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Effect of procyandin oligomers on oxidative hair damage

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Background: Procyanidins are a subclass of flavonoids and consist of oligomers of catechin that naturally occur in plants and are known to exert many physiological effects, including antioxidant, anti-inflammatory, and enzyme inhibitory effects. These possible inhibitory effects of the procyanidins were known to involve metal chelation, radical trapping, or direct enzyme binding.
Purpose: The purpose of this study was to investigate the effect of procyandin oligomers on hair damage induced by oxidative stress.
Results: In this study, several methods for evaluating oxidative damage in bleached hair are utilized to analyze the protective effect of procyandin oligomers against oxidative hair damage. It was observed that procyanidin oligomers strongly bind to keratin in hair and inhibit the breakdown of hair caused by oxidative damage in an analysis of hair using electrophoresis, transmission electron microscope, and fluorescence dye.
Conclusion: These results confirm that procyanidin oligomers can be applicable as a potential candidate to the development of hair care with protective effect on hair damage.

Key words: procyanidin oligomers – hair damage – bleaching – keratin

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HUMAN HAIR is composed of two components. One is keratin, which contributes to the mechanical properties of hair. The other is melanin located inside the cortex as hair pigments, whose type, size, and quantity establish hair color (1). Hair, as a type of keratin fiber, is the major component of our body image and functions as a physical barrier between us and the environment. Notably, the special feature of keratin is the presence of a large proportion of cystine. Cystine can bond two adjoining keratin polypeptide chains forming disulfide bonds. The crosslinks give a high degree of physical and chemical stability to the keratin fiber. These weaker bonds such as Van der Waals interactions, hydrogen bonds, and ionic bonds can be easily broken just by wetting the hair (2). Hair damage is created when hair has been exposed to environmental stresses and undergoes quite severe treatment. Hair damage refers to the breakdown or removal of structural components or parts of hair that either weaken it or make it more vulnerable to chemical or mechanical breakdown (3). The cuticle surrounding cortex, keratinized scales are also known to be damaged by exposure to ultraviolet radiation (4). In addition, oxidative stress such as bleaching and permanent coloring was reported to cause dryness, reduced strength, rough surface texture, loss of color, decreased luster, stiffness, and brittleness of hair. Especially, bleaching of hair causes damage of both hair proteins and melanins (5). The degradation of hair protein is be caused by oxidation of the sulfur-containing molecules within the hair shaft (6). Oxidation of the amide carbon of polypeptide chains also occurs with aging, producing carbonyl groups (7). This process has been studied extensively in hair, where it is known as photoyellowing. In our daily life, sunlight exposure, some cosmetic treatments like permanent perming, and bleaching are known to alter hair; however, the related quantitative data remain unclear. Hair melanins can immobilize many of the free radicals formed from oxidative stress, preventing the transport of these free radicals into the keratin matrix. However, in the process of protecting the hair proteins from attack of reactive oxygen species, the pigments are degraded or bleached. For hair damaged by oxidative stress, the amino acids of the cuticle are altered to a greater extent than those of the cortex (8). This oxidative exposure can cause rupture and detachment of the external layers, resulting in splitting of the ends. In this hair damage, the amino acids cystine and

methionine are those most degraded by reactive oxygen species (9) and greater damage occurs when hair is exposed to very high or very low relative humidity (10).

In the field of hair care, cationic oligomers are widely used in hair care products such as conditioners and shampoos and many of these oligomers are also applied in the form of emulsions. A number of studies have been carried out on the adsorption of cationic oligomers emulsions onto hair (11). However, to our knowledge no studies have been carried out on the adsorption of cationic colloidal particles onto hair. In this study, the adsorption of natural compounds onto hair is a subject for prevention of hair damage. In particular, the inhibitory effect of procyanidine oligomers on the oxidation of hair under bleaching condition was found in the present study. The procyanidins are a subclass of flavonoids composed of catechin as a monomer. They are reported to have a variety of biological effects such as antioxidant and anti-inflammatory effects (12, 13). Their effects are influenced by the number of catechine units within procyanidin oligomers. The possible inhibitory actions of the procyanidins are known to be due to metal chelation, radical trapping, or direct enzyme binding (14). These procyanidin oligomers are known to be abundant in Korean traditional medicine such as the elm cortex from the dried bark of *Ulmus pumila*, *Ulmus macrocarpa*, and *Ulmus davidiana*, which has been known to act against edema, gastric cancer, and inflammation such as arthritis and gastritis (15). In this study, the protective effect of procyanidin oligomer on hair damage induced by oxidative stress was investigated. Hair damage including the change of morphological structure, hair color, chemical components as well as tensile strength was studied with a view to collect more quantitative data. Therefore, the present study was undertaken to evaluate the protective effect of procyanidin oligomer on hair damage induced by oxidative stress by considering the above hair damage conditions.

