A New Method to Restore the Hair Crystalline and Amorphous Structures: Combination Treatments of Glycine Betaine and Macromolecular Hydrolyzed Keratin Proteins

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Abstract

Background: The objectives are not only to present novel hair processing method to repair the denaturation and efflux of intermediate filaments (IF) and keratin-associated proteins (KAP) that constitute the hair internal cortex, but also to enable detailed description of the repair mechanism.

Methods: The effects of treatments in sequence of glycine betaine and IF-or KAP-derived macromolecular hydrolyzed keratin protein on hair cortex processed by permanent waving were evaluated by tensile test, high-pressure differential scanning calorimetry, and small-angle X-ray scattering measurement. The interactions of glycine betaine with IF-or KAP-derived macromolecular hydrolyzed keratin proteins were also evaluated by circular dichroism spectroscopy.

Results: The combined treatments of glycine betaine and IF-derived macromolecular hydrolyzed keratin showed synergistic recovery effects on in-water tensile stress, enthalpy of denaturation, and IF regular structure, demonstrating a high repair effect on denatured IF crystalline structure. In contrast, the combined treatments of glycine betaine and KAP-derived macromolecular hydrolyzed keratin showed synergistic recovery effects with respect to in-air tensile stress, denatured peak temperature, and IF orientation, demonstrating a repair effect that replace the leaked KAP amorphous structure. Furthermore, the results of the circular dichroic spectra showed that glycine betaine and macromolecular hydrolyzed keratin proteins interact and restore the secondary structures of each keratin proteins.

Conclusion: Our study not only found a new hair processing method that showed a high repair effect on hair cortex structure, but also suggested a repair mechanism when the

thermodynamic theory of protein stabilization by glycine betaine was applied to a practical method of hair structure repair.

Keywords: permanent waving; hair structure repair; intermediate filament; keratin-associated protein; glycine betaine; macromolecular hydrolyzed keratin protein

1. Introduction

Chemical treatments, such as hair coloring and permanent waving, are known to denature and efflux intermediate filaments (IF) and keratin-associated proteins (KAP) that constitute most of the hair internal cortex [1]. At present, countermeasures against degeneration and spillage are inadequate, and replacement components to replace and improve the texture have been mainly studied. In contrast, to develop a repair method that "brings the damaged cortex closer to the original structure and restores the original function and texture of the hair" is more important. This will help to maintain the favorable texture of the natural elasticity and smoothness of the hair and improve the quality of life of the consumer.

We have focused on the mechanism by which glycine betaine (INCI name: Betaine) stabilizes the structure of proteins in aqueous solution; therefore, we reported on a method for restoring the hair crystalline structure denatured by hair-bleaching and permanent waving [2]. On the occasion, we speculated that treatment of damaged hair with high concentrations of glycine betaine depletes the water around the denatured cortex proteins and renders it entropically unstable, thereby converting it to a compact and stable structures that ejects water [3, 4, 5], resulting in restoration of the original crystalline structures of the hair cortex.

The objective of this study is to develop a repair method that sufficiently restores the native structure of cortex by further applying the protein stabilization effect of glycine betaine to improve the recovery of the denatured crystalline structure and to find a way to replenish the eluted amorphous structure.

To find the repair method, we evaluated the resilience of tensile stress when a damaged hair was treated with a mixture of glycine betaine and various ingredients; however, we found no method that showed a synergistic stress recovery effect. On the other hand, we found that the combination of glycine betaine and certain macromolecular hydrolyzed keratin proteins produced a synergistic high stress recovery effect.

Thus, in this paper, we examined the effects of the combination treatment of glycine betaine and certain macromolecular hydrolyzed keratin proteins on hair cortex structure, and the interaction of glycine betaine with certain macromolecular hydrolyzed keratin proteins. Thereby, the aims are not only to present a new hair processing method that demonstrates a high repair effect of the hair cortex structure, but also to enable a detailed description of the repair mechanism.

