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Effect of Heat Treatment on Human Hair Keratin Film

Toshihiro FUJII *, [1], Yuka TAKASHIMA[1], Shunsuke TAKAYAMA[1], Yumiko ITO[1], Tomoyuki KAWASOE[2]

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Abstract

Human hair keratin film consisting of fine fibrous and particle structures has been utilized as an alternative device to hair samples. Since application of heat is a well-known factor causing hair damage, the thermal effects on the keratin films and hair samples were examined focusing on color change, morphological observation, protein solubility, and oxidative proteins. The opaque white appearance of the keratin film changed to a yellow/light brown color by the thermal treatments at more than 170℃. This color change at 170℃ and higher was also detected by spectrophotometry. Scanning electron microscopy (SEM) observation showed that there was little change in the fine structures of the keratin films and hair samples even after a thermal treatment at 200℃ for 10 min. When protein solubility of heat-treated keratin films and hair samples were examined using a solution containing dithiothreitol (DTT) and urea, the amounts of proteins solubilized from heat-treated keratin films and hair samples were less than those from untreated keratin film and hair samples. The protein solubility decreased in a heating time- and temperature-dependent manner. A linear relationship between solubilized proteins and a heating temperature of 110 to 160℃ was found. Compared to hair samples, the degree of change was clearly evident in the keratin films. The solubilized proteins consisted of keratin and keratin-associated proteins (KAPs). The amount of carbonylated proteins increased in the films treated at 120–180℃. This data suggests that thermal treatment promotes protein oxidization and induce the formation of "Stable Structure" which has not yet been identified. In any event, hair thermal damage will be quantitatively evaluated using the keratin films in place of hair samples.

Key words: human hair keratin film, heat stability, thermal sensitivity, protein solubility, comparison with hair sample.

1. Introduction

Human hair is frequently subjected to thermal treatment when it is dried after washing, or when it goes through a process of hair styling. The use of hair irons at high temperatures in addition to hair dryers also causes hair damage. Thus, the effect of thermal treatments on hair has been a subject of research. The cuticles which make up the surfaces of hair are known to have "lift-ups" and to form "blisters" that are porous flares in hair caused by the heat treatment[1)–3)]. Therefore, the coefficient of friction in damaged hair by such factors increases[4)]. The technology to visualize flips and fuzz in damaged cuticles quantitatively has also been developed[4)–6)]. The formation of hollow spaces within end-cuticles was observed by transmission electron microscopy (TEM), which suggested the decrease of interaction between cuticles and cortex. Mechanical strength of hair mainly depends on the cortex, and it is known that when hair is treated by heat under wet conditions, its mechanical strength decreases[4)]. The changes within hair have been reported as follows: change in enthalpy and dynamic of water[7)], decomposition of tryptophan in the composing proteins[8), 9)], and cross-linkage among proteins[10)]. Therefore, it is considered that thermal changes in hair happen not only at the cuticle but also at the cortex consisting of keratin fibers.

In the hair research involving development of hair care related products, heterogeneous characteristics of hair samples due to individually different diameters, histories, and variations between tips and roots seem to be present in the accurate analyzer as noise. In order to obtain the accurate data from hair samples, numerous experiments are required. Thus, an alternative to hair samples, which can be the embodiment of average hair has been sought. Since hair mainly consists of filamentous proteins, we have solubilized them in the solution and succeeded in developing a film by inducing the self-assembly of proteins[11)]. This two-dimensional keratin film reacts in the same manner as three-dimensional hair samples: the formation of oxidative proteins when irradiated with UV[12), 13)], the formation of cysteic acid by bleach (oxidative) treatment[14)], and elution of low molecular weight proteins when treated with perm (reductive) solutions[15), 16)]. In each case, compared to hair samples, the films showed higher sensitivity in detection. The formation of cysteic acid was investigated using FT-IR (Fourier transform infrared spectroscopy) and the spectra were very similar in both the film and hair samples. When treating hair and film by commercial bleaching agent, the peak of cysteic acid (around 1,041 cm^{-1}) was detected[14)]. The peak height of the keratin

[1,*] Faculty of Textile Science and Technology, Shinshu University (3–15–1 Tokida, Ueda, Nagano 386–8567, Japan)
連絡先 E-mail: fujiit1@shinshu-u.ac.jp

[2] SHISEIDO Co., Ltd., Research Center (2–2–1 Hayabuchi, Tsuzuki-ku, Yokohamashi, Kanagawa 224–8558, Japan)

film was approximately 5 times as high as that of the hair sample. When the elution of proteins by perm treatment was investigated, the films indicated 2,000 times more sensitivity than hair[15), 16)]. These results suggest that the keratin film can serve as an alternative device to hair samples.