Materials and Methods

Preparation of procyanidin oligomers

The dried cortex of *U. macrocarpa* Hance was pulverized to 10–200 mesh and extracted with absolute ethanol at a ratio of 1:5 (w/v) at room temperature for 72 h. The extract was filtered, concentrated under a reduced pressure at 45 °C, resuspended in distilled water, and then consecutively fractionated with *n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol. The elm extract contained 20% of the procyanidin oligomer. The procyanidin oligomer used in this study was isolated from the elm extract and analyzed chemically. The procyanidin oligomers were composed of three to 12 flavan-3-ol monomers connected by single bonds, and found to have an average molecular weight of 1518 and an average oligomerization degree of 5.3. The procyanidin oligomer were purified by the method described by Kim et al. (16, 17). The elm extract was further subjected to Sephadex LH-20 column chromatography using a water–methanol mix as eluant with an increasing amount of methanol. The fraction containing the highest inhibitory activity was subjected to liquid chromatography–mass spectroscopy, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy, high-performance liquid chromatography/electron spray ionization, phloroglucinol acidolysis, and nuclear magnetic resonance spectroscopy. The elm extract and the procyanidin oligomers were dried under vacuum and dissolved in dimethyl sulfoxide (DMSO) at an appropriate concentration for use.

Binding ability of procyanidine oligomers to hair keratin

Procyanidine oligomers were assayed for the formation of insoluble protein–procyanidine complexes as follows. Hair keratin (5 mg) and procyanidine oligomers were incubated in a 50 mM phosphate buffer, pH 7.4, containing 250 mM NaCl. Total assay volume was 120 mL, and the samples were incubated at 37 °C for 1 h. The assays were performed in triplicate and each experiment was carried out three times. To assay whether the keratin can be bound to procyanidine oligomers, following incubation of the keratin with procyanidine oligomers for 30 min, the insoluble pellet was separated from the supernatant by centrifugation at $10000 \times g$ for 10 min and redissolved in electrophoresis sample buffer. The redissolved pellets were subjected to electrophoresis either on 15% SDS gels. The gels were stained with 0.25% Coomassie Blue R-250 in 30% methanol and 10% acetic acid, and de-stained in the same solution without the Coomassie Blue dye. Keratin bands were observed as the blue color and the intensity of the bands was estimated using

LAS3000® image analyzer and Multi Gauge V3.0 software (Fujifilm Life Science, Tokyo, Japan).

Analysis of hair keratin degraded by bleaching solution by electrophoresis

After hair keratin was pretreated with procyanidin oligomers, the complex was separated by centrifugation. The hair keratin bound with procyanidin oligomer was exposed to bleaching solution containing 6% hydrogen peroxide and 0.5% ammonia for 1 h. The reaction product were subjected to electrophoresis either on 15% SDS gels. The gels were stained with 0.25% Coomassie Blue R-250 in 30% methanol and 10% acetic acid, and de-stained in the same solution without the Coomassie Blue dye. Keratin bands were observed as blue color and the intensity of the bands was visualized using LAS3000® image analyzer (Fujifilm Life Science) and the protein expression was quantified by MULTI GAUGE V3.0 software (Fujifilm Life Science).

A sensitive fluorescence technique using dansyl chloride to assess hair damage

Dansyl chloride was obtained from Sigma Chemical Co. (St. Louis, MO, USA) all other chemicals used were of analytical grade. Hair samples used in the present investigations were purchase from beauty shop private individuals. All hair sample used in various experiments were washed with a mild (5%) solution of sodium laureth-2 sulfate in warm tap water, rinsed extensively with deionized water, and dried with a hair dryer.

Bleaching of hair samples

A 40-cm-long strand of untreated hair was cut into small pieces below 2 inches and pretreated with the proper concentrations of procyanidine oligomers. To bleach hair, samples (100 mg) were placed in a 100 mL beaker containing with 6% hydrogen peroxide and 0.5% ammonia solution. The bleaching process was allowed to proceed at room temperature. After 1 h, the hair sample were taken out, rinsed thoroughly with distilled water, and air dried.

Quantification of hair protein degraded by bleaching solution

After 20 mg of hair was pretreated with procyanidin oligomers, it was separated by centrifugation and thoroughly washed with distilled water. The hair was exposed to bleaching solution containing 6% hydrogen peroxide and 0.5% ammonia for 1 h. The reaction product were concentrated with Rapid-con protein concentration kit according to the manufacturer's guideline. The concentrated protein was quantified using the Lowry assay.

Quantifications of cysteine released from hair exposed to bleaching solution

After 500 mg hair was pretreated with procyanidin oligomers, it was separated by centrifugation and thoroughly washed with distilled water. The dried hair was exposed to bleaching solution containing 6% hydrogen peroxide and 0.5% ammonia for 15 min at room temperature. The reaction mixture (250 μL) was reacted with Ellman's reagent (50 μL) in 2.5 mL of reaction Buffer (0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA). Cysteine hydrochloride monohydrate was used as standard material to draw the standard calibration curve to quantify the amount of cysteine. The cysteine content released from hair exposed to bleaching solution was estimated using the above standard curve.

