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〈Research Paper〉

# Quality Improvement of the Human Hair by the Treatment of Protease Extracted from Earthworm

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**Abstract**— The feasibility of earthworm protease as a new cosmetic material for human hair care was investigated. The effectiveness of the earthworm protease treatment was assessed by thickness change of hairs, optical microscope examination, aminoacid analysis, surface morphology, angular resolution analysis through methylene blue staining method and tensile strength change. The protease treated hair became thinner and the soil on the surface removed in experimental groups unlike control group. Tensile strength decreased in experimental group in which the enzymes may decompose polypeptide bonds.

**Keywords:** *protease, earthworm, human hair, aminoacid analysis, cuticle, soil*

## 1. Introduction

By starting from Charles Darwin's publication a hundred years ago, it has been known that an earthworm, a polyphagous animal, digests the debris of all the plants or animal carcasses which can be very useful to agriculture. Biologists and zoologists conducted the research on the structure and life mode of the earthworm[1)].

Also, soil scientists studied[2)] on the soil purification capability of the earthworm and physical property change of soil. Agricultural scientists have carried out the study[3)] of utilizing the earthworm for the increase of agricultural products.

Proteases and many amylases are being used in various industrial fields of fibers, leathers, photo, papers, cosmetics, medicines, medical service and waste disposal[4)]. The enzymes had taken up more than 70% of the detergent market share in 1988 since the detergent products including protease appeared in Korea in 1985. The enzyme quantity consumed in Korea amounted to about 1,200 tons in 1994, and also enzyme market for detergents expanded because enzyme containing detergent products were more than 90%[5)].

Proteases are being used in the processing of leather and silk and wool, and it has been used to resolve the problems such as the discoloration and bad touch resulting from chlorination and oxidizing treatment to remove the scales in the non-shrink treatment of woolen goods in woolen cloth field[6-8)].

The activity of the proteases should be maintained over wide pH and temperature ranges as possible to be useful industrially[9,10)]. Because proteases have high catalytic activity and does not have to need to be removed after processing. The use of various enzymes is increasing rapidly.

The molecular weight of earthworm protease is 25~34 kDa and the optimum pH is known to be 3.7~4.0[11)].

As the researches using the enzyme from earthworm, Jeong[12)] reported the separation of thrombolytic protease from earthworm and the biochemical properties. Choi and Hur[13,14)] explored the possibilities for the sludge stabilization using earthworm. Also, Lee and others[15)] studied on the decomposition of sulfuretted hydrogen, the separation and properties of microorganisms from earthworm casts.

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Kwon and others[16] studied the surface performance variation after treating wool and silk fibers with protease extracted from earthworm.

However, there has been no research on human hair by using earthworm protease. About 80~90% of hair component is keratin protein, and it consists of 18 different aminoacids including much higher contents of cystine[17]. The aminoacids form polypeptide bonds and the hair have many S-S bonds[18].

One of main cause of hair damage is the physical stimuli in everyday life such as combing. The partially damaged hairs show rough surface and deteriorated feel. Also, absorption of various soils on the modification of hair surface becomes the factor to increase hair damage resulting from heavy metals. However, considering that hair is a keratin protein, it is considered that the hair quality can be improved by treating hair with protease[19].

In this study, the application of earthworm proteases as a new hair care product was investigated.

## 2. Materials and Methods

### 2.1 Hair sample

Sample hairs are woman's straight hair not to have performed the chemical treatment for recent 2 years among women in their twenties whose life practices are regular and who neither take drugs over the long term nor eat only what they like. Hair bundles of 1g were prepared by gathering hairs of 20cm from the laryngeal region. The samples were washed with the neutral shampoo and distilled water prior to use.

### 2.2 Protease extraction

Earthworm was washed with running wash to remove soil and foreign substances. The protease was extracted from the 1:1 mixture of earthworm and sodium phosphate buffer[20] of pH 7.4 after grinding it with homogenizer at room temperature. After centrifuging the extractant in 8,500 rpm for 15 min, the supernatant crude enzyme extract was used in the experiment.

