effect of AHYP on the lamellar structure and hydrocarbon chain packing structure of intercellular lipids using time-resolved X-ray diffraction measurements of human SC. We also determined the direct physicochemical effects of AHYP on the Orth ratio of the hydrocarbon-chain packing structure. Hence, the results of our human SC study suggest that AHYP preserves skin barrier function by maintaining the hydrocarbon-chain packing structure of intercellular lipids via electrostatic repulsion. These findings will facilitate the development of skincare formulations that can maintain the skin’s barrier function.

Key words stratum corneum; X-ray diffraction; intercellular lipid; amino acid; N-acetyl-L-hydroxyproline

Introduction

The stratum corneum (SC) plays an inside-out barrier function to inhibit water evaporation from the body and an outside-in barrier function to prevent the entry of external allergens. The barrier function of the SC is regulated mainly by intercellular lipids, which include ceramides (CER), cholesterol (CHOL), and free fatty acids (FFA).1) Intercellular lipids form lamellar structures with different periodicities.2) The lamellar structures are composed of different hydrocarbon-chain packing structures, including orthorhombic packing (Orth), hexagonal packing (Hex), and a fluid or liquid crystal state.3) The lamellar structure of intercellular lipids was influenced by SC water content.4) The ratio of Orth in the lateral packing has been reported to decrease in the surface layer of the SC.5) The outflow of constituent lipids due to detergent from the SC surface layer has also been proposed to occur.6) The barrier function of SC intercellular lipids is routinely exposed to various risks, including dehydration and excessive washing which can cause the failure of SC maturation. Barrier function has been proposed to be enhanced by a high Orth ratio.7) Based on the results of our previous study, we hypothesized that increasing the Orth ratio by applying non-toxic agents to defective SC will instantaneously improve barrier function. Some chemical agents were previously determined to be transdermal absorption enhancers that affect the intercellular lipid barrier by promoting fluidity and decreasing the Orth ratio.7–10) However, agents that can restore the intercellular lipid packing structures directly have thus far not been identified except for CER11–14) and FFA,15) which are known intercellular lipids.

We attempted to identify ingredients that can improve the hydrocarbon-chain packing structure from among known moisturizing ingredients. We focused on natural moisturizing factors (NMF)16) abundant in the SC. Amino acids are a major class of NMF, and their derivatives are widely used as moisturizing agents. In addition, we previously observed lamellar structures with different periodicities and hydrocarbon-chain packing properties using an intercellular lipid model (N-(a-hydroxyoctadecanoyl)-dihydrosphingosine (CER[ADS])/Palmitic acid (PA)/CHOL). This difference in lamellar structures is thought to be caused by the difference in whether or not FFA are contained in the membrane.17) In the lipid bilayer, FFA are subject to mutual control of the Born energy, which desorbs charged carboxyl groups from the lipid bilayer, and the hydrophobic interaction energy, which retains the protonated FFA in the lipid bilayer.7) It is assumed that the hydrocarbon-chain packing structure can be improved by controlling the position of FFA in the lamellae with charged substances such as amino acids.

Therefore, the effects of improving the hydrocarbon-chain packing structure of NMF were investigated using those models. N-Acetyl L-hydroxyproline (AHYP), which maintains barrier function,18) is widely used in cosmetics. In this report, we determined the effects of improving the hydrocarbon-chain packing structure of the amino acid derivative acetyl hydroxyproline (AHYP).
In this study, we determined the effects of improving the hydrocarbon-chain packing structure of amino acids by applying to an established intercellular lipid model. The amino acids used in this study were either neutral, acidic, or basic. In order to study their effects on our model, we first performed differential scanning calorimetry (DSC). DSC is a powerful methodology that can reveal structural changes of lipids in bilayers and lamellae by measuring phase transition enthalpies. The structural changes identified were subsequently analyzed in detail using small-angle (SAXS) and wide-angle X-ray diffraction (WAXD).