Analysis of hair proteins using electrophoresis

After 500 mg of hair was pretreated with procyanidin oligomers, it was separated by centrifugation and thoroughly washed with distilled water. The dried hair was exposed to bleaching solution containing 6% hydrogen peroxide and 0.5% ammonia for 15 min at room temperature. The hair then was lysed with lysis buffer (25 mM Tris-HCl, pH 9.5, 8 M Urea, 5% 2-mercaptoethanol) for 2 days at 50 °C. 0.2 mg of lyophilized hair proteins were added to 10 μL of 1 M Tris-HCl (pH 8.0) buffer containing 0.1 M EDTA and 1 M DTT. After adding 1 mL of 8 M urea solution, the reaction mixture was incubated for 30 min at 30 °C. Hair protein loss due to bleaching solution was quantified by the Lowry assay, modified by the addition of sodium lauryl sulfate 5% to improve dispersion. In order to prevent reduction of hair proteins, the equivalent amount of hair proteins was mixed with 0.25 M iodoacetamide in 0.25 M Tris-HCl, pH 8.0. After incubation of reaction mixtures for 15 min at room temperature, 200 μL was mixed with 4 μL of gel loading buffer and electrophoresis was carried out on 10% SDS gels. The gels were stained with 0.25% Coomassie Blue R-250 in 30% methanol and 10% acetic acid, and de-stained in the same solution without the

Coomassie Blue dye. Keratin bands were observed as blue color and the intensity of the bands was visualized using LAS3000® image analyzer (Fujifilm Life Science) and the protein expression was quantified by Multi Gauge V3.0 software (Fujifilm Life Science).

Transmission Electron Microscopy (TEM)

TEM micrographs were obtained using a Philips CM200® microscope, operating mainly at 160 kV. One centimeter segments of hair fibers were fixed using 2 mL of OsO_4 (Sigma) 2% (v/v) in 0.1 M sodium cacodilate buffer pH 7 solution, for 4 h in dark, followed by washing in water or buffer for 30 min. The segments were dehydrated with several ethanol solutions of increasing concentration (from 50 to 100%, v/v), for 15 min, two times with each one of the solutions. Then, the hair was exposed two times to a solution of ethanol and propylene oxide (1:1, v/v) for 5 min. Spurr resin was used as embedding media, in the following steps: (a) hair segments were added in closed flasks filled with Spurr and propylene oxide (1:1, v/v) solution, (b) flasks were placed in an acrylic rotor at 3 rpm constant rotation for 4–8 days, (c) the flasks were opened for propylene oxide evaporation during 24 h, and (d) the hair segments were transferred to proper inclusion molds and left for curing at 70 °C for 24 h. Ultra-thin sections were cut using a Sorvall Porter-Blum Mt2-B ultramicrotome, mounted in a 200 mesh grid and stained with a freshly prepared aqueous solution of uranyl acetate 2% for 15 min and lead citrate 1% for 8 min. Ten transmission electron micrographs were randomly chosen and considered as representative of the hair samples.

Dansylation of hair samples

Optimal conditions for dansylation are those under which the reactive groups most effectively compete for the limited amount of reagent. At pH values higher than this range, the reagent is hydrolyzed to o rapidly, whereas at pH values lower than 8, the unreactive protonated form of the amino acid predominates and slows the labeling reaction. Because dansyl chlorides is only slightly soluble in water, the dansylation reaction are usually performed in acetone:water mixtures. To dansylate 5.0 mg of hair samples were placed in a glass beaker containing 3 mL of 0.1 M sodium bicarbonate solution followed by 1 mL of freshly prepared dansyl chloride solution (20 mg/mL in acetone). The contents of the beaker were mixed well, covered with parafilm, and incubated in the dark at 37 °C for five hours. During this time, most of the excess reagent is hydrolyzed to sulfonic acid and the reaction mixture becomes colorless. At the end of the incubation period, each hair sample was rinsed extensively with DI water followed by a double rinse with acetone to remove traces of dansyl chloride. The samples were placed in a glass petri dish and stored in the dark. Under the above pH conditions, basic groups of peptides and proteins that will react swiftly with dansyl chloride include primary and second amines, phenolic hydroxyls, imidazoles, and thiol group.

Measurement of fluorescence intensity of dansylated hair

Fluorescence intensity of the dansylated hair was measured with LAS3000® image analyzer (Fujifilm Life Science, Tokyo, Japan). Hair fibers, 3 cm long, were mounted in lanes parallel to each other and 2 cm apart on a glass plate(200 × 200 mm) using double-stick Scotch tape to secure each fiber at both ends. The glass plate containing the test samples was then placed in the TLC unit with the hair fibers facing down. Each lane was then manually adjusted in such a way that the central portion of each hair fiber directly crossed the path of the incident light beam during the automatic scanning operation in Y-direction. The fluorescent light intensity of the dansylated hair was measured at an excitation of 350 nm and an emission of 465 nm.