2. Materials and Methods

2.1 Materials

Reagents: Ammonium thioglycolate solution, ammonia solution, sodium bromate, glycine betaine, and hydrolyzed keratin protein (from wool) were used in commercial cosmetic grades. Certain macromolecular Hydrolyzed keratin proteins were either IF-derived (INCI name: Keratin, molecular weight 40,000) or KAP-derived (INCI name: Hydrolyzed Keratin, molecular weight 20,000-40,000).

Hair samples: Healthy, chemically untreated hair collected from the same Japanese adult woman approximately 25 cm in length was used to make hair bundles approximately 1.0 g.

Purification of hair bundles: Immersed in a 5% sodium C12-13 pareth sulfate aqueous solution at a bath ratio of 1:100 at 25°C for 1 hour, then washed with deionized water and air dried. The processed bundles were named as “Untreated”.

2.2 Preparation of sample hair

2.2.1 Permed hair

Preparation of reduction solution: The ammonium thioglycolate concentration was 0.75 mol/L and adjusted to pH 9.25 with ammonia solution.

Preparation of oxidation solution: The sodium bromate concentration was 6% (w/w) and adjusted to pH 6.0 with phosphate buffer.

The permed hairs were prepared by reducing and oxidizing the Untreated, as follows:

Reduction Treatment Step: Untreated were immersed in a reduction solution (35°C) having 20 times the weight of the hair bundles for 15 minutes, washed with water for 3 minutes, and then towel dried to remove excess water.

Oxidation Treatment Step: The resulting reduced hair bundles were immersed in an oxidation solution (35°C) having 20 times the weight of the hair bundles for 15 minutes, washed with water for 3 minutes, and air dried. The processed bundles were named as “Permed”.

2.2.2 Glycine betaine treated hair

The Permed was immersed in a 60% (w/w) solution of glycine betaine (pH 8.0) for 30 minutes at 35°C, washed with water for 3 minutes, air dried. The processed hair bundle was designated "GB". GB is a positive control in the in-water tensile test and the high-pressure differential scanning calorimetry based on previous reports [2].

2.2.3 Macromolecular hydrolyzed keratin protein treated hair

The Permed were immersed in a 1% (w/w) solution of IF-or KAP-derived macromolecular hydrolyzed keratin protein at 35°C for 30 minutes, washed with water for 3 minutes, and air dried, the processed hair bundles obtained were named as "Ifp" or "Kap", respectively.

2.2.4 Combinatorial treated hairs of glycine betaine and Macromolecular hydrolyzed keratin protein

The Permed were immersed in a 60% (w/w) solution of glycine betaine (pH 8.0) at 35°C for 30 minutes, immersed in a 1% (w/w) solution of IF- or KAP-derived hydrolyzed keratin protein at 35°C for 30 minutes, washed with water for 3 minutes, and air dried. The processed hair bundles obtained were designated as "GB-Ifp" or "GB-Kap".

On the other hand, the Permed were immersed in a 1% (w/w) solution of hydrolyzed keratin protein derived from IF or KAP at 35°C for 30 minutes, immersed in a 60% (w/w) solution of glycine betaine (pH 8.0) at 35°C for 30 minutes, washed with water for 3 minutes, and air dried. The resulting treated hair bundles were designated as "Ifp-GB" or "kap-GB".

2.3 Hair tensile strength test

The cross-sectional area of the monofiber samples were calculated from the average of the short and long diameters of the hair measured by a hair diameter measuring instrument (SK2000, KatoTech) under the conditions of 20°C and 60% RH. Subsequently, the tensile load at the time of 15% elongation was measured using a high-sensitive tensile tester (KES-G1-SH, KatoTech) at 20°C in water or at 20°C in 60% RH to calculate the hair tensile stress per unit cross-sectional area f (N/m^2) in water or air.

2.4 CD spectra

A circular dichroism spectrometer (J-820, JASCO Corp.) was used to measure at 20°C, cell length of 1 mm, and measurement range of 190 to 250 nm. Glycine betaine was prepared at 0.30% (w/w) and IF- or KAP-derived hydrolyzed keratin protein at 0.005% (w/w) in distilled water. The interaction of glycine betaine with IF- or KAP-derived hydrolyzed keratin proteins was evaluated by alteration of the spectra.