Next, for any amino acid that showed significant effects in the intercellular lipid model, we performed structural analysis using time-resolved SAXS and WAXD measurements to determine if similar structural changes are observed in human SC.

**Experimental**

**Chemical Reagents** L-Serine (Ser, >99%), l-alanine (Ala, >99%), l-glutamine (Gln, >99%), and l-(+)-arginine (Arg, >99%) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). l-Glycine (Gly, >99%) and glutamic acid (Glu, >99%), were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). l-Hydroxyproline (Hyp, 98.5%), N-acetyl-l-hydroxyproline (AHYP, 99%), and N-acetyl-l-glutamine (AGLN, 98.5%) were purchased from Kyowa Hakko Co., Inc. (Tokyo, Japan). L-Hydroxyproline (Hyp, 98.5%) and glutamic acid (Glu, 99%), were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). L-Asparagine (Asn, 98.5%), L-Glutamic acid (Glu, 99%), were purchased from Kyowa Hakko Co., Inc. (Tokyo, Japan). L-Serine (Ser, >99%), l-alanine (Ala, >99%), l-glutamine (Gln, >99%), and l-(+)-arginine (Arg, >99%) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

**Preparation of Lipid Model** A lipid model containing CER[ADS], PA, and CHOL (CER[ADS]:PA:CHOL, 26.5:59.6:13.9, %mol) was prepared as previously reported.18 The preparation was similar to an established intercellular lipid model. The amino acids used in this study were either neutral, acidic, or basic. In order to study their effects on our model, we first performed differential scanning calorimetry (DSC). DSC is a powerful methodology that can reveal structural changes of lipids in bilayers and lamellae by measuring phase transition enthalpies. The structural changes identified were subsequently analyzed in detail using small-angle (SAXS) and wide-angle X-ray diffraction (WAXD).

Next, for any amino acid that showed significant effects in the intercellular lipid model, we performed structural analysis using time-resolved SAXS and WAXD measurements to determine if similar structural changes are observed in human SC.

**Preparation of Human SC Sheets** Human abdominal SC sheets were purchased from Biopredic International (Saint-Grégoire, France). The procedure to separate SC sheets from human skin was as follows. Skin was floated on 0.25% (w/v) trepsin dissolved in phosphate-buffered saline (PBS), pH 7.4, at 30°C for 4h. Thereafter, the SC was separated from the skin. Prepared SC sheets were rinsed three times in distilled water. Either 20% AHYP (w/w) or water as a control was applied to the dried SC.

**SAXS and WAXD Measurements** SAXS and WAXD profiles of our intercellular lipid model were obtained using BL40B2 located at the SPring-8 (Hyogo, Japan). Sample-to-detector distance and the X-ray wavelength were approximately 540 nm and 0.083 nm, respectively. The energy of the X-rays was 15 keV. X-ray scattering and diffraction data were collected using an R-Axis VII imaging plate detector (Rigaku, Tokyo, Japan); exposure time was 30 s. Sample temperature was maintained at 32°C using an FP-99 DSC (Mettler-Toledo, Tokyo, Japan). A sample cell containing intercellular lipid model was sealed with polyimide film (Kapton EN, Du Pont-Toray Co., Ltd., Tokyo, Japan) and placed in the X-ray diffractometer’s sample holder.

**Time-Resolved SAXS and WAXD** Time-resolved SAXS profiles of human SC sheets were obtained using the BL40B2 located at the SPring-8. Measurement conditions were the same as for our intercellular lipid model. An approximately 5 mg of the SC was placed in a 1-mm capillary glass tube. Immediately after the sample solution was applied to the SC, X-ray exposure for 60 s and repeated at 120 s intervals up to 60 min after application at 32°C.