In the course of our comparative study between the keratin film and hair, we have found that the keratin film demonstrated high thermostability for proteins. This finding indicates the need for further research on the films as to their suitability as an evaluation device for heat damage caused by hair dryers and irons. In this study, the thermal sensitivity of the keratin film was analyzed focusing on color, morphological observation, and biochemical analyses.

2. Materials and Methods

2–1. Preparation of Human Hair Keratin Film

All hair samples, free from any chemical treatments such as bleach, hair dye, or perm were collected from numerous Japanese volunteers. Proteins from human hair were extracted by the Shindai method[17)] and keratin films were produced from this protein solution by the Pre-cast method[11)]. Briefly, the hair samples were incubated with the solution containing 25 mM Tris–HCl, pH 8.5, 250 mM dithiothreitol (DTT), 2.6 M thiourea, and 5 M urea for 24 h at 50℃ in order to obtain solubilized hair protein solution. The hair protein solution (40–60 mg/m*l*) was mixed with acetic acid (final concentrations: 0–30%) and quickly poured into heat-resident glass petri dishes containing 5–6 m*l* of distilled water. After letting them stand for 1–2 h at room temperature, the resulting protein aggregates were washed by flowing water and distilled water. The keratin film was recovered after drying at room temperature[11), 16)].

2–2. Thermal Treatment on Keratin Film and Hair Samples and Color Measurement

Keratin films used for thermal treatment were prepared in the glass petri dishes. White hair of Japanese origin was cut into desired lengths for color change observation, and other hair samples were cut into small pieces by scissors for examining protein solubility. After treating keratin films and hair samples by an Aluminum heat block bath (Thermo Alumi Bath ALB-121, SCINICS Co., Tokyo, Japan), they were returned to room temperature. Thermal treatment temperature ranged from 80 to 200℃. Then, the color change of the keratin films were measured by a Handy Spectrophotometer (NF333, Nippon Denshoku Ind. Co., Ltd., Tokyo, Japan).

2–3. Protein Solubilization

Keratin films after heat treatment were removed from the glass dishes and ground to particles by a mortar and pestle to examine their protein solubility. Hair samples after cutting to 2–4 mm were also heat-treated using the Aluminum bath. Untreated samples were also used as controls. For each sample, the following solutions were added at 5 mg/m*l*: solution-A (50 mM Tris–HCl, pH 8.5), solution-B (50 mM Tris–HCl, pH 8.5 and 50 mM DTT), solution-C (50 mM Tris–HCl, pH 8.5 and 8 M urea), and solution-D (50 mM Tris–HCl, pH 8.5, 50 mM DTT, and 8 M urea)[14)]. Then each sample was incubated at 50℃ for 0–5 h. Samples were collected at suitable intervals and centrifuged at 12,000 *g* for 10 min, in order to measure the amount of proteins by the Bradford method[18)].

2–4. Scanning Electron Microscopy (SEM)

The morphology of the keratin films and hair samples after thermal treatment was examined by a scanning electron microscope (JCM-5000, JEOL, Tokyo, Japan). The samples were placed on specimen mounts using double-sided adhesive tape and were made electrically conductive through being coated with a thin layer of gold in a vacuum. The pictures were taken at the excitation voltage of 10 kV and 5,000-fold magnification[14), 15)].

2–5. Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[19)] using a 5–20% gradient polyacrylamide gel. Gels were stained with 0.1% Coomassie brilliant blue R-250, 10% acetic acid, and 40% ethanol for 2 h and destained in 10% acetic acid[11), 14), 17)].

