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"Discovery and analysis of a novel hair damage mechanism, 'Glyco-oxidation'"

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1. Introduction

Hair is an important organ in beauty care, as it has a significant impact on a person's appearance. However, hair damage can be caused by factors such as hair care, beauty treatments, and changes in the environment. There are many different types of damage, including bleaching [1, 2, 3], perming [4], UV exposure [2, 3], heat from hair irons and hairdryers [5], and friction from combing [6]. Most of this hair damage is caused by damage to the structure of the protein that makes up 80-90% of the hair [7, 8, 9]. For example, bleaching and exposure to UV light can lead to the breakage of cystine bonds, resulting in a decrease in the breaking strength of the hair [2]. Combing the hair can also cause the cuticle to peel off [6]. These damages are not experienced alone, but can be experienced independently and simultaneously, but there is a lack of knowledge about the state of hair when it is exposed to multiple types of damage at the same time. The absence of a convenient and reliable method for detecting hair damage, aside from limited indicators such as cysteic acid [10], presents a significant challenge in the comprehensive evaluation of hair damage.

We have attempted to quantify hair damage by focusing on the process of glycation, which is one of the structural changes of proteins. Glycation refers to a series of reactions in which reducing sugars such as glucose irreversibly react with the amino group side chains of proteins to generate advanced glycation end products (AGEs). Glycation occurs in all parts of the body and causes problems such as yellowing of the skin [11] and the formation of age spots [12]. Some AGEs have characteristic fluorescence (Ex 370 nm / Em 440 nm), and it is possible to easily and accurately quantify them by measuring the fluorescence intensity. In addition, pentosidine is being explored as a marker for osteoporosis diagnosis and can be measured by HPLC [13]. These AGEs are also expected to serve as indicators of glycation. We investigated whether substances exhibiting AGEs-like fluorescent behavior exist in hair and found a novel phenomenon that differs from conventionally recognized forms of hair damage, which we termed "glyco-oxidation." Furthermore, we examined the effects of glyco-oxidation on the physical properties of hair.

2. Materials and Methods

Chemicals

Hydrogen peroxide, ammonia and thioglycolic acid were used in cosmetic grade. Chloroform, methanol, urea, thiourea, dithiotreitol, sodium hydroxide and hydrochloric acid were used in reagent grade (FUJIFILM Wako Pure Chemical, Japan).

Quantification of AGEs-like fluorescent substances in hair

The following treatments A to D were applied to hair (hair from the same person of Chinese origin, black hair, no treatment experience, Beaulax, Japan). A) 6 times treatment with 4.0 % hydrogen peroxide and 2.5 % ammonia (bleaching treatment), B) 6 times treatment with perm solution containing 6.6 % thioglycolic acid and 1.2 % hydrogen peroxide (perm treatment), C) UV irradiation (UV treatment) at an intensity of 1500 W/m^2 for 28 hours, and D) both A and C. The hair was immersed in a chloroform:methanol solution (2:1) for 24 hours and then degreased. 15 milligrams of degreased hair was incubated in a urea/thiourea/dithiotreitol solution at 50 °C for 48 hours. The extract was dialyzed with 1.25 mM NaOH using a Visking tube (Nihon Medical Science, Japan) , and the resulting hair extract protein solution was obtained. The amount of AGEs-like fluorescent substances was measured as the fluorescence intensity (Ex 370 nm / Em 440 nm) per unit protein mass in this solution. Pentosidine and other AGEs-like fluorescent substances also were measured by HPLC fluorescence analysis (Ex 325 nm / Em 385 nm and Ex 370 nm / Em 440 nm) after hydrolyzing the protein extract with 0.5 M hydrochloric acid at 105 °C for 18 hours. InertSustain AG column was used, and 0.1 % formic acid/acetonitrile was used as the eluent. The protein concentration of the extract was measured using the BCA method (Pierce™ BCA Protein Assay Kits, Thermo Scientific™, USA).