**WAXD Profiles of Human SC Sheets** WAXD profiles of human SC sheets were obtained using BL19B2 located at the SPring-8. Sample-to-detector distance and the X-ray wavelength were approximately 769 nm and 0.049 nm, respectively. The energy of the X-rays was 25 keV. X-ray diffraction data were collected using PILATUS 2M semiconducting detector (Dectris Ltd., Baden-Daettwil, Switzerland). Sample temperature was maintained at 32°C using an FP-99 DSC. For WAXD measurement, approximately 0.2 mg SC were stacked and set at the edge of a 1 mm capillary glass tube so that X-rays were emitted perpendicularly to the glass tube. Each sample was exposed for 30 s at 150 s intervals up to 15 min after sample solution application. Diffraction intensity was standardized by the incident X-ray intensity in front of the sample.

The scattering and diffraction intensities I were plotted as a function of the scattering vector q, defined as \[ q = \frac{4\pi \sin \theta}{\lambda}, \] where θ is the scattering angle and λ is the wavelength. The periodicity of the lamellar phases d from the positions of series of equidistant peaks (\( q_n \)) using the equation \[ d = 2\pi n q, \] where n is the order number of the scattering peak. Spatial calibration was performed using silver behenate. The circular average of the scattering and diffraction patterns was calculated to obtain a radial intensity profile.

**Data Analysis** The resulting SAXS and WAXD profiles were analyzed using the software Origin (Light Stone Co., Tokyo, Japan) to determine the integrated intensity, peak position, and full width at half maximum (FWHM). SAXS profiles were fitted using the Lorentzian function and WAXD profiles were fitted using a Gaussian function. The increase in FWHM indicates disturbance of the lamellar structure. Due to weakening of the interference effect, the scattering peak becomes a broad peak.
The resulting DSC thermograms were analyzed using the software Origin (Light Stone Co., Tokyo, Japan) to determine the transition enthalpy and phase transition temperature. DSC thermograms were fitted using a Gaussian function. Transition temperatures were determined as the maxima of the endothermic transition peaks.

Lorentzian function: \[ y = y_0 + \frac{2A}{\pi} \frac{w}{4(x-x_0)^2 + w^2} \]

Gaussian function: \[ y = y_0 + \frac{A}{\sqrt{4\ln(2)w^2}} \exp\left(-\frac{4\ln(2)(x-x_0)^2}{w^2}\right) \]

Here, \( y_0 \) is the offset for baseline correction; the peak area is represented by \( A \), \( w \) is the full width at its half maximum, and the center of the peak is denoted as \( x_0 \).

**Results**

**Effect of Amino Acids on the Nanostructure of CER[ADS]/PA/CHOL Bilayers** We previously reported that the intercellular lipid model (CER[ADS]/PA/CHOL) used in this study contains two phases with different lamellar periodicities. We found that Phase A has a 4.6 nm periodicity lamellar structure with Orth and Hex packing. In contrast, Phase B has a 4.3 nm periodicity lamellar structure with packing structure different from that of Orth and Hex. The

![DSC thermograph and SAXS profiles of the CER[ADS]/PA/CHOL lamellae lipid model used in our study. (a) DSC thermograph of the lipid model. Three endothermic peaks generated by performing curve fitting based on the Gaussian distribution are shown as green lines. The cumulative fitting curve is shown as a red line. (b) Overlay plot of the change in normalized integrated intensity of each lamellar phase, namely Phase A, Phase B, and PA Phase as a function of temperature (25–85 °C). (Color figure can be accessed in the online version.)](image)

**Table 1. Phase Transition Temperatures and FWHM of Phase A, Phase B, and PA Phase Are Summarized**

| Measurement method | Phase A | Phase B | PA phase |
|--------------------|---------|---------|----------|
|                    | Temperature (°C) | FWHM | Temperature (°C) | FWHM | Temperature (°C) | FWHM |
| DSC                | 61.16 | 3.78 | 67.24 | 3.36 | 60.76 | 9.10 |
| SAXS               | 60–62.5 | 0.12 | 65–72.5 | 0.14 | 45–62.5 | 0.06 |