2–6. Fluorescence Intensity

The amount of carbonylated proteins was measured by a fluorescence reagent, fluorescein-5-thiosemicarbazide (5-FTSC) (Ana Spec, San Jose, CA, USA) which can label specifically carbonyl groups in proteins[12), 13)]. The hair keratin films were heated at 80–200℃ for 10 min and cooled to room temperature. Then the films were incubated with 3 m*l* of the staining solution, which consisted of 20 μM 5-FTSC in 100 mM 2-morpholinoethane sulfonic acid–NaOH (pH 5.5) at 25℃ for 2 h. To remove non-reacting 5-FTSC, the films were rinsed with 30 mM sodium citrate buffer, pH 7, 0.1% SDS, and 300 mM NaCl for 30 min at 50℃, then in 3 mM sodium citrate buffer, pH 7, 0.1% SDS, and 30 mM NaCl for 30 min at 25℃, and finally in distilled water for 10 min at 25℃. After rinsing, the films were dried at room temperature. All procedures were carried out in a dark room. The fluorescence intensity of the keratin films was measured by a microplate fluorometer (Fluoroskan Ascent FL, Labsystem, Helsinki, Finland). The average value was calculated from 110 different points per film.

3. Results

3–1. Effect of Thermal Treatment on the Color of Keratin Film and White Hair Samples

In order to examine the thermal effect on the appearance, the keratin films in glass petri dishes and white hair samples were heated for 10 min at 80 to 200℃, by raising the temperature every 10℃. Keratin films are normally light beige color in appearance, but after the thermal treatment at 170℃ or more, the color started to change to light yellow and then to light brown (Fig. 1A). The similar change of color was also observed in white hair samples, however, hair samples showed heterogeneous changes of color among themselves. Even within each piece of hair, differently changed colors were observed. Since keratin films demonstrated uniform

color change compared to hair samples, the measurement by spectrophotometry was carried out. A significant change in Lab values was observed at temperatures of 180℃ and higher (Fig. 1B). The keratin film did not separate from the dish, even when the film was treated at 200℃ for 60 min (data not shown).

3–2. Effect of Thermal Treatment on the Fine Structure of Keratin Film

It was observed by SEM, that keratin films which were prepared by the Pre-cast method consisted of reticular structure[11), 14), 15)]. After the thermal treatment of 10 min at 140, 170, and 200℃, morphology of each keratin film surface was compared to that of an untreated film surface. No significant change in the fine structure was observed among all the films, even though color of the films started to change at 170℃ and turned to light brown at 200℃ (Fig. 2a–2d). This result indicated that the reticular structure of the keratin films remains stable through thermal treatments. On the other hand, no evident change was observed in the morphology of cuticles, when dried hair samples were heat-treated at 200℃ for 10 min (Fig. 2e and f).

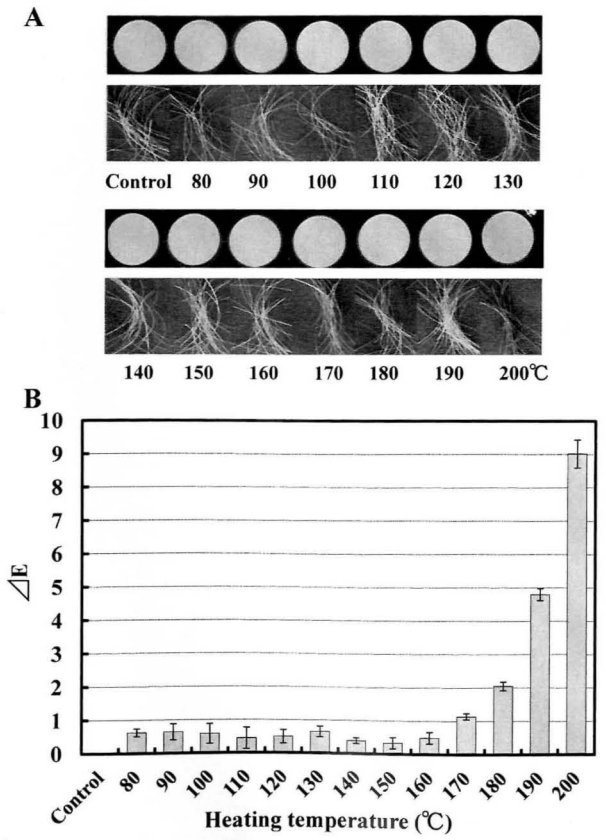

Fig. 1. Effect of thermal treatment on the color of keratin film and white human hair samples (A) and color difference (B). Keratin films prepared in the glass petri dishes and human hair samples were heated at 80–200℃ for 10 min by aluminum block bath. The colors of samples which had varied from initial white to yellow-brown by the end of the thermal treatment were measured by colorimeter and expressed as ΔE ($\sqrt{\Delta L^{*2}+\Delta a^{*2}+\Delta b^{*2}}$).