2.5 High-pressure differential scanning calorimetry (HPDSC)

After the sample hairs were moistened at 25°C and 60% RH, they were cut to approximately 0.5 mm, and 5 mg was accurately weighed into a stainless-steel pressure pan for DSC measurement. Then, 10 μL of water was added to the pan, and the container was sealed. The samples were left for 1 week to keep the moisture content and distribution in the hair constant. A differential scanning calorimeter (DSC 7020, Hitachi High-Tech Corp.) was used to raise the temperature from 30°C to 190°C at a rate of 10°C/min.

2.6 Small-angle X-ray scattering (SAXS)

Small-angle X-ray scattering was measured at BL8S3 of the Aichi Synchrotron Radiation Center. The measurement conditions were light source 8.2 keV, X-ray wavelength 0.15 nm, exposure time 300 s, camera length 1100 mm, beam size approximately 1.0 × 0.5 mm (width × height). We used the imaging plate detector (R-AXIS IV++, Rigaku Corp.); thereby, we analyzed the regular structure or the orientation of the IF, based on the reports of Briki et al. [6] or Kajiura et al. [7].

3. Results and Discussion

3.1 Verification of the effect of combined treatment on tensile strength in water or air

3.1.1 15% Elongation stress in water

Figure 1 shows the 15% elongation stress in water (20°C). Several studies on the tensile properties of hair to date [8-12], show that the behavior of hair elongation in water is explained by the elongation model of aggregates. The aggregates are considered hard IF components composed of α-crystals surrounded by gelled KAP components. Therefore, the

15% elongation stress in water at 20℃ approximates the characteristic value upon elongation of IF.

It is widely known that hair strength was reduced by permanent waving, and in Figure 1, the Permed was reduced in stress from Untreated. The GB showed a significant restoration of stress, like the results previously published by us, suggesting an effect of restoring the intensity of IF [2]. In contrast, hydrolyzed keratin proteins alone, such as the Ifp and Kap, recovered stress, but most recovered as much as or less than the GB. On the other hand, among the combinatorial treatments of GB and molecular hydrolyzed keratin proteins derived from IF, the GB-Ifp was found to be significantly more stress-resilient than the GB, suggesting a synergistic effect.

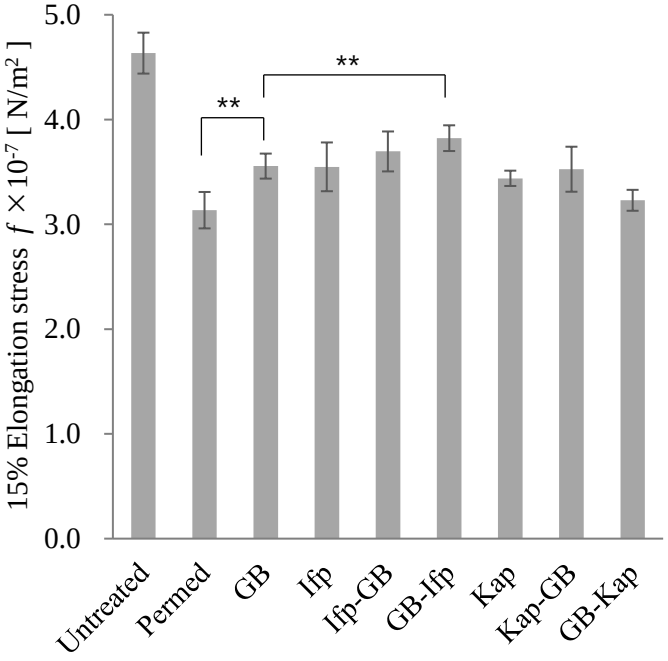

Fig. 1 15% Elongation stress in water (20℃)
Mean(*SD*) $n = 10$ *t*-test ** :$p < 0.01$

3.1.2 15% Elongation stress in air

Figure 2 shows the stresses at the time of elongation of 15% under dry conditions in air (20°C, 60% RH). As can be seen from the comparison with Figure 1, tensile stress in the air is more than twice that in the water, but the tensile stress of IF components is almost constant in the water and the air [8]. Therefore,

