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"Innovative protein cross-linking technology using natural polyphenols to restore hair structure: Recovery of hair strength using quinone cross-linking in hair dye treatments containing hydrogen peroxide"

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1. **Introduction**

Nowadays, many people dye their hair at beauty salons. Hair dyeing is not just an act of changing hair color, it is also said to effectively improve psychological well-being and has become an indispensable part of adding color to life. However, dyeing hair can cause serious damage to the structure of the hair.

To degrade the melanin in hair and as part of the coloring of the hair dye pigment, hydrogen peroxide is included in the bleaching agent and in oxidative hair dye. The hydrogen peroxide generates radical species during the hair coloring process. Repeated treatments can cause degradation of hair protein [1]. The damaged hair becomes wavy and hard in texture [2]. The cause of this phenomenon is thought to be the degradation of proteins inside the hair and the cleavage of cross-links such as disulfide bonds between keratin proteins [3]. In hair coloring and bleaching treatments, there is a need to suppress or repair these phenomena during treatment in order to achieve a higher quality finish.

In a previous study, we found that the combination of polyphenols and copper (II) ions produces quinones that form cross-links between proteins [4]. We also found that treating hair with this combination increases the value of the breaking stress of the treated hair in a tensile test. Treatment with this combination make it possible to improve hair shape retention and other physical properties by forming cross-link between keratin proteins through quinone bonds. It has been confirmed that treatment with this polyphenol is effective when applied as a cosmetic treatment of hair [4]. However, there is not enough information available about the effects of hydrogen peroxide in alkaline aqueous solutions, such as those used in hair coloring and bleaching treatments.

In this study, we investigate the effect of hydrogen peroxide on the binding reaction of quinones in an alkaline aqueous solution. We also study the effects on the physical properties of hair when treating with hair under similar conditions. In addition, we hypothesize that the cause of the effect on quinone binding is the decomposition of hydrogen peroxide present in the system. We also present the results of our investigation into the effects of using a chelating agent.

2. Materials and Methods

2.1. Samples and Reagents

Straight reddish-black hair was kindly donated by Japanese women. The hair fibers were neither bleached nor colored previously. Lysozyme from hen egg white (Sigma-Aldrich Japan, Tokyo, Japan) was commercially available. The gallic acid used was reagent grade (FUJIFILM Wako, Osaka, Japan). Copper (II) chlorophyllin (Japan Chlorophyll, Saitama, Japan) and 35 wt.-% hydrogen peroxide (ADEKA, Tokyo, Japan) were commercially available. Bleaching was conducted using a combination of a commercial bleaching agent and an oxidative agent; the former consisted of alkaline components and persulfate salt, while the latter contained 6 wt.-% hydrogen peroxide as an essential ingredient. The chelating agents used were ethylenediaminetetraacetic acid (EDTA), ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA-4Na), N-Carboxymethyl-N'-(2-hydroxyethyl)-N,N'-ethylene diglycine trisodium salt dihydrate (HEDTA), 1-hydroxyethane-1,1-diphosphonic acid (HEDP). These chelating agents were reagent-grade (FUJIFILM Wako, Osaka, Japan). The pentasodium;2-[bis[2-[bis(carboxylatomethyl)amino]ethyl]amino]acetate (DPTA-5Na) used was also reagent grade (TCI, Tokyo, Japan).

2.2. Preparation of Samples

Polyphenol treatment of lysozyme: Lysozyme (0.1 wt.-%), hydrogen peroxide (0–3 wt.%), gallic acid (1 wt.-%), Cu (II) chlorophyllin (0.0001 wt.-%), and a chelating agent (0–1 wt.-%) were mixed with phosphate buffer solution (0.1 wt.-%). This mixture was left to stand at 25°C for 30 minutes, and the reaction mixture was obtained.

Bleaching treatment: The previously mentioned bleaching and oxidizing agents were mixed in a ratio of 1:2 (w/w). After immersing the hair in the aqueous mixture at a bath ratio of 1:50 (w/w) at 25°C for 30 minutes, the fibers were washed well with water and dried at 60°C with a hairdryer. These operations were repeated three times. The resulting hair will hereafter be referred to as “bleached hair”.