### 2.3 Measurement of protease activity

Protease activity was determined by Kunitz method[21]. The mixture of crude enzyme extract(2 ml) and casein substrate(1 ml) was reacted at 40℃ for 20 min in a test tube. The sediment was deposited by leaving it at room temperature for 30 min after putting 5% TCA solution of 3 ml, and the undecomposed protein was filtered through filter paper (Whatman No. 2). Optical density at 280nm of the filtrate was measured at different temperatures using a spectrophotometer. The pH effect was determined by optical density by adding 1 ml substrate and the crude enzyme extract of 2 ml at 50℃ after adjusting pH with 0.2 M sodium acetate buffer (pH 7.4).

### 2.4 Method of protease treatment

The hair of a experimental group was added into the crude enzyme extract of the earthworm protease extracted by a buffer solution, and the hair of the control group was put into the buffer solution of the enzyme solution that was inactivated by inducing the degeneration of the enzyme by the heat after warming up the crude enzyme extract of earthworm protease in a double boiler at 100℃ for 15 min. Each hair and enzyme in 50 ml conical tube were treated in water bath at the agitating rate of 100 rpm at 30℃. The enzyme solution was washed with running water and then dried at room temperature.

### 2.5 Measurement of hair thickness

The sample hairs were dried at room temperature for 24 h at 24~25℃ and 50~55% relative humidity. The mean value of hair thickness was calculated after measuring repeatedly ten times with Mitutoyo digital micrometer (model no. IP 65, Saint Louis, America) after collecting each 20 pieces from each sample, and the measurement point was selected as the point of 5 cm from the silicon-treated hair root. To minimize the error of the hair thickness and to give the reliability of the experiment, the mean value and the standard deviation were calculated by using SPSS program after measuring the thickness ten times in the same way.

### 2.6 Optical microscope observation

Optical microscope (OLYMPUS BX50, New Jersey, America) was used. to observe the hair changes of the control group treated in the buffer solution and of the experimental groups.


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## 2.7 Aminoacid analysis

3 ml of 6N HCl was added into 0.5 g of earthworm protease solution and 0.5 g of the hair in 18 ml test tube. The prepared sample was sealed after the air in the test tube is replaced with nitrogen gas and hydrolyzed at 121℃ for 24 h. The quantity of aminoacid in the hydrolyzed sample was measured by using an aminoacid autoanalyzer (Sykam Co., S433H, Germany). pH 1, pH 2, pH 3, pH-RG, R-3 and ninhydrin solution (Wako Co., Osaka, Japan) were used as the mobile phase. Ion exchange column #2622SC PH was used at 50℃ and the temperature of reaction chamber remained at 135℃. Aminoacid calibration mixture (Sigma Co., AA-S-18, Saint Louis, MO, U.S.A.) was used as the standard solution[22].

## 2.8 Hair surface observation

The hair surface of the experimental group and the control group treated with protease was observed in 600 ~ 800 magnifications with a scanning electron microscope (Akaso Alpha 25A, Hitachi S-4700, Tokyo, Japan).

## 2.9 Measurement of the decomposition of protein by using methylene blue staining method

The hair samples were stained with methylene blue in order to measure the decomposition of protein. The stained samples were extracted with 5 ml mixture solution of 49% ethanol, 1% glacial acetic acid and 50% distilled water[23] for 5 min and their optical density at 660 nm was measured by using a spectrophotometer.

## 2.10 Tensile strength test

Tensile test (KS K0323) for the hair samples were carried out in the direction of the hair root with a universal materials testing machine (Instron, model no. 4301, England). For the reliability of the measurements, the mean values were calculated after excluding the measured values of which the deviation ranges were more than 30% after measuring 10 times each sample. The tensile strengths were measured under clamping distance of 20 mm and the tensile speed of 20 mm/min. The laboratory temperature was 20℃ and the laboratory humidity was 65%.

# 3. Results and Discussion

## 3.1 Change of hair thickness

About 80~90% of the hair consists of keratin protein[17]. Accordingly, since protease treatment may decompose keratin protein of the hair, it was expected that the hair thickness will change. The optimum temperature and pH were measured to investigate the properties of the enzyme after making the crude enzyme extract from earthworm as the pilot test first, and it showed high activity at 30~50℃, and the activity decreased at the higher temperature. The results corresponded with the report of Kwon[16]. Also, the optimum pH was 7.0 at 40℃ which is neutral, and it showed high activity even at pH 8.0. However, the activity of pH decreased rapidly in more than pH 9.0. The thickness changes were measured after treating the normal hair after extracting the protease from earthworm with the buffer solution of phosphoric acid of pH 7.0. Fig. 1 is the hair thickness change of the control group treated with the inactivated enzyme solution and the experimental group treated with earthworm protease. To measure the hair thickness of the control group, treated with the protease solution denatured first, was 0.0996±0.0104 mm.