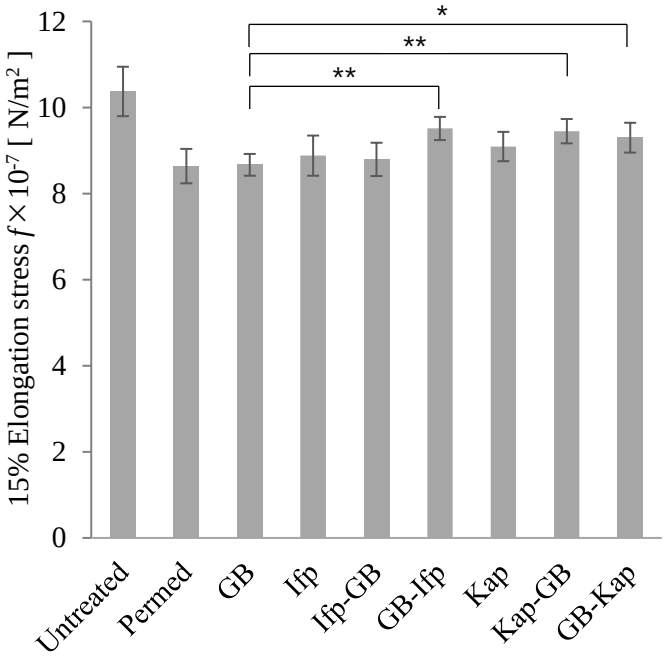

Fig. 2 15% Elongation stress in air (20℃ 60%RH)
Mean(*SD*) $n = 10$ *t*-test * :$p < 0.05$ ** :$p < 0.01$

comparing the 15% tensile stress in the water and the air makes it possible to infer the effect of each treatment on KAP.

In Figure 2, the stress of the Permed decreased from the Untreated, on the contrary the GB was comparable to the Permed. In the GB, it was expected that elongation stress would be recovered by stabilizing KAP as in IF. However, because the stabilization effect alone did not restore cystine binding abundant in KAP, it may not have been possible to recover the stress.

As with in water, the GB-Ifp significantly recovered from stress compared with the GB; however, the degree of recovery was like that in water. On the other hand, the Kap-GB and the GB-Kap showed significant stress recovery not seen in water. This suggests that the combination of glycine betaine and KAP-derived hydrolysis keratin protein can significantly alter and restore the state of KAP.

3.2 Verification of the interaction between glycine betaine and macromolecular hydrolyzed keratin protein by CD spectra

Figure 3 shows the CD spectra of glycine betaine and IF-derived hydrolyzed keratin protein, and Figure 4 shows the CD spectra of glycine betaine and KAP-derived hydrolyzed keratin protein. In Figure 3, glycine betaine alone did not show circular dichroism in the measured

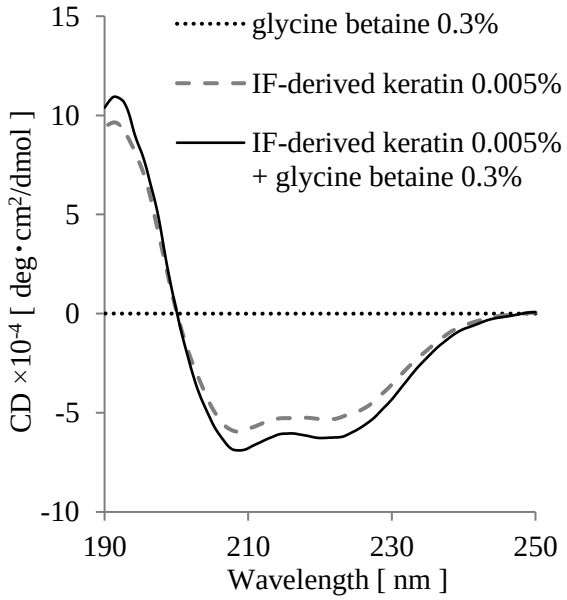

Fig. 3 CD spctra of glycine betaine and IF-derived macromolecular hydrolyzed keratin solution