3–3. Solubilization of Proteins from Keratin Film and Hair Samples

Solubilized proteins of untreated and heat-treated keratin films were as follows: solution-D≫solution-B>solution-C> solution-A≈0 (Fig. 3A). On the other hand, in the hair samples, the solubilized proteins were only confirmed in solution-D (Fig. 3B). The amounts of solubilized proteins from heat-treated films and hair were lower than those of untreated film and hair. In addition, the solubilized proteins from the films were twice as high as those from the hair samples.

The amounts of solubilized proteins from all samples increased in a time-dependent manner, and they were almost finished within 2 h (Fig. 4A and B). When the solubilized protein after incubation at 50℃ for 2 h in the solution-D was studied by SDS-PAGE, the main components were found to be keratin and keratin-associated proteins (KAPs), and no significant difference was observed between keratin films and hair samples (Fig. 4C). The solubilized proteins after incubation at 50℃ for 2 h in the solution-D was used as an indicator of the solubility in the following experiments.

3–4. Effect of Heating Time and Temperature on the Solubilization of Proteins

The solubilized proteins from keratin films and hair samples after treatment of 140℃ for 0 to 30 min, were investigated by incubating the samples at 50℃ for 2 h in solution-D. Keratin films and hair samples showed decreased amounts of

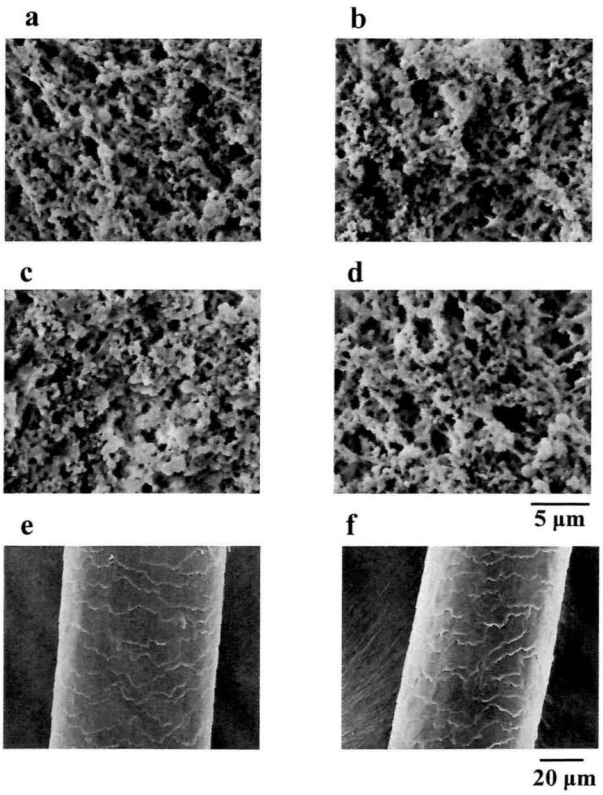

Fig. 2. SEM observation of keratin film (a–d) and hair samples (e and f) after heat treatments. a and e, untreated film and hair samples; b, 140℃ for 10 min; c, 170℃ for 10 min; d and f, 200℃ for 10 min.