Microscopic IR

Hair (from the same person of Chinese origin, black hair, no treatment experience, Beaulax) was immersed in a 4.0 % hydrogen peroxide + 2.5 % ammonia solution for 25 minutes, 6 times, and then exposed to a xenon weather meter at an illuminance of 180 W/m^2 for 120 hours. This was used as the glyco-oxidized hair. Water was dripped into the hair sample, and quick freezing was used for embedding, and transverse hair sections (10 mm) were prepared using a microtome for measurement. Measurements were taken at the following conditions at the SPring-8 BL43IR (Japan) synchrotron radiation facility: wavenumber range 7500-450 cm^{-1}, number of scans 128, wavenumber resolution 4 cm^{-1}, measurement interval 4 mm, aperture width 5 mm.

Calculation of bending modulus

Hair diameter measurement

In untreated and glyco-oxidized hair, the short and long diameters were measured at 2 mm intervals over a 16 mm section centered on the 150 mm section from the root using the SK-2000 Hair Diameter Measurement System (Kato Tech, Japan), and the average of each was taken as the short and long diameters of that hair.

Bending stress measurement

The bending stress at a curvature of 0.5-1.5 cm^{-1} was measured using a single-bending tester (KES-FB2-SH, Kato Tech). Measurements were taken for each treatment 18-20 times, and the average of the three measurements taken for each hair strand was used.

Exploration of anti-glyco-oxidation ingredients

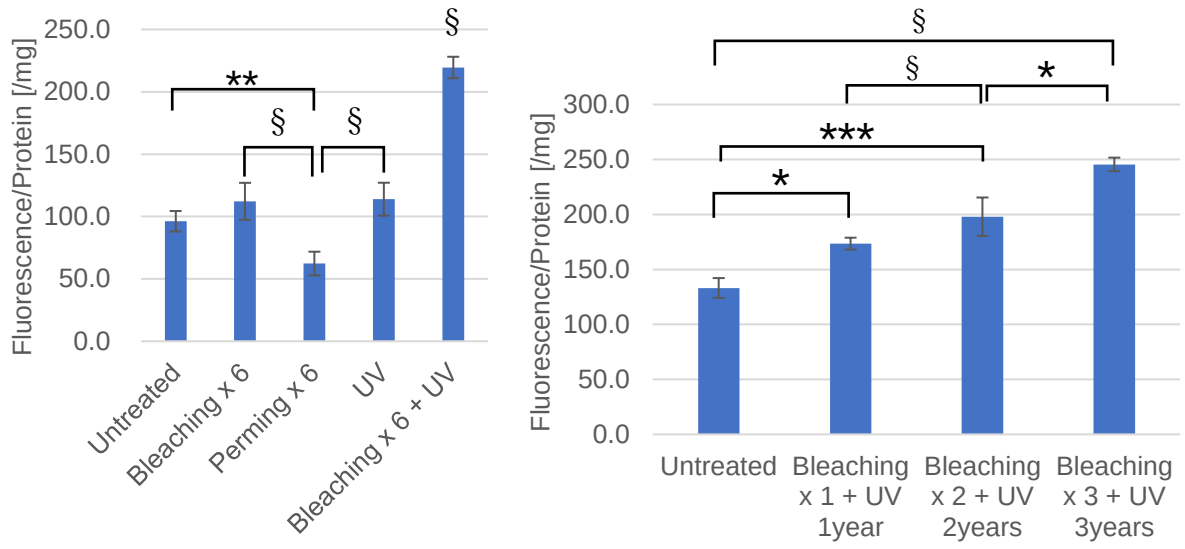
Hair was immersed in a 1 w/w% aqueous solution of various plant-derived extracts or purified gallic acid (MP Gokyo Food & Chemical, Japan) or aminoguanidine hydrochloride (FUJIFILM Wako Pure Chemical) for 18 hours. The hair was then subjected to glyco-oxidation, and the fluorescence intensity (Ex 370 nm / Em 440 nm) per amount of extracted protein was measured. These hair samples were also subjected to bending tests.

3. Results

1. Relationship between AGEs-like fluorescent substances and damage in hair

1.1. Quantification of AGEs-like fluorescent substances in hair

In order to confirm the presence of AGEs-like fluorescent substances within the hair, we examined the amount of substances exhibiting AGEs-like fluorescent behavior in the protein solution extracted from the hair. As a result, a certain amount of fluorescence was observed in untreated hair, suggesting that AGEs-like fluorescent substances are present within the hair (Figure 1a). In addition, in the hair that had undergone chemical or physical treatment, the fluorescence intensity of hair that had undergone a combination of bleaching and exposure to UV light treatment increased significantly, suggesting that these treatments generated AGEs within the hair. On the other hand, there was no significant increase in fluorescence intensity in hair treated with either bleaching or exposure to UV light, and there was a decrease in fluorescence intensity in hair treated with perming. Furthermore, as a more realistic damage amount, a single bleaching treatment and a year's worth of exposure to UV light calculated based on the total solar radiation in Tokyo [14] also showed a significant increase in fluorescence intensity, which increased further with the number of bleaching treatments and the amount of UV exposure (Figure 1b).