Polyphenol treatment of hair: The bleached hair fibers were immersed in pure water or a mixture of a phosphate buffer solution (0.1 wt.-%, pH 8) of hydrogen peroxide (0-3 wt.-%), gallic acid (0-1 wt.-%), Cu (II) chlorophyllin (0.0001 wt.-%) and chelating agent (0-1 wt.-%) at a bath ratio of 1:50 (w/w) at 25°C for 12 hours. The resulting hair samples were rinsed with water and dried using hairdryer.

2.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE was carried out in a manner similar to the previous method [5]. The treated lysozyme solution (10 μL) was diluted with Laemmli 2x Sample Buffer (Bio-Rad Laboratories, Hercules, California, USA) (10 μL). Each of the mixtures was heated at 100°C for 5 minutes, cooled to ambient temperature, and applied to the polyacrylamide gradient gel plate (10-20 wt.-%, Kishida Chemical, Osaka, Japan). The resulting gel plate was subjected to electrophoresis at 20 mA for approximately 90 minutes. Protein Molecular Weight Maker (TEFCO, Tokyo, Japan) was also electrophoresed at the same time. The protein bands were visualized by staining with Coomassie Brilliant Blue.

2.4. Measurement of the Tensile Testing of the Hair Samples

The treated hair samples (20 strands) were immersed in purified water for 12 hours and then placed in an automatic tensile tester (Dia-Stron, Hampshire, UK). Tensile measurement was performed at a pulling speed of 3 mm/min while the hair (30 mm in length) was immersed in purified water. Under the testing conditions, which were similar to those previous studies [6], we observed that the tensile data exhibited only minor variations. Twenty fibers per test cell were individually tested to estimate the breaking stress. The breaking stress (J/m^3) calculated

as the total work (J) per volume of sample hair ($((d/2)^2 \times \pi \times L_0)$). Here, d is the average value of the hair diameter at five points as measured with a laser contour measuring device (Dia-Stron; Hampshire, UK), and L_0 is the initial hair length.

2.5. Measurement of the Brightness of the Hair Bundle

The brightness of the hair was measured using a Spectrophotometer (Konica Minolta, Tokyo, Japan) in a manner similar to the method used in a previous study [7]. The measurement conditions included a 10-degree observation, D65 illuminant, and SCI mode. The brightness (L^*) was recorded, and three repetitions of measurements were taken to obtain an average value.

2.6. Electron Spin Resonance (ESR) Measurement

The mixture contains the hydrogen peroxide (1 wt.-%), gallic acid (0.1 wt.-%), Cu (II) chlorophyllin (0.0001 wt.-%), and chelating agent (0.1 wt.-%) was diluted with a phosphate buffer solution (0.1 wt.-%, pH 8). The 5,5-Dimethyl-1-pyrroline N-Oxide(DMPO, TCI, Tokyo, Japan) was diluted with the mixture solution. The resulting solution was stirred well and allowed to stand at 25°C for 5 minutes. The solution was placed in an ESR measurement tube and attached to an ESR measuring device (JEOL; Tokyo, Japan). The measurement conditions were: center field 330 mT; sweep width 20 mT; frequency 9.445 GHz; modulation frequency 100 kHz; and time constant 0.1 sec. Mn/MgO was used for the ESR signal correction.