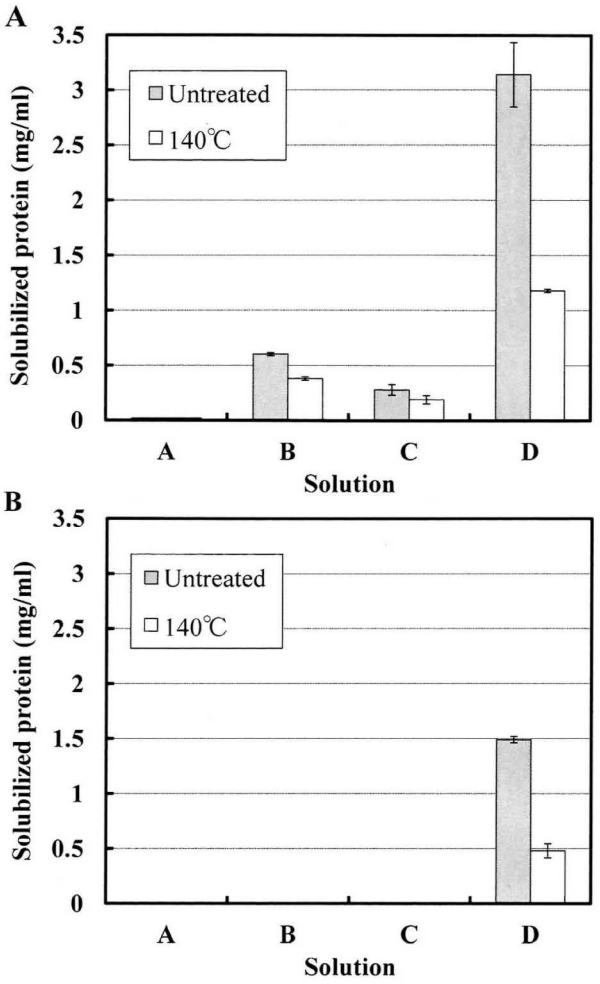

Fig. 3. Protein solubility of untreated and heat-treated keratin film (A) and of untreated and heat-treated hair samples (B) by various solutions. Samples were heated at 140℃ for 10 min. The obtained samples of keratin films and hair samples were each mixed with solution-A, solution-B, solution-C, and solution-D, at 5 mg/m*l* and incubated at 50℃ for 2 h. After centrifugation at 12,000 *g* for 10 min, the supernatants were recovered in test tubes and used for determination of the protein concentration.

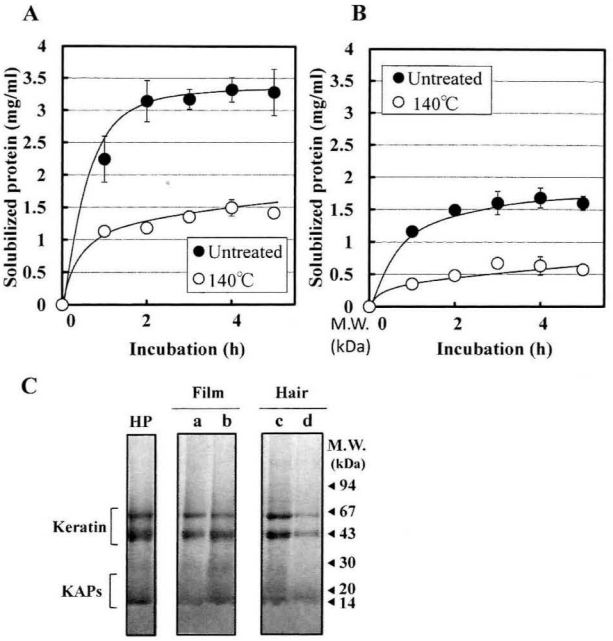

Fig. 4. Time course of protein solubility from untreated and heat-treated keratin film (A) and from hair samples (B) by solution D, and SDS-PAGE analysis (C). After treating keratin films and hair samples at 140℃ for 10 min, they were incubated in the solution-D at 50℃ to examine the amount of solubilized proteins at suitable time intervals. Untreated keratin film and hair samples were also used as controls. HP, hair proteins extracted by the Shindai method; a and c, untreated samples (2 h solubilization); b and d, 140℃ heat-treated samples (2 h solubilization). The assay conditions were the same as in Fig. 3. The obtained supernatants were subjected to 5–20% SDS-PAGE.

solubilized proteins in the heating time-dependent manner (Fig. 5A). Solubilized protein almost reached a plateau when the samples were heat-treated for 10 min. Time required for a 50% decrease in the changed amount of solubilized proteins was 2 min in keratin films and 3 min in hair samples.