(**a**) (**b**)

Figure 1. Quantity of AGEs-like fluorescent substances derived from hair extract. Quantity of AGEs-like fluorescent substances: Fluorescence intensity per protein quantity (Ex 370 nm / Em 440 nm). (a) Untreated and Bleaching x 6 + UV: n=3; others: n=6. In Tukey's multiple comparison test, **: $p<0.01$, §: $p<0.001$. Bleaching x 6 + UV was $p<0.001$ for all samples. (b) n=3. In Tukey's multiple comparison test, *: $p<0.05$, ***: $p<0.005$, §: $p<0.001$.

1.2. HPLC analysis of AGEs-like fluorescent substances in hair

In order to investigate in detail the origin of these fluorescent substances, the extract was hydrolyzed and subjected to HPLC fluorescence measurement. As a result, for AGEs-like fluorescent substances, a peak increase in area was observed at around 13.5 minutes retention

time with the combination of bleaching and exposure to UV light (Figure 2a). This peak area tended to increase as the amount of damage increased (Figure 2b), and it was thought that this peak was derived from AGEs-like fluorescent substances generated by these treatments. Pentosidine was not detected in these extracts. (Figure 2c).

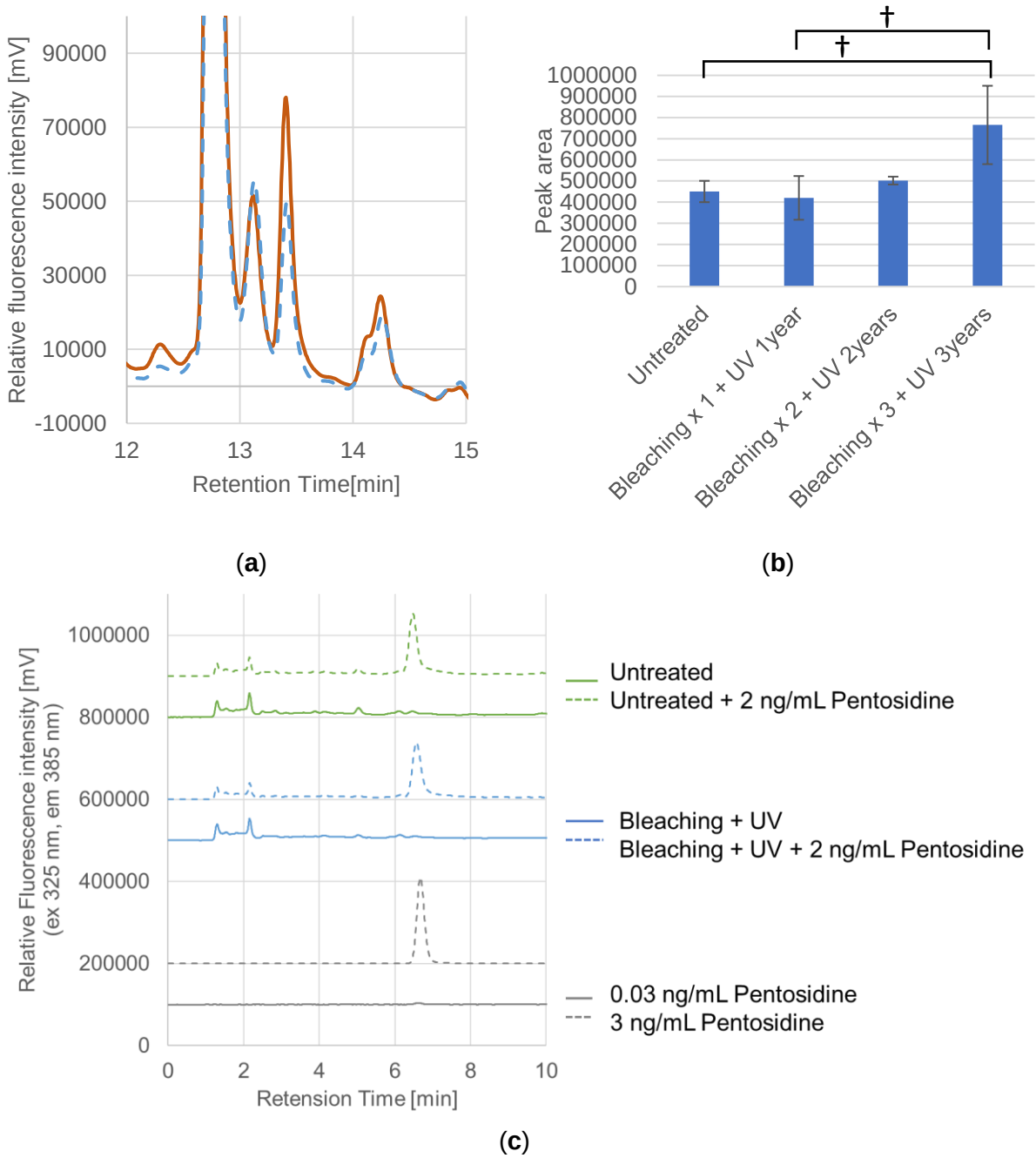