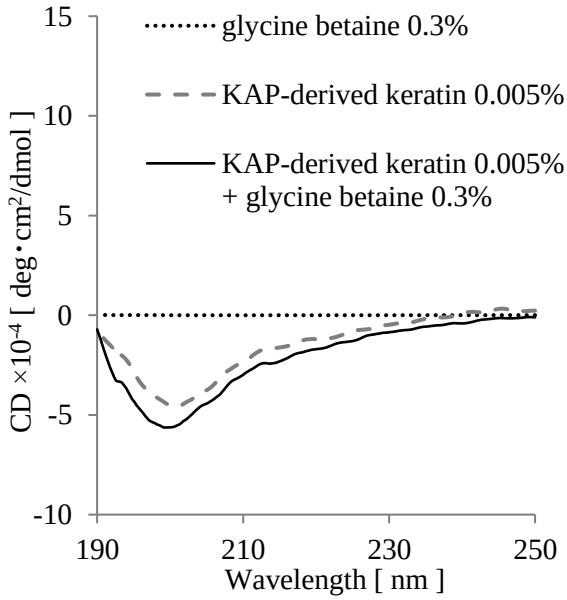

Fig. 4 CD spctra of glycine betaine and KAP-derived macromolecular hydrolyzed keratin solution

wavelength range; however, IF-derived hydrolyzed keratin protein showed a spectrum of α-helix structure (negative maxima at 208 nm, 222 nm, positive maxima at 191-193 nm). In Figure 4, the KAP-derived hydrolyzed keratin protein showed a spectrum of the random-coil structure (negative maxima between 195 nm and 200 nm). Moreover, it was found that a mixture of glycine betaine and each hydrolyzed keratin protein exhibited higher intensity of circular dichroism.

These results suggested that the hydrolyzed keratin proteins derived from IF or KAP regenerate a compact secondary structure through interaction with glycine betaine. This may prove that glycine betaine interacts with the proteins that constitute the hair cortex and restores the structure of the cortex. In addition, it was predicted that restoration of the secondary structure of the hydrolyzed keratin proteins would improve the penetration into the hair and its affinity for the hair cortex protein, which was thought to be the reason for synergistic restoration of hair tensile stress.

3.3 Verification of the effect of combined treatment on hair cortex structure by HPDSC

HPDSC, also referred to as Wet-DSC, analyzes hair cortex keratin containing excess water in sealed capsules to retain moisture during heating. In this method, the endothermic peak at 130-160 °C is attributed as the denaturation enthalpy ΔH of the α-helix of the keratin, depending on the amount and structural integrity of the helix segment in the IF [13, 14]. The denaturation peak temperature T_P is thought to control the melting peak by the amount and the crosslinking density of non-helical KAP surrounding the IF [15].

Figure 5 shows the HPDSC results. The Untreated shows that both ΔH and T_P are highest, and the hair has high structural integrity. In contrast, the Permed significantly decreased

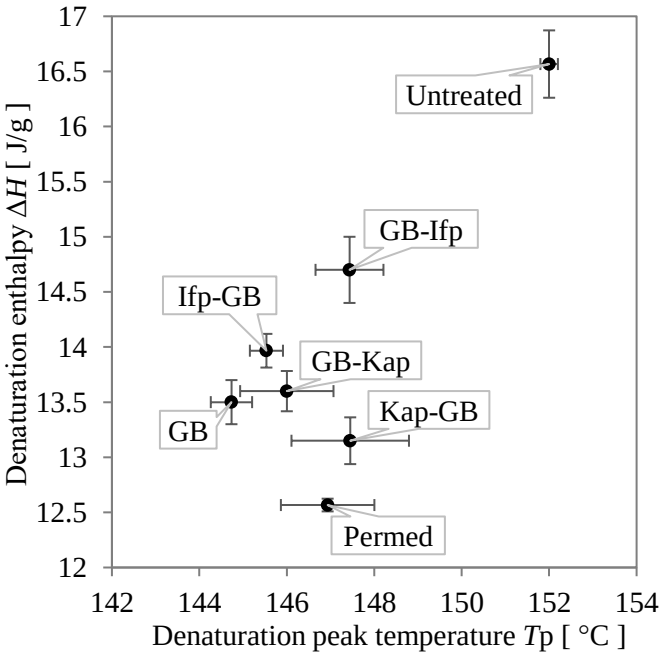

Fig. 5 Denaturation enthalpy ΔH and denaturation peak temperature Tp of hair samples