**Table 2. Sample Categories and DSC Measurement Parameters for Each Lamellar Phase**

| Sample Categories | ΔH (μJ/mg) | Phase A | Phase B | ΔH (μJ/mg) | Phase B | Total enthalpy change ΣΔH (μJ/mg) |
|-------------------|------------|---------|---------|------------|---------|---------------------------------|
| Control           | 352.50     | 61.16   | 3.78    | 200.85     | 67.24   | 553.35                          |
| Ser Neutral       | 278.00     | 63.80   | 3.80    | 151.88     | 69.79   | 429.88                          |
| Ala                | 380.27     | 63.16   | 3.89    | 164.26     | 68.75   | 544.52                          |
| Gly                | 159.95     | 63.62   | 4.62    | 156.88     | 70.29   | 316.83                          |
| Hyp                | 375.95     | 64.65   | 3.83    | 277.32     | 69.12   | 653.27                          |
| Gln                | 362.89     | 65.00   | 5.25    | 245.43     | 69.73   | 608.31                          |
| AHYP Acidic       | 305.95     | 65.88   | 12.00   | 258.54     | 74.61   | 564.49                          |
| AGLN Basic        | 361.12     | 67.10   | 12.39   | 289.51     | 74.49   | 650.63                          |
| Glu                | 275.90     | 65.56   | 9.77    | 370.99     | 73.16   | 646.88                          |
| Arg                | 109.64     | 62.94   | 2.90    | 266.53     | 69.59   | 376.16                          |
hydrocarbon-chain packing structure of Phase B also has a lower proportion of Orth relative to Phase A. Therefore, we concluded that Phase B has a weak barrier function.

Figure 1(a) shows a lipid model thermogram while Fig. 1(b) shows the change in normalized integrated intensity of each lamellar phase as a function of temperature. The original thermogram of our lipid model is shown in black while Phase A-derived, Phase B-derived, and mainly PA-derived fitted curves are shown in green. The phase transition temperature of the endothermic peak observed by DSC and the temperature range and FWHM of the scattering intensity of the lamellar structure observed by SAXS are summarized in Table 1. Based on their thermotropic behavior, endothermic peaks were classified as either PA-derived, Phase A-derived, and Phase B-derived in the order of the lower phase transition starting temperatures. The characteristic of lamellar structures with different periodicities determined by SAXS can be attributed to DSC measurements.

Amino acids were applied and evaluated during preparation of the intercellular lipid model. Table 2 summarized the transition enthalpies, phase transition temperatures, and FWHM of individual amino acids that were applied. Neutral amino acids, namely Ser, Ala, Hyp, and Gln, caused a large change in both Phase A and Phase B in terms of transition enthalpy, phase transition temperature, and FWHM. In contrast, a group of acidic amino acids comprising AHYP, AGLN, and Glu caused FWHM to increase, as well as both the phase transition temperature bands, Phase A and Phase B. Those compounds possess a negative charge on either their side chain or...
their α-carboxyl group. A basic amino acid, Arg, reduced the transition enthalpy in Phase A.

The thermogram that we obtained following AHYP application was shown in Fig. 2. The phase transition temperature of Phase A was 65.9 °C, higher than that of the control (61.2 °C). Similarly, the phase transition temperature of Phase B was 74.6 °C, higher than that of the control (67.2 °C). In contrast, the phase transition temperature zone corresponding to phase-separated PAs decreased to between 40–55 °C.

Since the amino acid derivative AHYP maintains barrier function,18) it is widely used in formulating cosmetics. We therefore decided to conduct a more detailed study focused on AHYP.