Figure 2. HPLC analysis of AGEs-like fluorescent substances. (a) HPLC chromatogram of AGEs-like fluorescent substances analysis in hair-derived proteins. Detection: Ex 370 nm / Em 440 nm. Orange solid line: glyco-oxidation treatment, Blue broken line: untreated. (b) Peak area values of AGEs-like fluorescent substances by damage amount. n=3. In Tukey's multiple comparison test, †: $p<0.1$. (c) HPLC chromatogram of pentosidine in hair. Detection: Ex 325 nm / Em 385 nm.

2. Relationship between the generation of cysteic acid and glyco-oxidation

It is well known that cysteic acid is generated in hair by bleaching [1, 3]. To investigate whether this type of change also occurs in glyco-oxidized hair, we conducted an analysis using micro-IR. As a result, the peak intensity at 1040 cm^{-1}, which is derived from the S-O stretching vibration of cysteic acid, increased throughout the hair (Figure 3), indicating that the generation of cysteic acid occurs in parallel with glyco-oxidation.

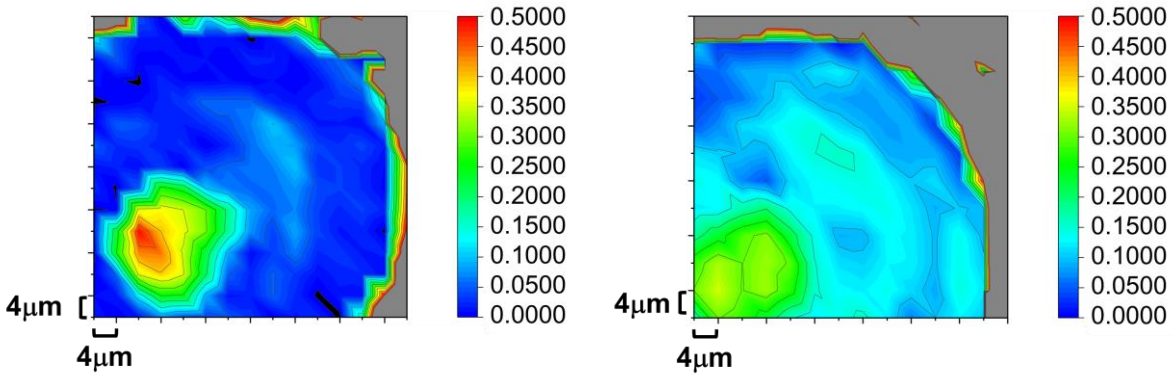


Figure 3. Location of cysteine acid. The intensity of the 1040 cm^{-1} peak is shown, normalized by the intensity of the 1540 cm^{-1} peak. (a) Untreated hair, (b) Glyco-oxidized hair.