Tensile strength test

The tensile strength of the collagen treated with procyanidin oligomers was measured by Instron 4411 with a 0.01 N load cell operating at 10 mm/min constant speed using a sample holder.

Statistical analysis

Comparisons of all data were performed using the two-tailed, unpaired Student's *t*-test. A P value <0.05 was considered statistically significant. Data are expressed as means ± SE.

Results

Binding ability of procyanidine oligomers to hair keratin

The binding ability of procyanidin oligomer with hair keratin was investigated by the centrifugation

method. In this assay, formation of insoluble protein–procyanidin complexes can be observed after centrifugation if procyanidin oligomers strongly bind to hair keratin. As shown in Fig. 1, while the blank group did not show any pellet, the pellet in groups treated with procyanidin oligomers was clearly observed at the bottom of microtube. The binding of procyanidin oligomer with hair keratin was increased in a dose-dependent manner. It was determined that procyanidin oligomer above 1250 μg/mL could bind to human keratin. These results indicate that procyanidin oligomers have a potential capability of protecting hair from damage. In the next experiment, the protective effect of procyanidin oligomers was examined in hair keratin exposed to bleaching solution. To do so, after hair keratin was pretreated with procyanidin oligomers, the complex was separated by centrifugation. The hair keratin bound with procyanidin oligomer was exposed to bleaching solution containing 6% hydrogen peroxide and 0.5% ammonia for 1 h. The reaction products were redissolved in electrophoresis sample buffer and were subjected to electrophoresis. As shown in Fig. 2, the clear keratin band was observed in the blank group, but disappeared in the control group treated with bleaching solution by breakdown of hair keratin. However, procyanidin oligomers dose dependently increased the protective effect on hair keratin. These observations confirmed that procyanidin oligomers have a possible capability of protecting hair damage when exposed to bleaching solution.

Effect of procyanidin oligomers on degradation of hair exposed to bleaching solution

In order to examine the protective effect on degradation of human hair exposed to bleaching solution with simulating the condition of hair bleaching, the amount of the proteins released from hair was quantified using Lowry assay. After hair was pretreated with 1% procyanidin oligomers, 68% ammonia or 6% hydrogen peroxide was used to bleach hair. After bleaching hair, the released proteins were concentrated and quantified. As shown in Fig. 3, first, the amount of the released proteins in the combined group was highly increased than those of hair treated with ammonia or hydrogen peroxide alone. However, the released proteins were decreased in hair pretreated with procyanidin oligomer. The above results demonstrate that procyanidin oligomers can protect hair damage caused by hair bleaching.

Analysis of cysteine released from hair exposed to bleaching solution

Cysteine is an amino acid abundant in hair keratin. Therefore, to confirm whether procyanidin oligomers have a protective effect on degradation of human hair exposed to bleaching solution, the amount of cysteine released from the oxidized hair was determined with Ellman's reagent. DTNB is reacted with SH group of cysteine, emitting visible wavelength at 412 nm. By this principle, by measuring the content of cysteine, hair damage can be evaluated. As shown in Fig. 4, the level of cysteine released from hair damage by bleaching was enhanced compared with the blank. Cysteine released from hair damage showed the same trend as that of the proteins released from hair damage. The combined group with ammonia and hydrogen peroxide exhibited higher breakdown of hair than single treatment group. Hair pretreated with procyanidin oligomer decreased the release of cysteine from hair exposed to bleaching solution.

Analysis of hair proteins using electrophoresis

After hair was pretreated with procyanidin oligomers, they were exposed to bleaching solution containing 6% hydrogen peroxide and 0.5% ammonia and the hair then was lysed with lysis

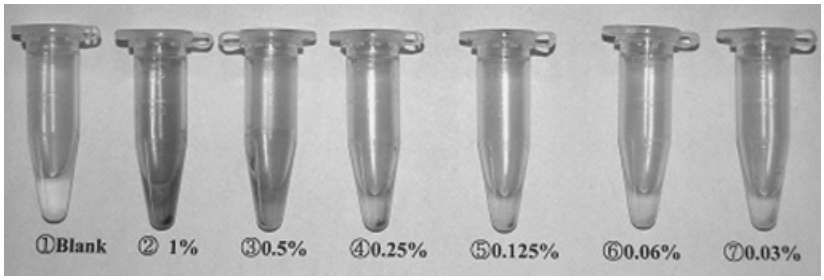

Fig. 1. Binding ability of procyanidine oligomers to hair keratin. Formation of insoluble keratin–procyanidin complexes can be measured by centrifugation after mixing keratin and procyanidin oligomers.

buffer. Lyophilized proteins was completely dissolved in buffer EDTA, DTT and urea solution, electrophoresis was carried out with these reaction mixtures, and electrophoretic patterns of hair proteins were analyzed. As shown in Fig. 5, it is observed that the proteins in hair exposed to bleaching solution were degraded. The hair pretreated with procyanidin oligomers showed clear protein bands corresponding to keratin proteins with molecular weight that ranged from 3000 to 5000 kDa. Therefore, the keratin level in hair treated with procyanidin oligomers was increased compared with that of the bleaching group. Above results indicate that procyanidin oligomers can protect keratin protein from hair damage.