**Effect of AHYP on the Nanostructure of CER[ADS]/PA/CHOL Bilayers**

We focused on AHYP, which had a significant effect on the intercellular lipid model CER[ADS]/PA/CHOL, using SAXS measurements. SAXS measurements are useful for analyzing lamellar structures and determining the lamellar period of lipid membranes. Figures 3(a) and 3(b) showed the scatter profiles of lipid model at 25 °C for application of AHYP and control. Scattering peaks observed at approximately $q_1 \approx 1.3$, $q_2 \approx 2.7$, and $q_3 \approx 4.1 \text{ nm}^{-1}$ were derived from lamellar structures with a lamellar period of 4.6 nm, and the scattering patterns indicate the presence of a Phase A of short-period lamellar structures. Scatter peaks from other structures were also observed in the vicinity of scatter peaks from Phase A. This distinct scatter peak was observed at approximately $q_1 \approx 1.4$, $q_2 \approx 3.0$, and $q_3 \approx 4.5 \text{ nm}^{-1}$, with a lamellar period of 4.3 nm derived from the lamellar structure, indicating the presence of Phase B in the short-period lamellar structure.

The peak positions, peak intensities, and FWHM of Phases A and B are summarized in Table 3. In the AHYP-applied model, the FWHM of Phase A was reduced to 0.075 compared with 0.122 for the control. Similarly, FWHM of Phase B was reduced to 0.119 compared to 0.138 for the control. In addition, we observed no change in the ratio of Phase B/Phase A in the integrated intensity comparison and not intensity comparison, but this ratio decreased to 0.36 following AHYP application compared with 0.51 for that of the control. SAXS analysis, which complemented the DSC measurement, showed a decrease in the intensity of Phase B relative to Phase A (Table 3). Additionally, AHYP application decreased Phase B. Next, we investigated the effect of AHYP on human SC by

### Table 3. Peak Position, Peak Intensity, FWHM, and Peak Intensity Ratio Determined by Simultaneous SAXS and WAXD Measurements

|                | Phase A (25 °C) | FWHM | Phase B (25 °C) | FWHM | Ph B/Ph A peak ratio* (25 °C) |
|----------------|----------------|------|----------------|------|-----------------------------|
|                | Peak position (nm$^{-1}$) | Peak intensity (a.u.) | Peak position (nm$^{-1}$) | Peak intensity (a.u.) | Peak position (nm$^{-1}$) | Peak intensity (a.u.) | |
| Control        | 1.345          | 10289 | 0.122          | 1.474          | 5214           | 0.138          | 0.51 |
| AHYP           | 1.371          | 63028 | 0.075          | 1.486          | 22530          | 0.119          | 0.36 |

* The abbreviations PhA and PhB correspond to Phase A and Phase B, respectively.

Fig. 4. SAXS Profile of Phase X

SAXS profiles collected from the CER[ADS]/PA/CHOL lamellae used in our study are shown. Note the changes in X-ray scattering peak intensities of the lipid model following application of either AHYP or water. SAXS intensity profiles collected from 0 to 60 min are indicated by a change in intensity from dark to light blue (black line: 0 min) (a) SAXS profiles with AHYP applied. (b) SAXS profiles of a control lipid model. Roman numerals (III) indicate the scattering 3rd order of the LPP. Arabic numerals indicate the different scattering orders of the SPP. (Color figure can be accessed in the online version.)
measuring SAXS and WAXD simultaneously.

**Effect of AHYP on the Human SC Determined by SAXS**

Either AHYP or water, as a control, was applied to SC at 25°C and changes were determined by time-resolved SAXS. The result revealed the changes in lamellar structure as shown in Figs. 4(a) and 4(b). The scatter peaks observed at approximately \( q_1 \approx 0.97, q_2 \approx 1.85, \) and \( q_3 \approx 2.80 \) nm\(^{-1}\) were from short-period lamellar structures with a period of 6.4 nm. Scattering peaks from other structures were also observed in the vicinity of peaks from short-period lamellar structures. This

![Figure 5](image-url)

**Fig. 5.** SAXS Profiles Collected from 0 to 60 min after Application of Either AHYP or Water

(a) Parameters for integrated intensity where scattering peak was observed at approximately \( q \approx 1.53 \) from Phase X. (b) Overlay plot of the change in normalized integrated intensity from Phase X of each application sample.