both ΔH and T_P, reduced the amount and crystallinity of IF, and reduced the amount and the density of the crosslinking of KAP. The GB showed a significant increase in ΔH and a decrease in T_P, the results of which were similar to previous reports [2]. Considering the DSC chart (not shown), it is considered that although the amount of crystalline structures and crystallinity of IF recovered by glycine betaine treatment, the temperature of the denatured peak decreased apparently due to the increase of crystalline structures that melt at relatively low temperature.

In the GB-Ifp and the Ifp-GB, ΔH and T_P was elevated, ΔH was particularly prominent. The combined treatment of glycine betaine and IF-derived hydrolyzed keratin proteins appears to have greatly restored crystal abundance and crystallinity. As a result, the T_P appeared to increase because of the increase in crystals that melt at relatively high temperatures.

In the GB-Kap and the Kap-GB, a significant increase in T_P was observed in the Kap-GB, where the increase in the in-air tensile stress was large; however, the difference in T_P from the GB was not large. This is probably because HPDSC is an excess water condition, i.e., KAP cross-linking (cystine binding) is evaluated by heating KAP in the gelled state, but the combined treatment of glycine betaine and KAP-derived hydrolyzed keratin proteins does not involve repairing cross-linking. Although, the combined treatment should have some effect of increasing T_P. Therefore, the following small-angle X-ray scattering was performed to evaluate the conformational changes in the IF level of hair cortex.

3.4 Evaluation of the Effect of Combined Treatment on the Hair Cortex Structure by Synchrotron Small-Angle X-Ray Scattering

In Figure 6, the relationship of the scattering vector q (nm^{-1}) to the equatorial small-angle X-ray scattering intensity were extracted from the two-dimensional SAXS patterns. Based on the IF distribution model proposed by Briki et al. [6], the shape of the first peak on the equator at $q = 0.7$ nm^{-1} provided information on the regularity structure of the IF in the fiber axis.

The shape of the first peak is clear for the Untreated, and IFs appear to be regularly arranged at regular intervals (about 9 nm). In the Permed, the shape of the first peak became unclear and it became a shoulder peak. This is considered to be a disturbance in the IF interval due to the loss of the crystallinity of the IF by permanent waving.