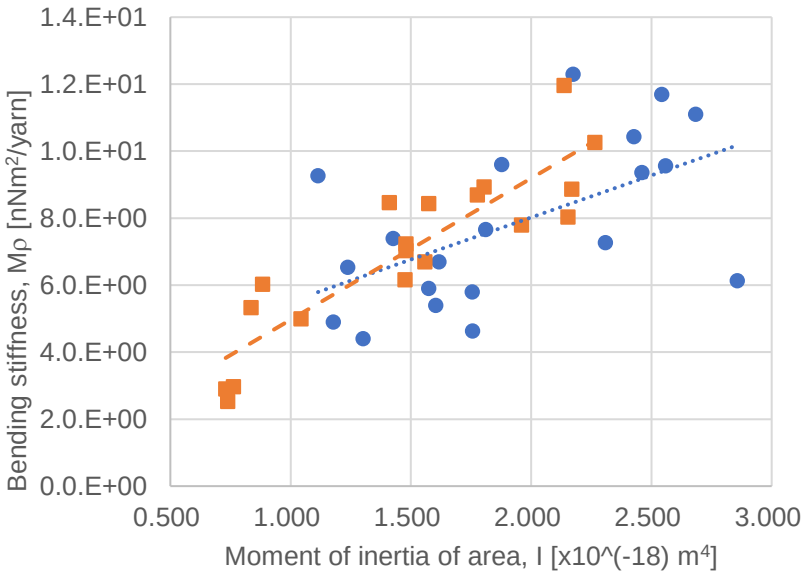


Figure 4. Bending stiffness in relation to the second moment of area. The blue circles and dotted lines show untreated hair (r = 0.56), and the orange squares and broken lines show glyco-oxidized hair (r = 0.89). The standardized residuals for each system were calculated, and any values with an absolute value of 2.5 or more were excluded as outliers.

3. The relationship between hair bending modulus and glyco-oxidation

In order to investigate the changes in the physical properties of hair caused by glyco-oxidation, the bending modulus E was calculated from the bending stiffness of untreated hair and glyco-oxidized hair [15, 16]. In general, it is known that the force exerted when hair is bent has the following correlation.

$$M = \frac{E \cdot I}{\rho}$$

Here, M is the bending moment, E is the bending modulus, I is the second moment of area, and ρ is the curvature. If the cross-section of a hair is approximated as an ellipse, and it is assumed that the hair bends on the minor axis when it bends, I is expressed by the following formula.

$$I = \frac{\pi \cdot a^3 \cdot b}{64}$$

a and b are the short and long diameters of the hair. By measuring the bending stiffness M / (1/ρ) and calculating I from the diameters, it is possible to obtain the bending modulus E. From the slope of the plot of the bending modulus and the second moment of area, the bending modulus of untreated hair was 2.51 GPa, and that of glyco-oxidized hair was 4.22 GPa, showing that the bending modulus E increased due to glyco-oxidation (Figure 4).

4. Examination of anti-glyco-oxidation ingredients

4.1. Exploration of anti-glyco-oxidation ingredients

With the aim of developing hair care products that prevent damage from glyco-oxidation, we investigated plant-derived extracts. As a result, extracts from *Punica granatum*, *Phyllanthus emblica*, *Sapindus mukorossi*, and *Terminalia bellerica* were found to have an effect of suppressing the increase in fluorescence intensity due to glyco-oxidation (Figure 5). Surprisingly, the anti-oxidant effect of gallic acid [17] and the anti-glycation effect of aminoguanidine hydrochloride [18] did not have an effect of suppressing the increase in fluorescence intensity.