The hair thickness did not change much throughout treatment time when treating it for 48 h as time goes by.

However, the hair of the experimental group treated with the protease not inactivated became thinner from 0.0959±0.0085 mm to 0.0792±0.0086 mm after 48 h.

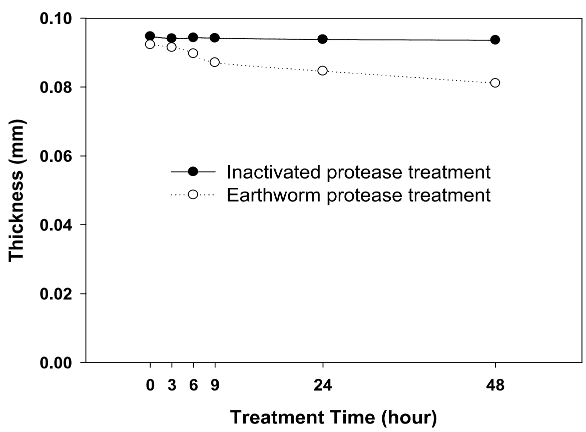


Fig. 1. Hair thickness change by protease treatment.

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The results correspond to the results of Hyun's research[24] that hair gets thinner according as some keratin protein of hair decomposed by alkaline reagent prescribed in permanent wave lotions, which can be considered to be the phenomenon to occur because earthworm protease decomposes partially the hard keratin protein of the cuticle which is the outermost layer of hair.

## 3.2 Optical microscope observation of protease treated hair

Kwon and Jeong[12,16] separated thrombolytic protease from earthworm. This enzyme assisted the decomposing protein and removing soils of fibroin. The results of the hair treated with inactivated enzyme solution and the hair with the other enzyme solution are shown in Fig. 3 and Fig. 4. Fig. 2 shows the normal hair that was not treated with enzyme. We can see a threadlike soil absorbed on the upper part and a soil absorbed on the hair surface of the lower part in the hair that was not treated with enzyme as shown in Fig. 2. In case of human hair, the sebum secreted within the scalp is not removed completely by being absorbed on hair owing to bad shampooing habits and so on even after shampooing hair.

As shown in Fig. 4, in case of experimental group which was treated with earthworm protease, the soils absorbed on the hair began to be removed gradually in 30 min and removed completely in 3 hours.

This result is consistent with that of Lee's research[25]. The protease removes the soils of the fiber in the detergency test of fiber. However the hair of the control group (Fig. 3), no change was observed. Kwon[16] reported that earthworm protease removed the soils on silk. Earthworm protease decomposed partially keratin protein of the hair and removed various soils absorbed on the hair after being secreted from the scalp.

## 3.3 Aminoacids analysis

Keratin protein of the hair consists of 18 kinds of aminoacids with polypeptide bonds, and is used as the index in aminoacid analysis of the hair because it includes much cystine and glutamic acid, serine, arginin. In particular, cystine does not exist or if any, only a very small amount in other proteins while hair contains about 16~18% among the aminoacids[26].

Fig. 2. Untreated normal human hair.

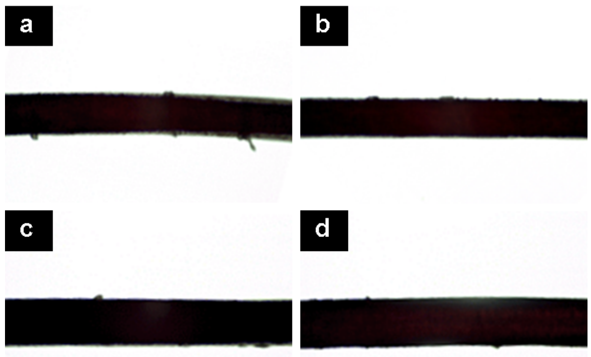


Fig. 3. Optical microscope observations of the hair treated with inactivated earthworm protease.
(a) hair treated with inactivated enzyme for 30 min,
(b) hair treated with inactivated enzyme for 1 h,
(c) hair treated with inactivated enzyme for 3 h and
(d) hair treated with inactivated enzyme for 6 h.