![Figure 6](image-url)

**Fig. 6.** WAXD Profiles Collected from 0 to 15 min after the Application of Either AHYP or Water

(a) Parameters for integrated intensity where scattering peaks were observed at approximately \( q = 15 \) nm\(^{-1}\) from Hex \((I_{Hex}; I_{17} = 2 \times I_{15}/3)\) and \( q = 17 \) nm\(^{-1}\) from Orth \((I_{17});\) with either AHYP applied or (b) water as control. (c) Overlay plot of the change in \( I_{17}/I_{Hex} \) ratio. (d) Normalized integrated intensity from data shown in (a). (e) Normalized \( I_{17}/I_{Hex} \) ratio from data shown in (c).
distinct scatter peak was observed at approximately $q_1 \approx 0.44$ and $q_3 \approx 1.33$ nm$^{-1}$, and the lamellar period was derived from a lamellar structure of 14.2 nm, indicating the presence of a long-period lamellar structure.

Those lamellar structures showed values close to those previously reported for the short periodicity phase (SPP) in SC, but they were slightly longer than the 13.4 nm lamellar period reported for the long periodicity phase (LPP) in SC. No significant changes in SPP and LPP were observed when AHYP and water were applied. However, we observed a different lamellar structure (Phase X) with a lamellar period of 4.1 nm, whose scattering peaks were observed at approximately $q_1 \approx 1.53$, $q_2 \approx 3.06$, and $q_3 \approx 4.59$ nm$^{-1}$. We considered the phase transition temperature of Phase X to be around 65°C (Fig. S2), and it had the same thermal property as the phase transition of Phase B at around 65°C in the intercellular lipid model. Since the peak position of Phase X differs depending on individual differences in the SC, the peak position $q_1 \approx 1.47$ (Fig. S2) that we observed in this study is thought to be derived from Phase X. This idea is consistent with the peak position $q_1 \approx 1.47$ (Table 3) for Phase B. Based on these observations, we believe that Phase X corresponds to Phase B in the intercellular lipid model. We therefore focused on this Phase X. Figure 5 showed the consequences of the integrated intensity due to the change in Phase X. When water was applied, the integrated intensity from Phase X was temporarily increased. However, the scattering intensity of Phase X did not change between before and 60 min after applying water. When AHYP was applied, however, we verified that the scattering intensity derived from Phase X decreases by half over time. Figure 5(b) showed the results of comparing the integrated intensity change (Fig. 5(a)) as $I/I_0$. Even when the initial values were uniform, the scattering integrated intensity derived from Phase X decreased following AHYP application.

**Effect of AHYP on Human SC Determined by WAXD**

We determined AHYP’s effects on the hydrocarbon-chain packing structure, which significantly altered the CER/ADS/PA/CHOL ratio of intercellular lipid model, was determined by WAXD. Supplementary Fig. 1 shows the WAXD profiles of AHYP and water applied to human SC at 25°C. Figure 6 shows the consequences of the change in the integrated intensity derived from Hex ($I_{Hex}$) and Orth ($I_{Orth}$). $I_{Hex}$ was calculated from the integrated intensity of diffraction peaks observed around $q \approx 15$ nm$^{-1}$ ($P_{18}$) and $q \approx 17$ nm$^{-1}$ ($P_{17}$). $I_{Orth}$ was calculated from the integrated intensity of $P_{37}$. These results are consistent with the sample data from human SC with AHYP measured by WAXD profiles (Fig. S1).

We verified that $I_{Orth}$ derived from Orth increased 9 min after the start of dehydration, but returned to the same integrated intensity as before application 15 min after the start of dehydration. When water was applied, however, the $I_{Orth}$ decreased with dehydration after application. After 15 min of dehydration, the intensity was decreased, as was the Orth ratio. Therefore, we propose that the application of water disrupted the hydrocarbon-chain packing structure of intercellular lipids during the dehydration process.