Transmission electron microscopy (TEM)

In order to further examine the protective effect of procyanidin oligomer on hair damage by bleaching visually, the internal images of the hair samples were analyzed with TEM. As shown Fig. 6A, the number of melanosome in hair damaged by bleaching was highly decreased compared with normal hair. However, procyanidin oligomer inhibited degradation of melanosome in hair exposed to bleaching solution, increasing the number of melanosome in hair compared with the bleached hair. It is observed that protein layer in microfibril of hair oxidized by bleaching solution was not more denser than that of normal hair in Fig 6B, indicating that proteins in hair were degraded and released from hair. In contrast to the bleaching group, the protein layer in microfibril of hair pretreated procyanidin oligomers was more denser than that of bleaching group. Above results confirmed that procyanidin oligomers clearly inhibited hair damage induced by bleaching solution.

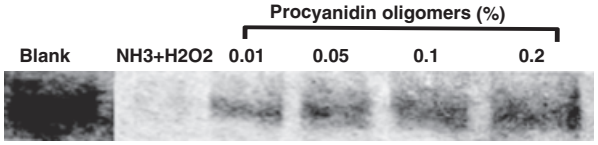

Fig. 2. The protective effect of procyanidin oligomers on hair keratin degradation by oxidative damage. Hair keratin was pretreated with procyanidin oligomers, the complex was separated by centrifugation. The hair keratin bound with procyanidin oligomer was exposed to bleaching solution containing 6% hydrogen peroxide and 0.5% ammonia for 1 h. The reaction product were redissolved in electrophoresis sample buffer and were subjected to electrophoresis.

Effect of procyanidin oligomer on fluorescence intensity of damaged hair

Fluorescence intensity of the damaged hair was used as a marker to evaluate the level of hair damage. The higher the hair damage caused by bleaching solution, the clearer the fluorescence intensity of hair was measured. As shown in Fig. 8, fluorescence intensity of normal hair was much higher than that of damaged hair. Procyanidin Fig. 7. Fluorescence intensity of dansylated hair treated with procyanidin oligomers. Fluorescence intensity of the dansylated hair was measured with LAS3000® image analyzer. The fluorescent light intensity emitted by the dansylated hair was measured as peak heights on the chart recorded and expressed arbitrary emission units. The fluorescence emission of hair was measured at excitation of 350 nm and emission of 465 nm. Oligomers decreased the fluorescence intensity of the damaged hair in a dose-dependent manner, indicating that hair damage can be

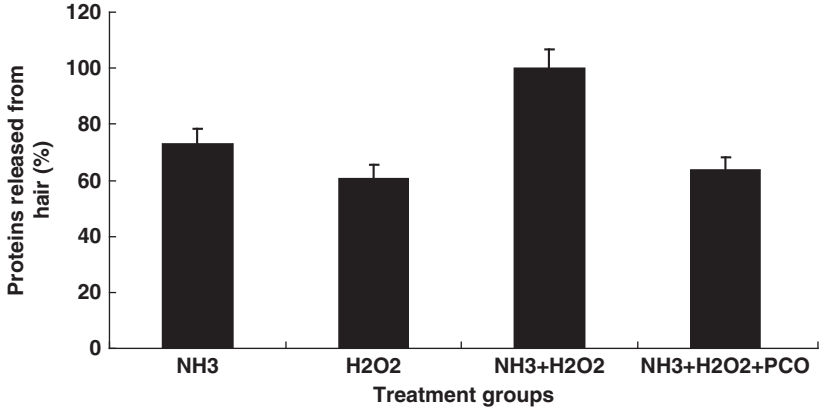

Fig. 3. Effect of procyanidin oligomers on degradation of hair exposed to bleaching solution. The protective effect on degradation of human hair exposed to bleaching solution with simulating the condition of hair bleaching, the amount of the proteins released from hair was quantified using Lowry assay.

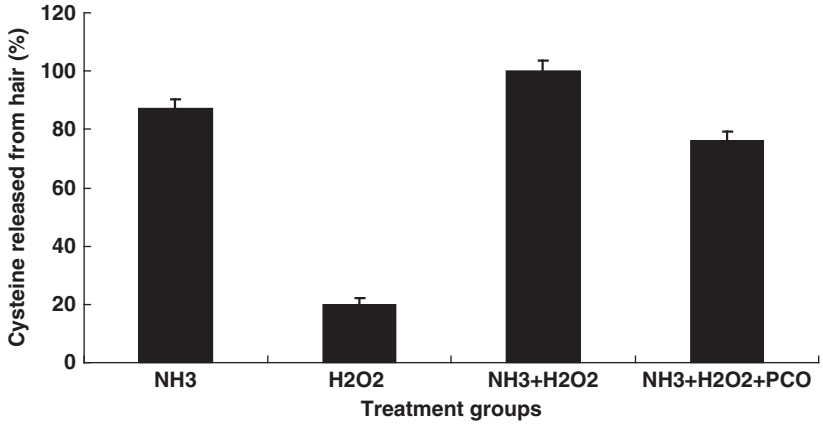

Fig. 4. Analysis of cysteine released from hair exposed to bleaching solution. Protective effect of procyanidin oligomers on degradation of human hair was analyzed in view of the amount of cysteine released from hair exposed to bleaching solution measured with Ellman's reagent.