The keratin films and hair samples were heat-treated for 10 min at 80–200℃ by raising temperatures every 10℃ in order to examine the amount of solubilized proteins. In both films and hair samples, the amount of solubilized proteins decreased in a temperature-dependent manner. Interestingly, a linear decrease of solubilized proteins were observed in the heat-treated keratin films at 110–160℃, and in the heat-treated hair samples at 120–160℃ (Fig. 5B).

3–5. Effect of Thermal Treatment on the Formation of Carbonylated Proteins

The effect of thermal treatment on the formation of carbonylated proteins was examined. After treating keratin films with heat as shown in Fig. 6, they were reacted with 5-FTSC in order to measure the fluorescence intensity. Up to 120℃, the fluorescence intensity remained the same as untreated films, however, starting at 120–140℃, the intensity linearly increased until it was reached a plateau at 180℃ (Fig. 6). The amount of carbonylated proteins formed at 180℃ was almost equal to that of 2 h irradiation by a solar simulator (15 mW/cm^2).

4. Discussion

In this study, the effects of the thermal treatment on the keratin films and hair samples were examined and compared in detail. When white hair samples and light beige colored keratin films were heated for 10 min, both of them turned light yellow in color at 170℃ and light brown at 200℃. Similar observations made in color change indicated that keratin films resembled hair in thermal detection tendency. These results indicate that the keratin films can be used for evaluating the effect of thermal treatment by observing color changes.

When heating human hair or wool, it is known that the degradation of aromatic amino acid and cross-linkage among proteins take place[8),9)]. The cysteine residues in keratin films were speculated to be in both reductive and oxidative states. When treating keratin films with hydrogen peroxide, urea in

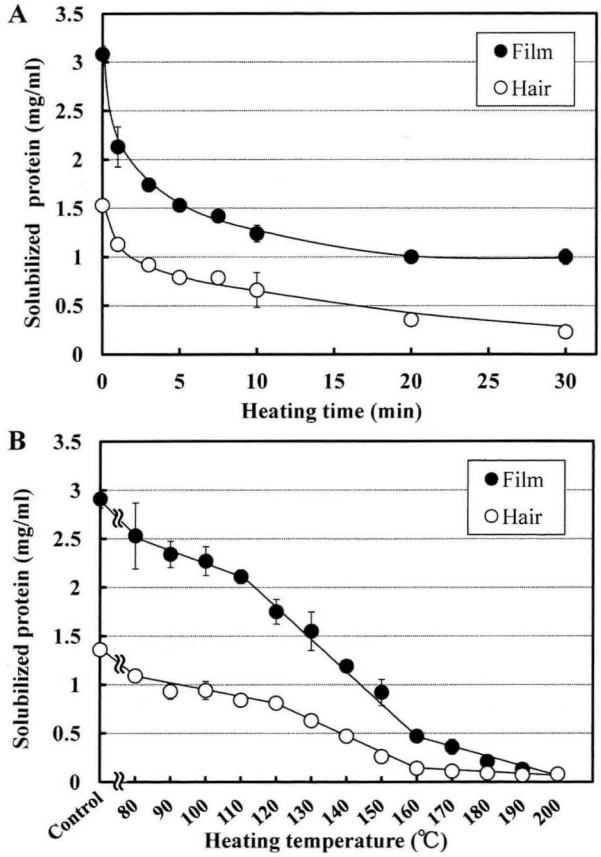

Fig. 5. Effect of heating time (A) and temperature (B) on the protein elution from untreated and heat-treated keratin films and hair samples. The assay conditions were the same as in Fig. 4. Protein solubilization was carried out at 50℃ for 2 h.

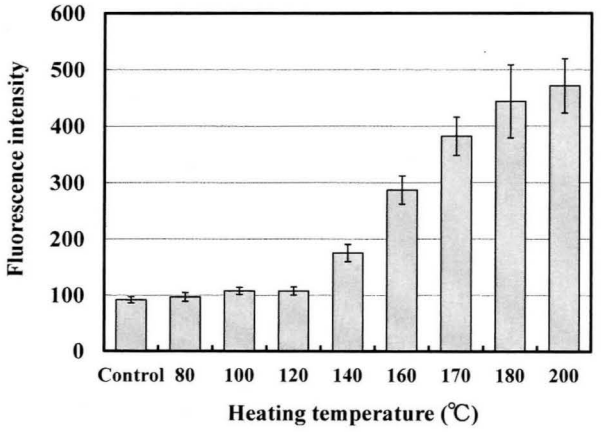

Fig. 6. Effect of heating temperature on the formation of carbonylated proteins in keratin film. The heat treatment was performed at 80, 100, 120, 140, 160, 170, 180, and 200℃ for 10 min. The keratin films reacted with 20 μM 5-FTSC and the fluorescence intensity was calculated at 110 points per film by microplate fluorometer.