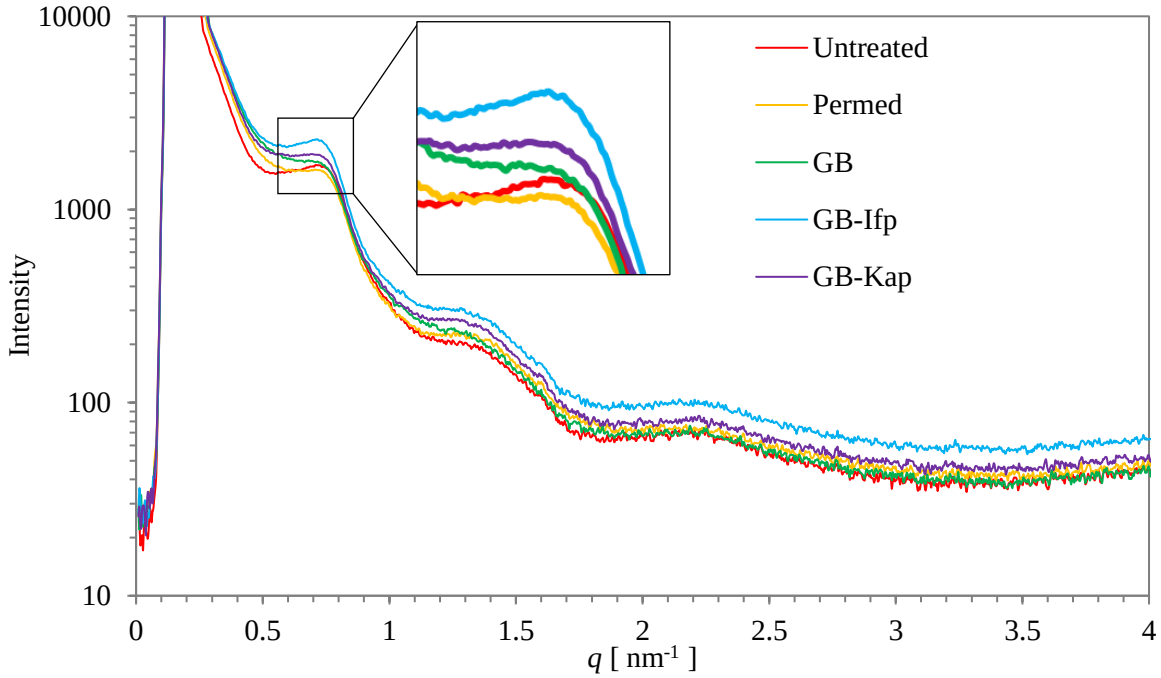

Fig. 6 The relationship of the scattering vector q (nm-1) to the equatorial small-angle X-ray scattering intensity

In the GB, the scattering intensity is generally higher, but the first peak is the still shoulder peak. Although some structural changes may have occurred to increase the scattering intensity, there was no clear effect on the regular structure of the IF. The GB-Kap has a slight peak shape compared to the GB and may have some effect of aligning the IF. Surprisingly, the shape of the first peak of the GB-Ifp was clearly restored, suggesting that the regular structure of the IF was restored. In other words, the combined treatment of glycine betaine and IF-derived hydrolyzed keratin proteins restored the original peak shape because it restored the original structure of the IF and increased the crystallinity.

Figure 7 shows the results of extracting the first peak (q = 0.7 nm^{-1}) intensity that were integrated as a function of azimuthal angle from the two-dimensional SAXS patterns. The full width at half maximum W of each orientation peak reflects the mean IF inclination to the hair fiber axis [7], and the orientation degree F (%) was calculated from the following equation and is shown in Table 1.