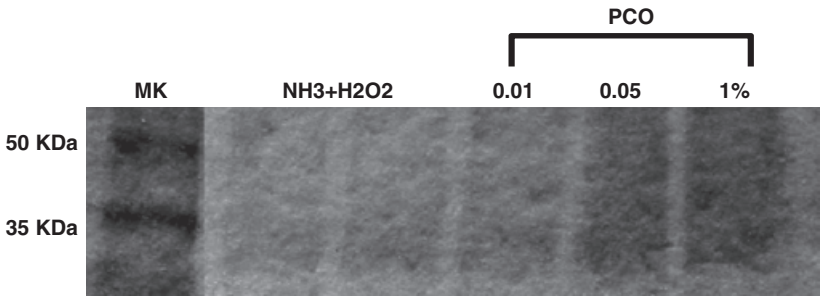

Fig. 5. Analysis of proteins of hair treated with procyanidin oligomers using electrophoresis. After hair was pretreated with procyanidin oligomers, the hair was lysed for 2 day at 50°C. The lyophilized hair proteins were mixed with 8 M urea solution, the reaction mixture was incubated for 30 min at 37°C. Hair proteins was carried out electrophoresis either on 15% SDS gels.

inhibited in the presence of procyanidin oligomers.

Tensile Strength

Finally, it was investigated whether procyanidin oligomers can influence the tensile strength of proteins. To do so, after procyanidin oligomers were mixed with collagen, the tensile strength of the collagen gel formed is measured. The actual value of tensile strength was displayed as shown in Fig 8A. Procyanidin oligomers increased the tensile strength of collagen in a dose-dependent manner, as shown in Fig 8B. This suggests that procyanidin oligomers can strongly bind to proteins.

Discussion

Human hair is a very complex fiber whose chemical composition varies somewhat with its water content (18). The main component of hair is made up of keratin, water, lipids, pigment, and trace elements. The special feature of keratin is to contain a large proportion of cystine. These cross-links give a high degree of physical and chemical stability to hair. In addition, the polypeptide chains of hair are linked by other weaker bonds such as Van der Waals interactions, hydrogen bonds, and ionic interactions that result from the attraction of positively and negatively charged groups (11). They can be easily broken just by wetting the hair (2). Hair damage also occurs owing to free radicals, leading to disulfide bond breakage (19). Bleaching involves degradation of melanin. First, there is a dispersion and dissolution of melanin granules, which is involved in a change in color from black to brown (20). Following this dissolution phase, there is a much slower de-coloring phase. The exact biochemistry based on these processes is not completely understood, but it is thought that the dissolution phase involves the destruction of different bonds maintaining melanin granules while the de-coloring phase involves the breakdown of the oligomeric structure of melanin (21). The oxidation reaction associated with bleaching not only alters melanin. It will also destroy some