the solution cause the solubility of proteins to decrease, and when treating keratin films with commercial permanent solution, reductive agents such as thioglycolic acid cause the film to appear transparent[15), 16)]. These results suggest that the keratin film is a dynamic material, which responds to its changing environment. SEM observation showed that reticular networks accompanied by granular structure in the film collapsed and changed to a smooth structure by the reductive treatment. The fine structure of the keratin film exhibited flexibility; however, heat treatment had little effect on the translucent and reticular networks of the keratin film.

As we focused on the formation of cross-linkage within fibers of heat-treated hair and wool, it was considered possible for the protein solubility to change, if the similar interaction or cross-linkage was taking place among proteins in both keratin films and hair samples[9), 10), 14), 20)]. Thus, the thermal effect at the molecular level was investigated by the protein solubilization from the keratin film and hair samples. Both samples were heat-treated at 140℃ for 10 min, and their solubilized protein amounts were measured and compared to those of untreated keratin film and hair. As a result, in the untreated keratin film, solubilized proteins by the solution containing both reductive agent (DTT) and denaturant (urea) showed the highest amount of solubilized proteins; it was four times greater than the sum of solubilized proteins by solution-B and solution-C. Regarding the heat-treated keratin film, a synergistic increase in protein solubilization was also observed, however when compared to that of the untreated film, the values were 40–50% lower.

These results lead to the hypothesis that thermal treatment prompts the formation of "Stable Structure" which is not as big as the reticular networks detected by the SEM observations. The "Stable Structure" may consist of complex and entangled higher-order structure including S–S bonds at the molecular level. This indicates insoluble characteristic even to the solution containing S–S bond disconnecting reductive agent DTT, and higher-order structure destroying denaturant urea. Protein solubilization of heat-treated keratin film after the incubation by solution-D reached a plateau after 2 h. Even after an incubation of up to 5 h, an increase of protein solubility was barely observed. Furthermore, the "Stable Structure" was speculated to be formed according to heating time and temperature. Especially the linear decrease of protein solubility from 110–160℃ indicated these "Stable Structure" to be formed in the continuous manner in a wide range of temperatures.

On the other hand, regarding the hair samples, protein solubility was only detected in the solution containing both reductive agent and denaturant. Protein solubility from heat-treated hair decreased to 35%. Similar phenomena were observed in both keratin film and hair in regard to incubation time for protein solubilization, heating time, and temperature. These results suggest that thermal treatment passes through cuticles and reaches to inner parts of hair, forming similar "Stable Structure" which is insusceptible to DTT and urea, as in the case of keratin films. Since solubilized proteins from film and hair contain keratin and KAPs at approximately the same rate, cross-linkage of keratin–keratin, keratin–KAPs, and KAPs–KAPs can occur in keratin films and hair by thermal treatment. This hypothesis needs further proof in the future, however the effectiveness of measuring an amount of solubilized proteins using solution containing reductive agent and denaturant, for the evaluation of thermal sensitivity and heat

damage has been demonstrated.

It is known that the oxidative proteins are formed when treating keratin films or hair samples with UV or bleach agents[13), 14)]. Therefore, the induction of oxidized protein formation by thermal treatment was investigated and found the significant formation of carbonyl groups was observed at the heat treatment of 140℃ or more. Even though the amount of solubilized proteins started to decrease once the film or hair was heated at 80℃ or more, the formation of carbonyl groups did not occur until they were treated at 120℃. Thus the amount of solubilized proteins and formation of carbonyl groups seemed unrelated.