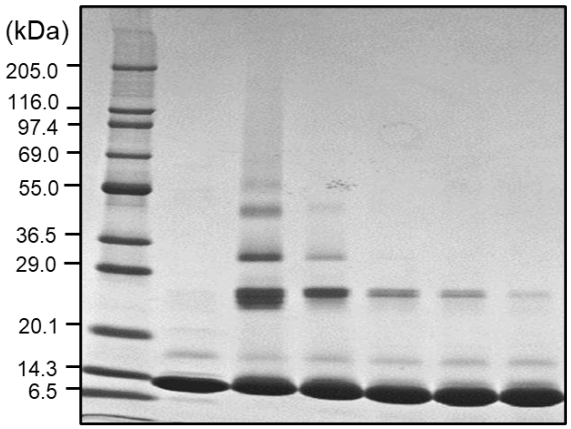
3. Results and Discussion

3.1. Polyphenol Treated Protein and Hair Sample Under the Presence of Hydrogen Peroxide

Figure 1 shows the results of the SDS-PAGE analysis of the products obtained by mixing lysozyme with gallic acid, copper chlorophyllin, and hydrogen peroxide. As shown in Figure 1-a, the lysozyme derived from hen egg white used in this study produced a band at approximately 14 kDa. When the lysozyme was treated with copper chlorophyllin and gallic acid, ladder-like bands were observed at approximately 28, 30, and 50 kDa (Figure 1-b). These bands indicate that lysozyme molecules are cross-linked via gallic acid to produce dimers, trimers, or higher polymerization products. When hydrogen peroxide was added to this system, the intensity of each band gradually decreased in proportion to the concentration of hydrogen peroxide (Figure 1-c, d, e, f). It is known that hydrogen peroxide decomposes via bimolecular or unimolecular chemical reactions in an alkaline solution [8]. In the unimolecular chemical reaction, radical species are generated from hydrogen peroxide. It is known that polyphenols have antioxidant properties and are oxidized to form homopolymers as oxidation products [9]. However, proteins contain many amino groups that are easily oxidized and are easily modified by hydroxyl radicals and other reactants [10]. These results suggest that the excess radical species generated from hydrogen peroxide promoted the oxidative polymerization of gallic acid, which result in a decrease in its binding reaction with proteins.

Figure 2 shows the breaking stress values of the hair samples treated with gallic acid, copper chlorophyllin, and hydrogen peroxide for bleached hair. In the case of the solution that did not contain hydrogen peroxide, the value of the breaking stress of the hair obtained after treatment was approximately 7% higher compared to before treatment. For the solution with the addition of 0.5 wt.-% hydrogen peroxide, the increase in the value of the breaking stress of the hair treated with the solution decreased to 4%.Furthermore, for the solutions containing 1, 2, and 3 wt.-% hydrogen peroxide, the value of the breaking stress decreased with increasing amount. When 3 wt.-% hydrogen peroxide was added, the amount decreased the in the value of the breaking stress of the hair treated with the solution decreased to

approximately 4%. From the results of the SDS-PAGE in Figure 1 and the tensile tests of the treated hairs, it is considered that there is a correlation between the amount of lysozyme polymerization and the change in the breaking strength of the hair. Keratin proteins in hair contain free amino and thiol groups, and it is thought that polyphenols increase hair strength by binding to the keratin proteins and cross-linking them [4]. In the presence of hydrogen peroxide, these groups are also oxidized [10], making it difficult for them to bind with polyphenols. For this reason, it is considered that the polyphenol treatment did not provide a sufficient effect of increasing hair strength.



No.	a	b	c	d	e	f
H_2O_2(wt.-%)	0		0.5	1	2	3
Gallic acid(wt.-%)	0	1	1	1	1	1

Figure 1. SDS-PAGE assay result for the reaction mixtures obtained with lysozyme (0.1 wt.-%), H_2O_2, gallic acid (0-1 wt.-%) and Cu-chlorophyllin (0.0001 wt.-%). The concentration of H_2O_2 was varied in the range of 0-3 wt.-% to obtain the five mixtures. These reaction mixtures were placed at 25°C for 30 min.

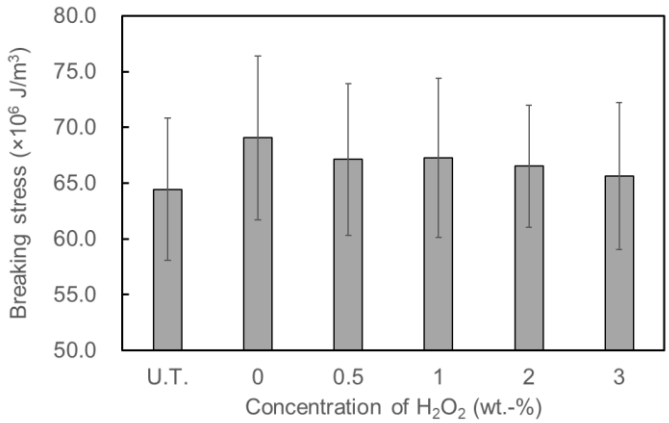


Figure 2. Relationship between the concentration of H_2O_2 (wt.-%) in the hair treatment solutions containing chelating agent, H_2O_2, gallic acid (0.1 wt.-%) and Cu-chlorophyllin (0.0001 wt.-%) and the breaking stress of the treated hair obtained using the hair treatment solutions. Here, “U.T.” stands for untreated bleached hair. These breaking stresses were obtained from tensile tests of single hair fibers. The error bars indicate standard error.