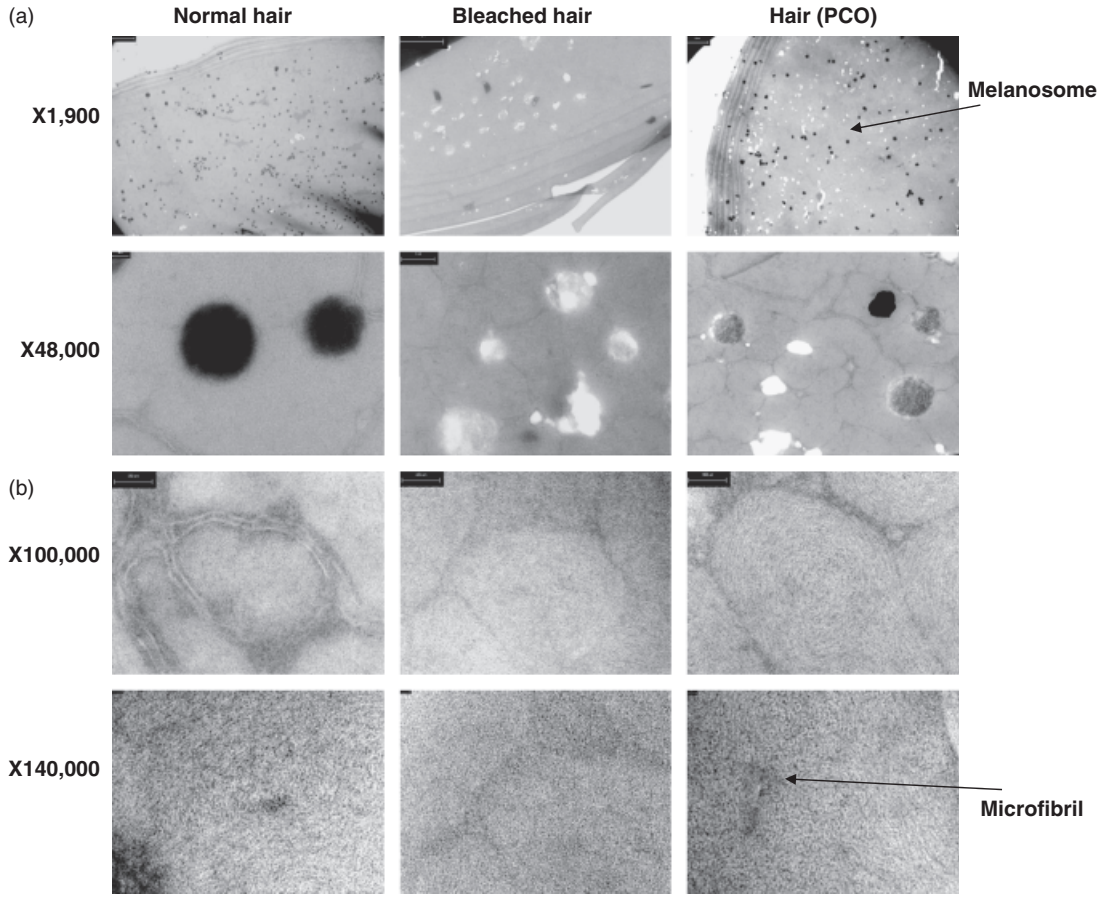

Fig. 6. Analysis of hair treated with procyanidin oligomers using Transmission Electron Microscopy. The internal images of the hair samples were analyzed with TEM. to examine the protective effect of procyanidin oligomer on hair damage by bleaching visually.

disulfide bonds within the keratin, which will lead to a weakening of the hair structure. Damage also occurs to the cuticle and will make the hair more porous. Following bleaching, the hair will often be different in texture, will break more easily, and will be more susceptible to humidity. To palliate these problems, a conditioner will often be used either within the bleaching solution or after bleaching (22). In fact, chemically treated hair, particularly after bleaching and perming, needs protection to prevent further damage that may cause severe weathering. Therefore, the necessity developing a protective material against hair damage has been growing in recent years. Before searching for them, several methods to evaluate hair damage should be examined and tested. First of all, the most important factor to prevent hair damage is to protect hair protein by binding to keratin abundant in hair. In our study, procyanidin oligomers derived from Ulmus radicies were identified to strongly bind to keratin of hair protein. Formation of insoluble protein–procyanidin complexes can be observed after centrifugation, indicating that procyanidin oligomers have a potential capable of protecting hair damage. This finding is consistent with a report that the physiological effect of tannins such as procyanidin oligomers is demonstrated by their protein-binding or precipitation capacity (23). However, it is difficult to expect that this binding can play a key role in the protection of hair damage. In order to clarify the protective effect of proctanidin oligomers on hair damage, after keratin pretreated with them was exposed to bleaching solution, the reaction product were subjected to electrophoresis. Procyanidin oligomers increased the protective effect on oxidative damage of hair keratin induced by bleaching solution, suggesting that they have a possibility to protect hair damage by being exposed to bleaching solution. This result was supported by the fact that procyanidin oligomers exhibit cell membrane protective effect by antioxidant activity (24). Thus, the upcoming question is whether this binding effect of proctanidin oligomers to keratin can inhibit the degradation of hair

proteins induced by oxidative stress. As a result of the protective effect on degradation of human hair exposed to bleaching solution with simulating the condition of hair bleaching, the amount of the released proteins and cysteine was decreased in hair pretreated with procyanidin oligomer. Therefore, procyanidin oligomers can protect hair damage caused by hair bleaching. In a subsequent experiment, it was determined whether hair is damaged by oxidants, disulfide bond is broken, and cysteine is produced because the level of cystine can be a criteria to evaluate hair damage. The level of cystine in hair was analyzed after the hair was pretreated with procyanidin oligomers before exposure to bleaching solution. The content of cystine in the hair damaged by bleaching was increased in the presence of procyanidine oligomers, suggesting that procyanidin oligomers exert a protective effect on hair damage exposed to bleaching solution. This activity is caused by the reducing power of procyanidin oligomers that contains a high amount of phenol (25). In the next step, it is necessary to visualize the protective effect of procyanidin oligomers on hair damage. The hair pretreated with procyanidin oligomers showed clear protein bands corresponding to keratin

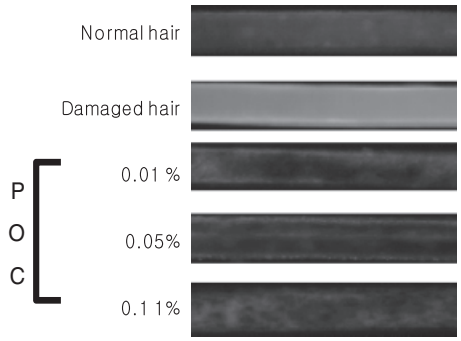

Fig. 7. Fluorescence intensity of dansylated hair treated with procyanidin oligomers. Fluorescence intensity of the dansylated hair was measured with LAS3000® image analyzer. The fluorescent light intensity emitted by the dansylated hair was measured as peak heights on the chart recorded and expressed arbitrary emission units. The fluorescence emission of hair was measured at an excitation of 350 nm and an emission of 465 nm.