The characteristics observed in thermal sensitivity of the keratin film are; ① Keratin film does not separate from the dish under thermal treatment up to 200℃. ② The color of keratin films changed due to thermal treatment in the same manner as human white hair, and such changes in color were easily quantified with a spectrophotometer. ③ Solubilized proteins from heat-treated keratin films decreased linearly in a temperature-dependent manner. ④ The decreased amount of protein solubility measured using keratin films were twice as high as those of the hair samples. ⑤ The formation of carbonylated proteins was detected in a temperature-dependent manner. ⑥ Since the keratin film has a plane structure, it is easier to handle and observe various responses compared to hair samples.

Taking into account the changes of color, protein solubility, and carbonylation, the chemical modifications in the keratin film were similar to those in the hair samples.

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References

1) Gamez-Garcia, M.: The cracking of human hair cuticles by cyclical thermal stresses. *J. Cosmet. Sci.*, 49: 141–153, 1998.
2) Okamoto, M., Yakawa, R., Mamada, A., Inoue, S., Nagase, S., Shibuichi, S., Kariya, E., Satoh, N.: Influence of internal structures of hair fiber on hair appearance. III. Generation of light-scattering factors in hair cuticles and the influence on hair shine. *J. Cosmet. Sci.*, 54: 353–366, 2003.
3) Ruetsch, S. B., Kamath Y. K.: Effects of thermal treatments with a curling iron on hair fiber. *J. Cosmet. Sci.*, 55: 13–27, 2004.
4) Ikuyama, R.: Heat damage and the prevention technology of the hair. *Fragrance J.*, 38(11): 45–50, 2010.
5) Gamez-Garcia, M., Lu, Y.: Patterns of light interference produced by damaged cuticle cells in human hair. *J. Cosmet. Sci.*, 58: 269–282, 2007.
6) Gamez-Garcia, M., Basilan, J.: The use of image analysis to assess the role of polymers on the thermal protection of Asian hair. *Fragrance J.*, 38(11): 77–79, 2010.
7) Milczarek, P., Zielinski, M., Garcia, M. L.: The mechanism and stability of thermal transitions in hair keratin. *Colloid Polym. Sci.*, 270: 1106–1115, 1992.
8) McMullen, R., Jachowicz, J.: Thermal degradation of hair. I. Effect of curling irons. *J. Cosmet. Sci.*, 49: 223–244, 1998.
9) McMullen, R., Jachowicz, J.: Thermal degradation of hair. II. Effect of selected polymers and surfactants. *J. Cosmet. Sci.*, 49: 245–256, 1998.
10) Asquith, R. S., Otterburn, M. S.: Self-crosslinking in keratin under the influence of dry heat. *Applied Polymer Symposium*, 18: 277–287, 1971.
11) Fujii, T., Ogiwara, D., Arimoto, M.: Convenient procedures for human hair protein films and properties of alkaline phosphatase incorporated in the film. *Biol. Pharm. Bull.*, 27: 89–93, 2004.
12) Kawasoe, T., Watanabe, T., Fujii, T.: Visualization of modified human hair by artificial sunlight with carbonylated proteins as an indicator of hair damage. *J. Jpn. Cosmet. Sci. Soc.*, 34: 287–291, 2010.
13) Kawasoe, T., Watanabe, T., Fujii, T.: A novel method using a keratin film for quantifying the photo-modification of hair proteins. *J. Soc. Cosmet. Chem. Jpn.*, 45: 100–107, 2011.
14) Fujii, T., Ito, Y., Kawasoe, T.: Effects of oxidative treatments on human hair keratin films. *J. Cosmet. Sci.*, 63: 15–25, 2012.
15) Fujii, T.: Evaluation of damage caused by perm treatment using the hair keratin films. *Fragrance J.*, 39(7): 46–52, 2011.
16) Kawasoe, T., Takayama, S., Ito, Y., Fujii, T.: Effects of reductive and/or oxidative treatment during permanent wave procedure on human hair keratin films. *J. Jpn. Cosmet. Sci. Soc.*, 35: 306–311, 2011.
17) Nakamura, A., Arimoto, M., Takeuchi, K., Fujii, T.: A rapid extraction procedure of human hair proteins and identification of phosphorylated species. *Biol. Pharm. Bull.*, 25: 569–572, 2002.
18) Bradford, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, 72: 248–254, 1976.
19) Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680–685, 1970.
20) Horvath, A. L.: Solubility of structurally complicated materials: 3. Hair. *Scientific World J.*, 9: 255–271, 2009.