$$F\,[\%] = (360 - \Sigma\, W) \diagup 360 \times 100$$

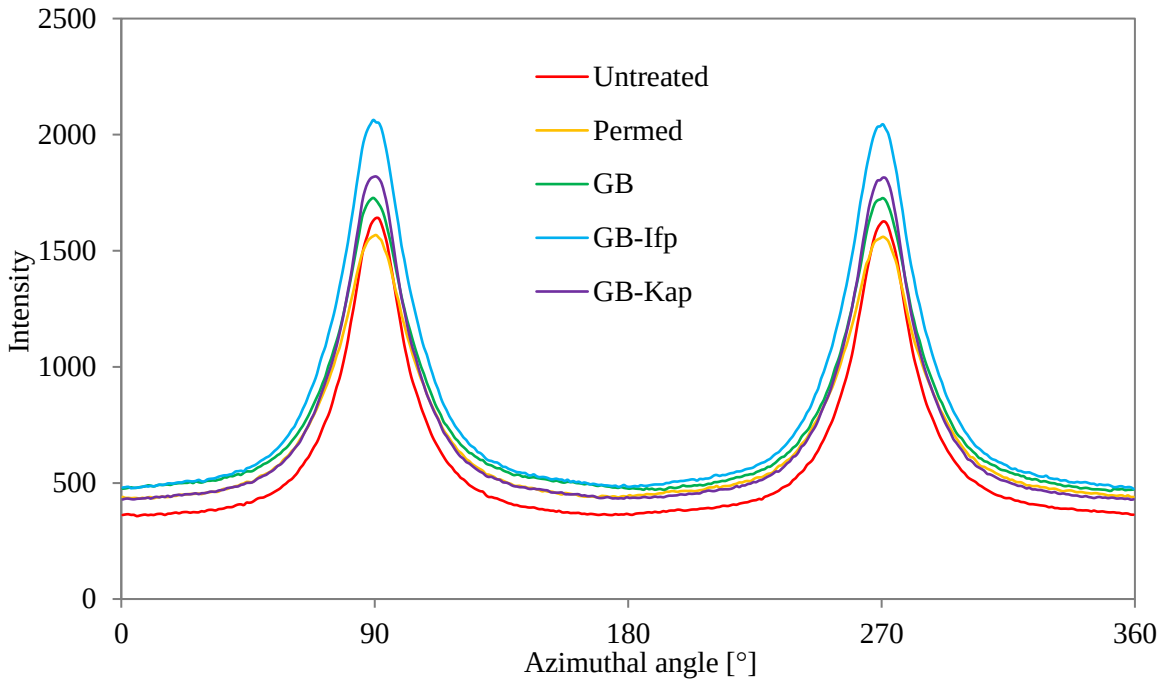

Fig. 7 The first peak (q = 0.7 nm^{-1}) intensity that were integrated as a function of azimuthal angle from the two-dimensional SAXS patterns patterns

Table 1 The orientation degree F (%) of hair samples

Hair sample	Untreated	Permed	GB	GB-Ifp	GB-Kap
Orientation degree F [%]	85.1	82.5	83.2	84.1	84.5

The Untreated shows the highest orientation, indicating that IF is aligned along the fiber axis. In the Permed, the crystallinity of the IF was reduced, and the orientation was misaligned with respect to the fiber axis.

The GB was thought to have a slightly higher orientation and increased orientation by restoring the crystal structure [2]. The orientation of the GB-Ifp was clearly restored compared with that of the GB, and it is considered that the orientation was restored by recovering the original crystalline structure of IF. The GB-Kap had the most restored orientation. This is thought to increase the orientation of the IF because the KAP-derived hydrolyzed keratin protein infiltrating the inside of the hair may densely filled between IF, and it is considered to be a substitute for the original KAP of the hair flowed out by permanent waving.

4. Conclusion

In this study, we evaluated the effects of the combination treatments of glycine betaine with IF-or KAP-derived macromolecular hydrolyzed keratin protein as a method to further enhance the ability to repair crystalline or amorphous structures, based on a previously reported study of the hair crystalline structure repair by glycine betaine [2].

As a result, the combined treatment of glycine betaine and IF-derived macromolecular hydrolysis keratin showed a synergistic recovery effect with respect to in-water tensile stress, enthalpy of denaturation, and IF regular structure, demonstrating a high repairing effect of IF crystalline structure denatured by permanent waving. On the other hand, the combined treatment of glycine betaine and KAP-derived macromolecular hydrolysis keratin showed a synergistic recovery effect with respect to in-air tensile stress, denatured peak temperature, and IF orientation, demonstrating a repair effect that could replace the leaked KAP amorphous structure.

Although the results are not shown, the combination treatment is considered important since no synergistic effect was seen when treated with a mixture of glycine betaine and macromolecular hydrolyzed keratin protein. The reason for this was inferred from the results obtained in the dichroic spectrum. Specifically, glycine betaine interacts with macromolecular hydrolyzed keratin proteins around and within the hair; therefore, glycine betaine leads to the restoration of secondary structures of not only the hydrolyzed keratin proteins but also the cortex protein. This can have resulted in a highly synergistic repair effect against denaturation and runoff to facilitate the recovery of the crystalline or amorphous structure of cortex to its original compact and stable structure.

This study not only found a new hair processing method that showed a high repair effect of hair cortex structure, but also suggested a repair mechanism when the thermodynamic theory of protein stabilization by glycine betaine was applied to a practical method of hair structure repair. These results suggest that this is an important example of a solution to the problem of restoring the cortex of damaged hair to its original structure, which was considered impossible in the past.

In this paper, the hair processing method is simplified for ease of discussion, but by further devising the treatment method and conditions, it is possible to find a method with a higher effect on the hair cortex structure repair.

5. Acknowledgments

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6. Conflict of Interest Statement

The authors declare no conflicts of interest associated with this manuscript.

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