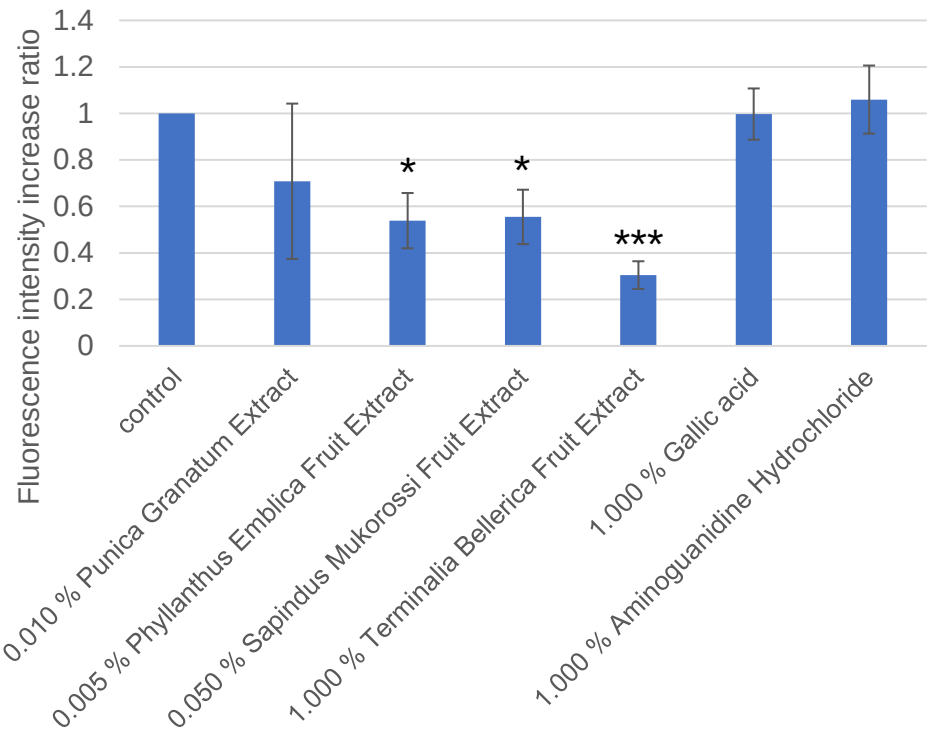


Figure 5. Fluorescence intensity increase ratio of hair treated with extract. Detection: Ex 370 nm / Em 440 nm. n=3. In Dunnett's multiple comparison test, *: p<0.05, ***: p<0.005 compared to control. Fluorescence intensity increase ratio = (extract-treated, (glyco-oxidized hair

fluorescence intensity) - (untreated hair fluorescence intensity)) / (extract-untreated, (glyco-oxidized hair fluorescence intensity) - (untreated hair fluorescence intensity))

4.2. Effect of anti-glyco-oxidation ingredients on bending modulus

In order to investigate the relationship between the generation of AGEs-like fluorescent substances and changes in hair properties after glyco-oxidation, we examined the bending stiffness of hair treated with extracts that had been shown to suppress the increase in fluorescence intensity. As a result, the increase in bending modulus after glyco-oxidation treatment was suppressed in hair treated with the glyco-oxidation suppressing extract and gallic acid, but there was no effect of suppressing with aminoguanidine hydrochloride (Figure 6).

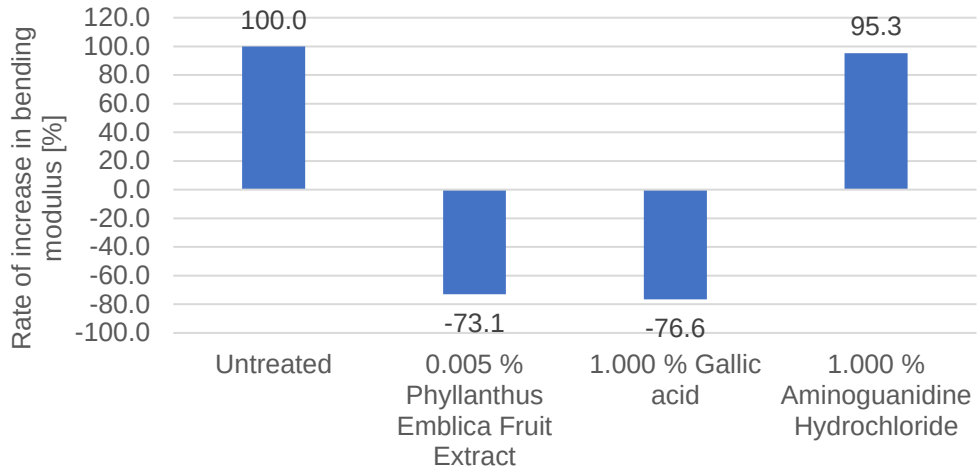


Figure 6. Rate of increase in bending modulus. The rate of increase was derived using the following formula. Rate of increase = (((bending modulus of extract-treated, glyco-oxidized hair) - (bending modulus of extract-treated, untreated hair)) / ((bending modulus of glyco-oxidized hair) - (bending modulus of untreated hair))) * 100.