3.2. Effect of Chelating Agents on the Cross-linking Reaction of Polyphenols in the Presence of Hydrogen Peroxide

To investigate the effect of the decomposition of hydrogen peroxide on cross-linking between proteins by polyphenols, a test was carried out in which a chelating agent was added to the reaction system. Figure 3 shows the results of the SDS-PAGE analysis of the reaction products obtained by adding 0.1 wt.-% of various chelating agents to a solution containing 0.1 wt.-% lysozyme, 0.1 wt.-% gallic acid, 1 wt.-% hydrogen peroxide, and 0.0001 wt.-% copper chlorophyllin. Figure 3-A and B indicate the result of the lysozyme solution and the solution containing lysozyme, gallic acid, and Cu-chlorophyllin, respectively. When no chelating agent was added (Figure 3-C), a clear band at approximately 14 kDa derived from lysozyme and a faint band at 28 kDa were observed. It was observed that the addition of a chelating agent to this system increased the concentration of the band around 28 kDa (Figure 3-D, E, F, G, H). The positions of these bands correspond to the molecular weight of the lysozyme dimers, which suggests that cross-linking between the lysozyme molecules occurs, and that polymers were formed. Figure 4 shows the breaking stress values obtained from tensile testing of hair that was treated with a solution containing 0.1 wt.-% of one of various chelating agents, 0.1 wt.-% gallic acid, 1 wt.-% hydrogen peroxide, and 0.0001 wt.-% copper chlorophyllin and left at 25°C for 30 minutes. In this case, the highest breaking stress was observed when EDTA-4Na was added as a chelating agent, and the lowest value was observed in the system where HEDP was added. These results show that the effect of a chelating agent on fracture varies depending on the type of chelating agent. Figure 5 shows the breaking stress value for the hair samples obtained using solutions containing 0.01-1 wt.-% EDTA-4Na, 0.1 wt.-% gallic acid, 1 wt.-% hydrogen peroxide and 0.0001 wt.-% copper chlorophyllin. This treatment revealed that the breaking stress gradually increased with increasing concentration of EDTA-4Na in the reaction mixture.

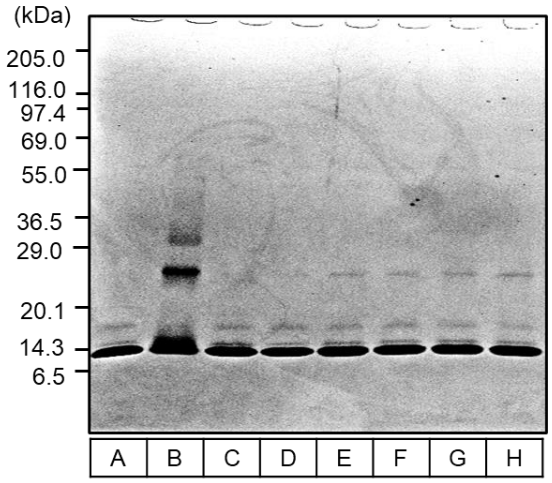


Figure 3. SDS-PAGE assay results for the reaction mixtures comprising lysozyme (0.1 wt.-%), H_2O_2 (1 wt.-%), gallic acid (0.1 wt.-%), chelating agents (0.1 wt.-%) and Cu-chlorophyllin (0.0001 wt.-%). These reaction mixtures were left at room temperature for 24 hours. A: lysozyme solution. B: lysozyme, gallic acid, and Cu-chlorophyllin. C: lysozyme, H_2O_2, gallic acid, and Cu-chlorophyllin. D: lysozyme, H_2O_2, gallic acid, Cu-chlorophyllin, and EDTA. E: lysozyme, H_2O_2, gallic acid, Cu-chlorophyllin, and EDTA-4Na. F: lysozyme, H_2O_2, gallic acid, Cu-chlorophyllin, and HEDTA. G: lysozyme, H_2O_2, gallic acid, Cu-chlorophyllin, and DPTA-5Na. H: lysozyme, H_2O_2, gallic acid, Cu-chlorophyllin, and HEDP.