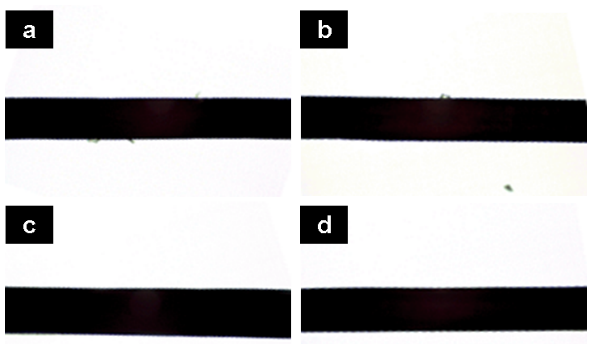


Fig. 4. Optical microscope observations of the hair treated with earthworm protease.
(a) hair treated with protease for 30 min,
(b) hair treated with protease for 1 h,
(c) hair treated with protease for 3 h and
(d) hair treated with protease for 6 h.

Table 1 is the result of aminoacids analysis in the hair not treated with protease and earthworm protease solution, and the experimental group's hair was treated with protease for 1 h. Total content of aminoacids in earthworm protease was low as 614.525 mg/100 g.

This is because aminoacids in earthworm was about 75~90% of earthworm weight consisted of water. The dry weight of 0.5 g enzyme solution from earthworm decreased about 50% as 0.24 g.



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Table 1. Aminoacids contents in earthworm protease and human hair

| Sample hair / aminoacid | Earthworm protease | Virgin Hair (Control) | Earthworm protease treatment for 1 h |
|---|---|---|---|
| Aspartic acid | 35.4 | 5353.3 | 6823.4 |
| Threonine | 17.4 | 5875.6 | 4040.7 |
| Serine | 13.2 | 7656.5 | 4753.7 |
| Glutamic acid | 37.3 | 11974.9 | 9750.8 |
| Proline | 22.4 | 5818.7 | 4534.9 |
| Glycine | 35.6 | 3049.8 | 2423.3 |
| Alanine | 35.7 | 2730.3 | 2212.7 |
| Cystine | 5.0 | 10531.6 | 7312.3 |
| Valine | 45.6 | 3951.7 | 3599.7 |
| Methionine | 13.2 | 475.9 | 457.0 |
| Isoleucine | 43.5 | 2206.8 | 1899.3 |
| Leucine | 43.6 | 3867.8 | 3212.2 |
| Tyrosine | 5.5 | 1195.9 | 603.4 |
| Phenylalanine | 26.0 | 1550.1 | 1284.7 |
| Histidine | 24.12 | 1093.6 | 1049.2 |
| Lysine | 13.4 | 2441.5 | 2064.3 |
| Ammonium chloride | 184.6 | 3753.0 | 4684.6 |
| Arginine | 9.9 | 7009.2 | 5407.7 |
| Total | 614.5 | 80536.4 | 66113.8 |

Unit: mg/100 g

As shown in Table 1, the total aminoacids of the normal hair were 80530.446 mg/100 g, of which the following aminoacids were higher content: glutamic acid was 11974.92 mg/100 g, cystine 10531.550 mg/100 g, serine 7656.535 mg/100 g, arginine 7009.227 mg/100 g.

However, in the hair treated with earthworm protease for 1 h, the total contents of aminoacids decreased to 66113.784mg/100g by 17.9%. Also, glutamic acid decreased about 18.5%, and cystine decreased about 30.5%, and serine decreased about 37.9%, respectively.

As seen in above results, protease extracted from earthworm had an excellent effect on the decrease of hair thickness and the removal of the soils that were not removed by conventional washing the hair surface. Also, the protease of earthworm decreased aminoacids such as glutamic acid and cystine, serine which proved that earthworm protease decomposes keratin protein as was reported in Kwon's research[16].

According to Kim's research[22], protein aminoacid was reported to be 76.4% hair, withdrawal efficiency is assumed to be about 90~95%.