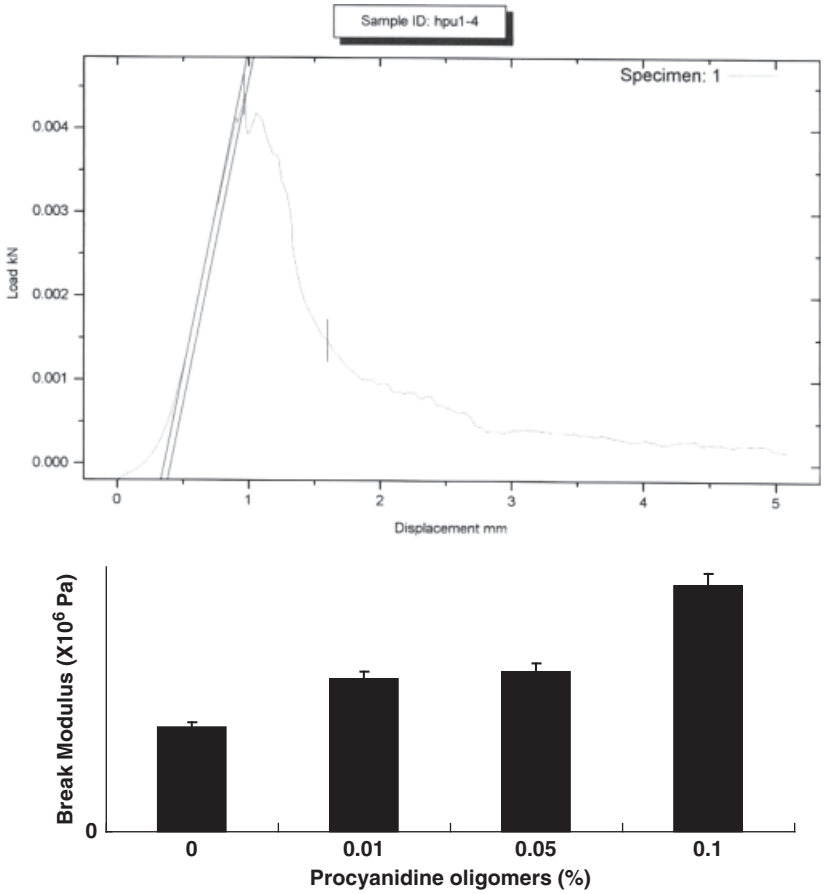

Fig. 8. Effect of procyanidin oligomers on tensile strength of hair. After procyanidin oligomers were mixed with collagen, the tensile strength the formed collagen gel was measured. The tensile strength of the collagen treated with procyanidin oligomers was measured by Instron 4411 with a 0.01 N load cell operating at 10 mm/min constant speed using a sample holder.

proteins with molecular weight ranging from 3000 to 5000 kDa. Furthermore, unlike analysis of hair damage using scanning electron microscope (26). The internal images of the hair samples exposed to procyanidin oligomers and bleached solution were analyzed with TEM. In this study, it was for the first time found that the protein layer in microfibril of hair pretreated procyanidin oligomers was more denser than that of bleaching group, demonstrating that procyanidin oligomers clearly inhibited hair damage induced by bleaching solution. In addition, fluorescence intensity of the damaged hair was used as a marker to evaluate the level of hair damage. Previous study reported that fluorescence intensity increases as a result of oxidative damage of hair (27). The higher the hair damage caused by bleaching solution, the clearer the fluorescence intensity of hair is. Procyanidin oligomers decreased the fluorescence intensity of the damaged hair, indicating that hair damage can be inhibited in the presence of procyanidin oligomers. Finally, it was investigated whether procyanidin oligomers can influence the tensile strength of proteins. They increased the tensile strength of collagen, suggesting that procyanidin oligomers can strongly bind to proteins. This finding is related to a report that the tensile properties of hair fiber play an important role in determining the efficacy of hair treatments such as permanent waves, permanent hair colors, bleaches and permanent hair straightners (28).

In conclusion, procyanidin oligomers strongly bind to keratin in hair and inhibits the breakdown of hair caused by oxidative damage in analyses of the proteins, cysteine, and cystine by quantification and electrophoresis, transmission electron microscope and labeling fluorescence dye. The above results confirmed that procyanidin oligomers clearly inhibit hair from oxidative damage induced by bleaching solution as a potential candidate for development of hair care with protective effect on hair damage.

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