**Discussion**

In this study, we found that AHYP maintains lipid packing structure by using intercellular lipid model, and verified its effects on human SC. We propose that AHYP altered the intercellular lipid packing structure by electrostatic interactions. We also propose that applying AHYP to the intercellular lipid model increased the phase transition temperature of Phase A and improved the membrane’s thermal stability. We had proposed two possible mechanisms: either the lipid composition of Phases A and B has changed, or that AHYP application changed the lipid configuration.

Bouwstra et al. previously reported that cholesterol sulfate reduces the ratio of lamellar phases with similar lamellar period to that of Phase B in their intercellular lipid model. They reported that the sulfonic acid group of dissociated cholesterol sulfate increases the interface area per lipid molecule and decreases the packing density, thereby increasing the solubility of CHOL. In our study, we inferred that AHYP did not enhance CHOL solubility based on the absence of observed reduction in phase-separated CHOL in Fig. 3(a). We assumed that the carboxylic groups of acidic amino acids act as negative charge donors in our intercellular lipid model. This idea was supported by not only the increase in phase transition temperature for Phases A and B, but also the increase in FWHM of both phases. In our previous study, we considered Phase A as being composed of CER, CHOL, and PA, whereas Phase B was composed of only CER and CHOL. Moore et al. determined that in the PA-based domain, the phase transition temperature range of perdeuterated PA in their intercellular lipid model was 42.5–62°C, which agrees with the phase transition temperature range derived from the scatter peak at $q \approx 1.73$ nm$^{-1}$. We concluded that this scattering peak was derived from the crystalline phase composed mainly of PA (i.e., the PA Phase). The carboxylic groups of acidic amino acids were negatively charged in acetate buffer with a pH of about 5.3. We thought that the negative charges of these acidic amino acids suppressed PA elimination from Phase A due to electrostatic repulsion. We hypothesized that maintaining PAs within Phase A leads to local lipid composition changes and increases the phase transition temperature; however, suppressing PA elimination also leads to non-uniformity in the hydrocarbon-chain packing structure, resulting in increased FWHM.

We observed that acidic amino acids elevate the phase transition temperature and FWHM, especially in N-acetylated amino acids, AHYP and AGLN (Table 2), whose amino groups were protected by the acetyl group. We hypothesized that the electrostatic interaction between negatively charged acidic amino acids and dissociated PA suppresses PA elimination from Phase A. In contrast, the positively charged amino acid, Arg, reduced not only the transition enthalpy but also Phase A (Table 2). We assumed that electrostatic attraction between positively charged Arg and negatively charged PA promotes PA withdrawal from Phase A.

Amino acids without charged side chains, namely Ala, Hyp, and Gln, did not cause major changes in either Phase A, Phase B, or the phase transition temperature of FWHM. Those absence of major changes was probably due to the absence of electrostatic repulsion against PA. In contrast, Gly was observed to have a decreased transition enthalpy in both Phases A and B. Most amino acid NMFs have uncharged side chains. The observed decrease in enthalpy may be caused by stereological affinity with Gly. Since acidic amino acids were less abundant than other major amino acids among NMFs in the SC, the effects that acidic amino acids exert...
must be distinguished from the moisturizing effect of NMF.\(^{26}\) Lamellar conformation may also be maintained by highly dissociative lipids such as cholesterol sulfate.\(^{22,27}\)

In this study, we determined that AHYP exerted a prominent effect using an established intercellular lipid model. AHYP application to human SC halved the integrated intensity of Phase X (Fig. 4), which was considered a hydrocarbon-chain packing structure with low barrier function.\(^{10}\) In contrast, the integrated intensity of Phase X was temporarily increased by applying water, although it eventually returned to its original condition (Fig. 5). Similar changes in water content were reported in the short periodicity phase.\(^{4,28}\) AHYP application increased the Orth ratio, suggesting that the ordered hydrocarbon-chain packing structure was maintained. Based on those data, we proposed that AHYP improves barrier function by reducing Phase X in human SC.