## 3.4 Analysis of proteolytic activities by using methylene blue staining method

We conducted methylene blue dyeing[23] to measure the degree of the protein decomposition in the hair by treating with earth worm protease solution. Kang[23] reported that more damaged hair showed higher dye uptake. The hair with repeated alkaline reagents such as perm or bleaching is known to lose keratin protein and matrix[23]. Although it did not have a big effect on optical density values despite the passage of time in the control group. As shown in Fig. 5, the increase in optical density values was found in the experimental group treated with earthworm protease. The results can be explained by that earthworm protease decomposed keratin protein.

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## 3.5 SEM observation of the hair treated with enzyme

The healthy hairs have the uniform cuticle which never gets loose nor is exfoliated because CMC (cell membrane complex) exists within the hair cuticle[27].

Also, the feel of the hair may be deteriorated because the soils are not removed even after shampooing and they are absorbed among cuticles. There are also cuticle fractions exfoliated or excluded by the stimuli in everyday life.

Therefore, the hair surfaces were observed by using a SEM to investigate the effect of earthworm protease on the scale refining of the hair.

Fig. 6 is SEM observation taken after shampooing the normal hair, and it shows that soils are still attached partially on the surface. The experimental group 1 and 2 of Fig. 7 are the normal hair treated with protease. The various soils absorbed on the hair surface were removed and the scale on the hair surface was cleaned up as shown in the experimental group 2. The earthworm protease both cleaned up the hair surface by decomposing the cuticles and soils getting loose on the hair giving smooth feel of the hair and that does not have an effect on the morphological properties of the hair.

The control groups 1 and 2 of Fig. 7 are the results of the hair teat treated with inactivated earthworm protease after shampooing a woman's in her twenties hair which did not have no chemical treatment.

Rough scales and soils are still on the hair surface treated with inactivated protease, and the scale intervals of hair did not change, and it is similar to the normal hair of Fig. 6. Kwon and others[16] studied the surface performance of the wool and silk treated with protease extracted from earthworm. This enzyme was reported to excel in decomposing protein, and to remove soils and to have an excellent refining effect by being treated to wool and silk. Accordingly, the treatment makes cuticles smooth by removing and decomposing the soils on the hair like on wool and silk.

## 3.6 Changes of the tensile strength in the hair treated with protease

Keratin protein has high strength because it has high physical or chemical resistance[28]. Fig. 8 shows the changes of the tensile strength of the hair treated with earthworm protease.

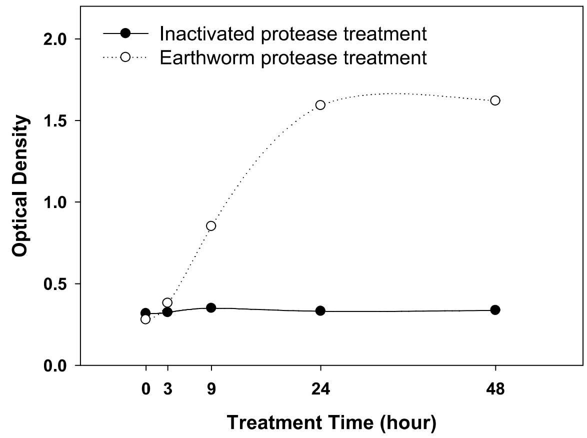


Fig. 5. Measurement of proteolytic activities of protease treated human hair by methylene blue staining method.

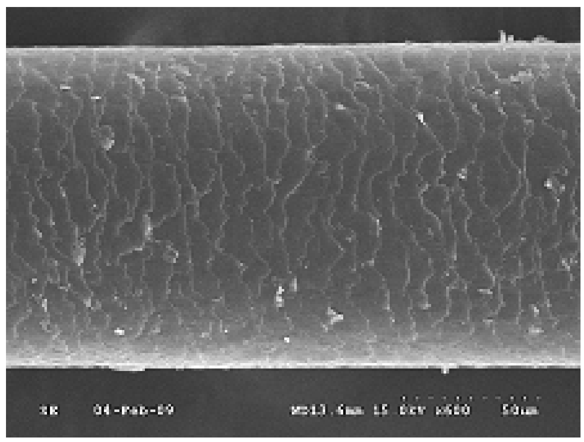

Fig. 6. SEM image observation of normal hair.