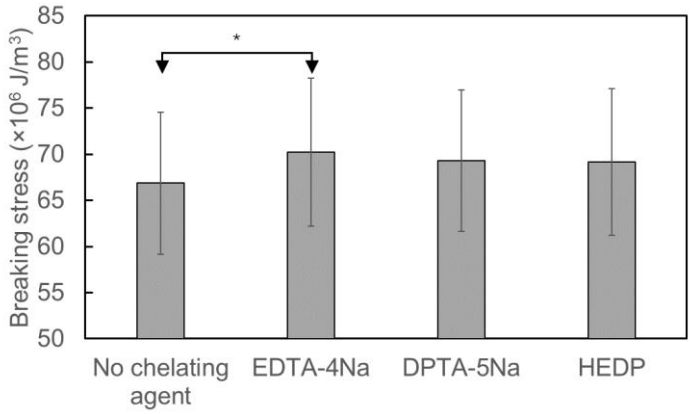


Figure 4. Relationship between various chelate agents in the hair treatment solutions containing chelating agent (0.1 wt.-%), H_2O_2 (1 wt.-%), gallic acid (0.1 wt.-%) and Cu chlorophyllin (0.0001 wt.-%), and the breaking stress of the treated hair. These breaking stresses were obtained through tensile tests of single hair fibers. The following chelating agents were used: EDTA-4Na, DPTA-5Na, and HEDP. These error bars indicate standard error. A t-test was used to compare between the two groups, which are shown with arrows. The difference was assessed with a two-side test. A total of 20 hair fibers were tested. The asterisk indicates a significant difference (* $p < 0.01$).

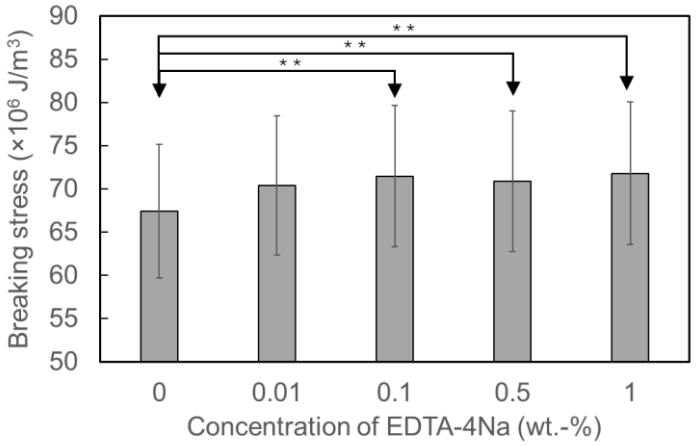


Figure 5. Relationship between the breaking stress of treated hairs and the concentration of EDTA-4Na in the hair treatment solutions containing EDTA-4Na, H_2O_2 (1 wt.-%), gallic acid (0.1 wt.-%) and Cu-chlorophyllin (0.0001 wt.-%). These breaking stresses were obtained through tensile tests of single hair fibers. The error bars indicate standard error. A t-test was used to compare the two groups, which are shown with arrows. The difference was assessed with a two-side test. A total of 20 hair fibers were tested. The asterisk indicates a significant difference (** $p < 0.005$).

This result indicates that EDTA-4Na affects the formation of cross-links between proteins in hair due to gallic acid. Figure 6 shows the results of the brightness (*L**) measurements on the same hair samples used in Figure 5. It was found that the brightness value decreases slightly with increasing concentration of EDTa-4Na. In the results of the student t-test between the

hair samples, there was no significant difference between these samples. These results suggest that the decomposition of hydrogen peroxide was inhibited by the chelating agent, inhibiting the polymerization of polyphenols and the oxidation of amino groups in proteins and promoting binding with lysozyme or keratin protein.