Considering the mechanism of Phase X change in human SC, AHYP inhibits Phase B formation in the intercellular lipid model used in this study. Inhibiting CER conformation changes was thought to increase the order within the hydrocarbon-chain packing structure due to the elimination of FFAs.

At pH 5.3, about 25% of FFA in human SC is dissociated\(^{24}\) based on the Henderson–Hasselbalch equation.\(^{29}\) Making the pH of the superficial layer of the SC more acidic should therefore suppress FFA dissociation. The pH deep inside the SC is believed to be approximately 7.4,\(^{23}\) and that is where FFAs are thought to be more than 90% dissociated\(^{27,29}\) from intercellular lipids, which suggests that Phase X was more likely to occur. Since the pH of deep inside the SC is difficult to control, delivery of electrostatically interacting agents that can inhibit FFA dissociation is the key factor to reducing Phase X. Small molecules such as AHYP and acidic amino acids are ideal for those purpose.

To date, moisturizing agents are thought to exert their effects based on hydration, occlusion, and physiological effects. No agents are known to maintain barrier function by physicochemical action on intercellular lipids. In this study, we verified that the proportion of intercellular lipids in human SC is decreased by applying water. This finding suggested that SC barrier function may be impaired even by washing one’s face without cleaning agents. AHYP application was proposed to maintain the ordered hydrocarbon-chain packing structure of intercellular lipids, and the Orth ratio. AHYP, an N-acetylated acidic amino acid, was previously proposed as a useful agent that maintains the barrier function of intercellular lipid by physicochemical action.

Narangifard et al. reported that the lamellar structure and lipid arrangement of intercellular lipids change during human SC maturation.\(^{10}\) This report suggested that the intercellular lipid hydrocarbon-chain packing structure is not rigid but is, instead, plastic. It also proposed that the efflux of lipids by the lavage agent also occurs in plastically intercellular lipids. Lipid efflux might be appeared to depend on lipid solubility, and the order of lipid elution from intercellular lipids is FFA, CHOL, and CER.\(^{8}\) Short-chain FFAs have been found to influence the formation of hydrocarbon-chain packing structures\(^{31}\) and the homogeneity of lipid membranes.\(^{32}\) The proportion of PA has been reported to increase in cases of atopic eczema.\(^{33}\) Because of the nature of PA and short-chain FFA, the intermolecular forces between the hydrophobic chains are thought to be weak.\(^{17}\) PA’s solubility is relatively high compared to other intercellular lipids. Thus, PA is easily withdrawn from intercellular lipid bilayers.

Taking advantage of the fact that electrostatic repulsion from amino acids inhibits the withdrawal of short-chain FFA from the lipid bilayer was valuable for improving the barrier function of pathological skin, which contains a higher proportion of short-chain FFA.\(^{33}\) This approach was also expected to contribute to preventing the formation of rough skin due to excessive washing during the current (COVID-19) pandemic. We believe that this approach may provide an important avenue for developing new skincare products.

**Conclusion**

In this study, we used an intercellular lipid model consisting of CER[ADS]/PA/CHOL with coexisting lamellar structures of different periodicities to determine the effects of AHYP and acidic amino acids using DSC measurements. We found that AHYP maintains the reduction in Phase X and Orth ratio. We also found that AHYP and acidic amino acids are useful agents for maintaining the hydrocarbon-chain packing structure of intercellular lipids through electrostatic repulsion. In the future, we will expand the scope of this study, which may eventually lead to the development of skincare products that can maintain the skin’s barrier function.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Supplementary Materials**

The online version of this article contains supplementary materials.

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