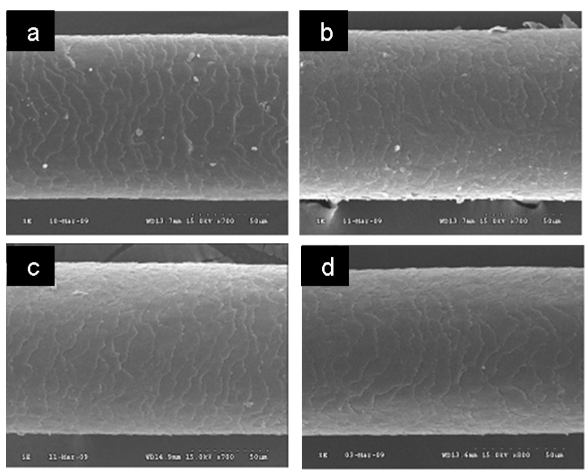


Fig. 7. Hair treated with earthworm protease of the experimental and the control groups.
(a) control group 1, (b) control group 2,
(c) experimental group 1 and (d) experimental group 2.


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As shown in Fig. 8, the tensile strength of the control group was 0.167 kgf, and the tensile strength of the experimental group's hair with enzyme activity decreased down to 0.162 kgf after 3hours' treatment, 0.154 kgf after 9 hours' treatment, 0.146 kgf after 48 hours' treatment. On the contrary, the control group showed little change in the tensile strength as compared to the normal hair.

Similar to the results of Doe[29], are the tensile strength in the experimental group decreased gradually because fiber protein in the hair decomposed keratin protein in the hair by protease treatment. Inactivated enzyme does not have a big effect on the physical change of the hair[19].

Choi[30] reported that about 40% in the tensile strength was lost after treated with permanent wave. This was attributed to the loss of the matrix within the hair. This is the representative damage in hair occurring because alkaline reagent like permanent wave lotion brings about the desquamation or missing of cuticles, along with the loss of matrix within the hair. However, earthworm protease used in this experiment is expected not to have a serious effect on the physical change of hair and is better in removing the soils on the hair surface. From the overall results, it is thought that earthworm protease shows the quality improvement effect by refining the hair cuticles and removing soils on the hair surface, and it could be applied to the scalp, at the enzyme treatment time of about 1 h. Also, it would be possible to get the effect of soil removal and the hair quality improvement in even less than 30 min at higher temperature.

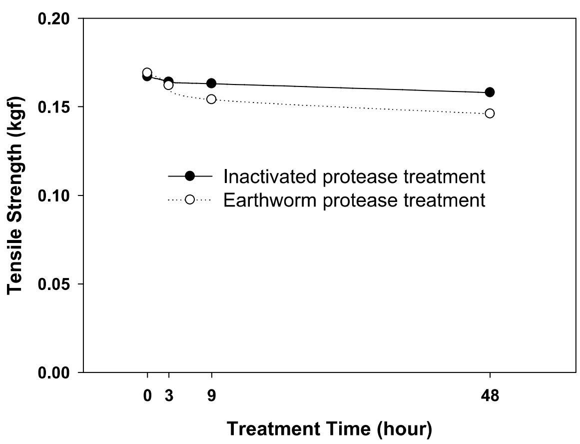

Fig. 8. Tensile strength change by protease treatment.

## 4. Conclusions

The feasibility of earthworm protease as a new cosmetic material for human hair care was investigated. The activated earthworm protease decomposed partially keratin protein of the hair and removed various soils absorbed on the hair after being secreted from the scalp, which was verified by microscopic examination. The thickness of active protease treated samples was decreased compared with that of inactivated protease treated samples due to the loss of aminoacid contents.

Also, the removal of impurities and keratin by protease treatment increased the dye uptake probably because of the improvement of dye accessibility to the hairs via cell membrane complex. However, the tensile strength did not show considerable differences for 48 hours of the protease treatment, between the treated hair samples and the control samples treated with the inactivated protease.

It could be concluded that the earthworm protease has the quality improvement effect by refining the hair cuticles and removing soils on the hair surface. Therfefore, it would be possible to develop the hair care product made from the natural materials rather than the use as the raw material of the conditioner or treatment of the scalp and hair.

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