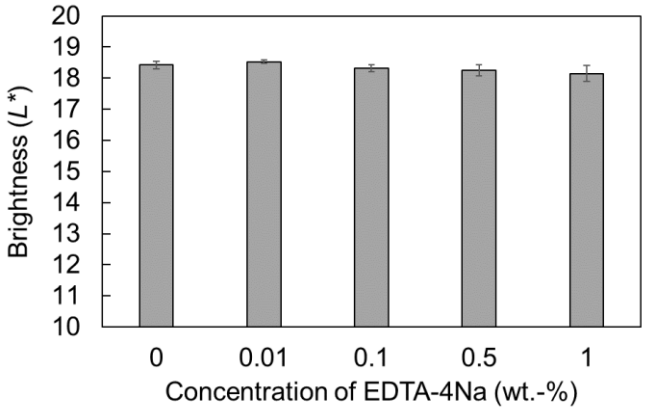


Figure 6. Relationship between the brightness (*L**) of the treated hairs and the concentration of EDTA-4Na in the hair treatment solutions containing EDTA-4Na, H_2O_2 (1 wt.-%), gallic acid (0.1 wt.-%), and Cu-chlorophyllin (0.0001 wt.-%). These breaking stresses were obtained through tensile tests of single hair fibers.

3.3. ESR Measurements of Solutions Containing Hydrogen Peroxide, Gallic Acid and Chelating agent

To investigate the radical species generated by the decomposition of hydrogen peroxide in the experimental system, DMPO was used as a spin trap and the ESR spectra were measured using an ESR spectrometer. Figure 7 shows the ESR spectrum of a solution containing0.1 wt.-% lysozyme, 0.1 wt.-% gallic acid, 1 wt.-% hydrogen peroxide, and 0.0001 wt.-% copper chlorophyllin, to which 0.1 wt.-% EDTA-4Na or HEDP was added as a chelating agent, and 600 mmol/L DMPO was added as a spin trap. In Figure 7-A, a spectrum was observed that is thought to be derived from the radical species generated from hydrogen peroxide [11].When gallic acid and copper chlorophyllin were added, a complex spectrum was observed, as shown in Figure 7-B. Furthermore, in the solution to which EDTA-4Na was added as a chelating agent, a complex spectrum was found, but its intensity was much lower (Figure 7-C). When EDTA-4Na was replaced with HEDP (Figure 7-D), the intensity of the spectrum was slightly lower compared to the spectrum produced without the chelating agent. From these results, it was found that the addition of a chelating agent to the aqueous solution containing hydrogen peroxide, gallic acid, copper chlorophyllin, and lysozyme was effective in reducing the generation of radical species.

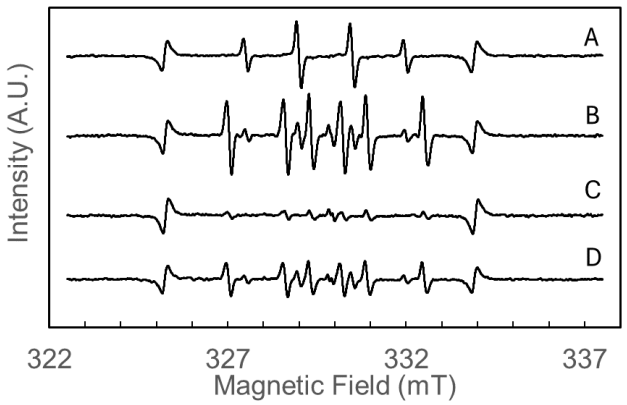


Figure 7. ESR spectra of DMPO adducts of free radicals generated by H_2O_2 (1 wt.-%) under the presence of lysozyme (0.1 wt.-%), gallic acid (0.1 wt.-%), chelating agents (0.1 wt.-%), Cu-chlorophyllin (0.0001 wt.-%) and DMPO (600 mmol/L). Cu-chlorophyllin is present in all mixtures. A: lysozyme and H_2O_2. B: lysozyme, H_2O_2 and gallic acid. C: lysozyme, H_2O_2, gallic acid, and EDTA-4Na. D: lysozyme, H_2O_2, gallic acid, and HEDP.