4. Discussion

Hair comprises a complex assembly of protein-based structures, which are susceptible to various forms of damage. In this study, we attempted to elucidate this phenomenon from the perspective of glycation. AGEs have been identified within hair and are known to accumulate with aging [19], as well as progressively increase along the hair shaft from the root to the tip over time [20]. In general, hair immersed in a sugar solution is used as a model for AGEs generation associated with aging, and under these conditions, typical AGEs such as pentosidine have been detected [19]. However, our tests showed that there was almost no pentosidine in the hair, regardless of whether or not glyco-oxidation treatments were applied (Figure 2c). This may indicate that AGEs generated in the hair over time are not derived from sugar. It is known that AGEs can also be generated from lipids [21, 22]. Hair contains around 10% lipids [7, 8, 9], and it is conceivable that the glycation reaction progresses due to the intense oxidative action of bleaching and exposure to UV light. However, since aminoguanidine hydrochloride, which is known to inhibit the generation of lipid-derived AGEs [18], showed no effect. These findings suggest that the glyco-oxidation we discovered involves a reaction distinct from previously known glycation processes, despite the similarity of its end products to AGEs.

When hair is treated with H_2O_2, it has been shown that the number of COH, NH_2, COOH and SH groups increases significantly [23]. In addition, exposure to UV light also causes oxidative damage to hair, such as the breakdown of amino acids, oxidation of lipids, and cleavage of cystine bonds [24]. It is known that the melanin contained in hair absorbs UV light and protects the hair [24, 25], and when the melanin is disrupted by bleaching, the effects of UV light become more pronounced [26]. This explains why the increase in fluorescence intensity is limited when treated with UV light alone (Figure 1a). Hypothetically, bleaching reduces melanin content, thereby exposing highly reactive functional groups. Under such conditions, oxidative stress from UV light may induce irreversible changes, such as the generation of AGEs-like fluorescent substances derived from lipid peroxides. Further investigation is required to validate this hypothesis.

The bending modulus of the hair treated with glyco-oxidation was greater than that of untreated hair (Figure 4). This is consistent with the previously reported results for hair that had been exposed to UV light after bleaching treatment, and this change is greater than that seen with either bleaching treatment or exposure to UV light alone [26]. This is the same as the behavior of the generation of AGEs-like fluorescent substances due to glyco-oxidation treatment, and it is suggested that there is a correlation with these phenomena. In fact, this change could be prevented by the anti-glyco-oxidation extract (Figure 6). Surprisingly, however, the increase in bending modulus was also suppressed by gallic acid, which has no anti-glyco-oxidation effect. According to Akiyama et al. [26], the reason that bleaching treatment and exposure to UV light make hair stiffer is that the water molecule binding sites inside the hair are lost due to the elution and denaturation of the matrix protein, and the hydrogen bonds between proteins are strengthened. This hypothesis is consistent with the report that the bending modulus decreases under conditions of high humidity [27]. Gallic acid has antioxidant properties and a high free radical scavenging capacity [17], so it is thought to have an effect of preventing protein denaturation caused by bleaching and UV light. *P. emblica* contains gallic acid [28], so it is possible that it has a similar effect of suppressing the bending modulus. However, since *P. emblica* demonstrates the same suppression effect in an extremely small amount compared to gallic acid alone, it is possible that other components demonstrated antioxidant effects, or that the anti-glyco-oxidation effect also contributed to suppressing the increase in bending stiffness. We plan to conduct additional tests to make this clear.

5. Conclusion

We discovered that the combination of bleaching and exposure to UV light generates AGEs-like fluorescent substances in the hair, and named this phenomenon “glyco-oxidation”. It was found that glyco-oxidation treatment increases the bending modulus of hair. To investigate whether the formation of AGEs-like fluorescent substances contributes to such damage, we employed extracts that suppress glyco-oxidation. As a result, the increase in bending modulus was effectively suppressed by these anti-glyco-oxidation extracts. This suggests that, in addition to the well-known damage caused by cystine bond cleavage, the accumulation of AGEs-like fluorescent substances may also contribute to such changes. Quantification of AGEs-like fluorescent substances could serve as an effective method for measuring this type of damage. Furthermore, incorporating anti-glyco-oxidation extracts into hair care products may help maintain supple and beautiful hair even after bleaching treatments.

6. References

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