4. Conclusion

In this study, we investigated the effect of hydrogen peroxide in alkaline aqueous solutions on the quinone cross-linking reaction of proteins. It was found that in the presence of hydrogen peroxide, the quinone cross-linking reaction was inhibited in proportion to the hydrogen peroxide concentration in the system. Treatment of hair with hydrogen peroxide also reduced the breaking stress in the tensile tests compared to the results for untreated hair. This is considered to be because hydroxyl radicals generated from hydrogen peroxide caused the polymerization of polyphenols and oxidation of amino groups in proteins, resulting in insufficient cross-linking between proteins. It was found that adding a chelating agent to this aqueous solution facilitates binding between lysozymes. Similarly, when a solution containing a chelating agent was applied to hair, the value of the breaking stress of the treated hairs increased compared to that of the untreated hair. Furthermore, when EDTA-4Na was added as a chelating agent, the value of the breaking stress increased in proportion to the amount of added EDTA-4Na. The brightness of the treated hair was influenced slightly by the addition of EDTA-4Na. The ESR measurements showed that radical species were generated in the alkaline aqueous solution including hydrogen peroxide, and the generation of the radical species was suppressed by adding EDTA-4Na to this solution. These radical species are important for oxidizing polyphenols to quinone forms [12]. However, the results of this study suggest that EDTA-4Na acts to inhibit the binding of polyphenols to proteins.

Bleach and hair dye products that contain hydrogen peroxide can seriously damage human hair. This study suggests that adding a chelating agent to a product containing hydrogen peroxide in an alkaline aqueous solution, such as a hair dye or a bleaching agent, is effective in promoting the quinone cross-linking reaction of polyphenols. It is expected that the application of this method will make it possible to develop hair dyes and bleaching products that cause less damage during the bleaching or dyeing process.

References

[1] Berlett, B.S., Stadtman, E.R., *Protein oxidation in aging, disease, and oxidative stress*, J. Biol. Chem., 272, 33 (2018) 20313-20316.
[2] Tanamachi, H., *Temperature as a moisture cue in haptics on hair*, Int. J. Cosmet. Sci., 33, 1 (2011) 25-36.
[3] Feughelman M., *Basic wool physics*, Proc. 6th Int. Wool Text. Res. Conf., 1 (1980) 35-57.
[4] Yoshida, M., Maruyama, R., Yamauchi, A., *Repairing Bleach-Damaged Hair by Treating with Polyphenol in the Presence of Cu (II) Ions*, J. Cosmet. Sci., 74, 3 (2023) 43-157.
[5] Laemmli, U. K., *Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4*, Nature, 227, 5259 (1970) 680-685.
[6] Naito, S., Arai, K., Hirano, M., Nagase, N., Sakamoto, M., *Crosslinking structure of keratin. V. Number and type of crosslinks in microstructures of untreated and potassium cyanide treated human hair*, J. Cosmet. Sci., 61, 11 (2012) 1913-1925
[7] Kim, B., Kim, M., Choi, H., Suk, H.-J., *Brightness and hue judgment of dyed hair color*, Acta Psychol., 242 (2024) 104120.
[8] Koubek, E., Haggett, M.L., Battaglia, C.J., Ibne-Rasa, K.M., Pyun, H.Y., Edwards, J.O., *Kinetics and mechanism of the spontaneous decompositions of some peroxoacids, hydrogen peroxide and t-butyl hydroperoxide*, J. Am. Chem. Soc., 85, 15 (1963) 2263-2268.
[9] Hirose, Y., Yamaoka, H., Nakayama, M., *Oxidation product of (-) -epicatechin under radical reaction*, J. Oleo Sci., 39, 11 (1990) 967-969.
[10] Toda, T., Nakamura, M., Morisawa. H., Hirota, M., Nishigaki, R., Yoshimi, Y., *Proteomic approaches to oxidative protein modifications implicated in the mechanism of aging*, Geliatr. Gerontol. Int., 1, S25-31.
[11] Ueda, J., Shimazu, Y., Ozawa, T., *Reactions of copper(II)-oligopeptide complexes with hydrogen peroxide: Effects of biological reductants*, Free Radic. Biol. Med., 18, 5 (1995) 929-933.
[12] Leatham G. F., King, V., Stahmann M. A., *In vitro protein polymerization by quinones or free radicals generated by plant or fungal oxidative enzymes*, Phytopathology, 70, 12 (1980) 1134-1140.