Application of an Enzymatic Method for Identifying Hair Treatments

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The purpose of this research was investigating the use of an enzymatic method for identifying various treatments, such as permanent waving, hair dyeing and bleaching used on hair from hair samples. Morphologically only negligible difference was observed between untreated hair and hair permed *in vitro*. However, after protease treatment, the degradation of permed hair was accelerated and a significant difference was observed between permed and untreated hair in morphology and degradation extent. The degraded fraction of untreated hair was confined to cuticle region of hair surface, whereas in hair samples permed and dyed *in vitro*, the microfibril protein of hair was degraded. Furthermore, there was a high correlation between the extent of degradation and the hair damage resulting from these chemical treatments. When we tested the method on human hairs from live subjects, no significant difference was observed on untreated hair in different hair sections. However, the tip section of permed and dyed hair showed much higher degradation than that of root section from morphology and degradation extent. Our findings for practical uses revealed that the enzymatic method can be applied to identify the chemical treatment used on hair.

Key words —— hair, chemical treatment, identification, protease, degradation

INTRODUCTION

Hair analysis can provide much information, such as diagnosis of a disease and personal history of drug usage based on the analysis of specific component.[1, 2] However, the accuracy of these hair analyses can be affected by the sampling method. For example, different results can be obtained between the tip and root section of hair sample.[3, 4] Furthermore, it has been known that popular chemical treatments such as permanent waving, dyeing and bleaching affect the analytical results.[5] Therefore, it is important information to known the past chemical treatments on hair samples.

Permanent wave treatment is a two step operation: reduction and subsequent oxidation.[6] Hair dye and bleach treatment is based on a oxidation operation with alkaline hydrogen peroxide.[7] Although these chemical treatments may cause a damage to hair and reduce hair components, high concentration of external chemicals was sometimes detected from enhanced sorption capacity.[5, 8]

Generally a personal history of these chemical treatments on hair can be detected by chemical or physical method as hair damage.[9, 10] For this purpose, FT-IR method was previously reported to detect from hair damage.[11] However, such hair damages from weathering can be detected from the tip section of untreated hair by using this method, it cannot be used to determine the chemical treatment made on hair sample. Although a biochemical method have been reported for detecting the chemical treatment by comparing extracted microfibril protein and matrix protein which existing in cortex,[4] this technique is not practical due to its very complex operations.

Previously we assessed the damage of hair permed *in vitro* with a protease and showed that the hair degradation with protease is closely related to the denaturation of microfibril protein.[12] In addition, our interests paid on searching appropriate protease to detect the cosmetic treatment on hair. This paper deals with the identification of

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the chemical treatment on hair. For this purpose, permed, dyed and bleached hair *in vitro* was treated with protease, then its degradation behavior were investigated. Subsequently, the method was verified with practical cases from live subjects with different chemical treatment.

MATERIALS AND METHODS

Reagents —— 50% ammonium thioglycolate (ATG), DL-Cystein (CYS), *p*-Phenylenediamine (PPD), sodium sulfite, 28% ammonium hydroxide, sodium bromate, 35% hydrogen peroxide used were a reagent grade and a special reagent grade sodium dodecyl sulfate (SDS) were used without further purification. Pronase E was supplied by Sigma (St. Louis, MO, U.S.A., for *Streptomyces griseus*, 12.4 U/mg).

Hair Samples —— Hair samples chemically treated *in vitro*: Virgin hair (73 μm average diameter) from Japanese woman in their 20's who had never had chemical hair treatments was used. Hair samples were soaked in 1.0% SDS aq. (w/v) for 10 min at 25°C, washed with water for 30 min and air dried. In permanent wave treatment, about 1.0 g of the hair was soaked for 15 min in 0.50 M ATG aq. (adjusted pH 8.6 with ammonium hydroxide) or 0.50 M CYS aq. (adjusted pH 8.6 with ammonium hydroxide) using 10 : 1 solution to hair ratio at 30°C. Immediately the hair was soaked for 15 min in 0.40 M sodium bromate aq. at 30°C, using 10 : 1 solution to hair ratio. Then hair was washed with water for 30 min and air dried. This process was repeated to prepare damaged hair samples. In hair dye treatment, about 1.0 g of the hair was soaked for 30 min in the 1 : 2 (v/v) mixture of 2.5% ammonium hydroxide aq. (containing 0.15% PPD and 0.3% sodium sulfite) and 6.0% hydrogen peroxide aq. using 10 : 1 solution to hair ratio at 37°C. Then hair was washed with water for 30 min and air dried. This process was repeated to prepare damaged hair samples. In bleach treatment, about 1.0 g of the hair was soaked for 30 min in 3.0% hydrogen peroxide aq. containing 1.0% ammonium hydroxide using 10 : 1 solution to hair ratio at 37°C. The hair was then washed with water for 30 min and air dried. This process was repeated to prepare damaged hair samples.

Hair samples from live subjects: Hair samples were collected from two Japanese women in their 20's who regularly had permanent wave treatment with CYS every two months and had hair dye treatment every two months. Untreated hair samples were collected from three Japanese women in their 5 olds, 20's and 40's who had never had chemical treatment. These hair samples were soaked in 1.0% SDS aq. (w/v) for 10 min at 25°C, washed with water for 30 min and air dried.

Protease Treatment —— Hair samples were cut to 2.0 cm length and about 100 mg of the hair sample was incubated in 2.0 ml Tris-HCl buffer (20 mM, pH 8.0) containing 0.05% Pronase E at 37°C for 100 hr.[12] After centrifugation at 12000 rpm for 5 min of this solution, the supernatant was used for amino acid analysis. Residual hair was washed with water 4 times. After drying, the residue was weighed and the degree of degradation was calculated as follows:

$$W\ (\%) = [(A-B)/A]\times 100$$

Where W is the percent degradation. A and B are the weights of hair before and after Pronase E treatment, respectively. The value represents an average from triplicate experiments.

Amino Acid Analysis —— Samples were hydrolyzed in 6.0 M HCl at 105°C for 24 hr under a nitrogen atmosphere. After removal of the acid and water in a rotary evaporator at 45°C, the residue was diluted with 20 mM HCl. Then the hydrolyzates were analyzed on a HITACHI (Tokyo, Japan) amino acid analyzer, Model L-8500A equipped with a ninhydrine detector. Half-cystine content was determined as cysteic acid after performic acid treatment.[13] The value represents an average from triplicate experiments.

Scanning Electron Microscopy (SEM) —— Hair samples before and after Pronase E treatments were mounted on stainless steel stubs and sputtering with gold (10 mA, 5 min). Morphology of the hair were examined using a JEOL (Tokyo, Japan) JSM-5200 scanning electron microscope (accelerating voltage: 15 kV, magnifications were 750 and 1000).

RESULTS AND DISCUSSION

Effect of Protease Treatment Time and the Frequency of Hair Treatments from *In Vitro* Hair Samples

To determine the effect of protease treatment time, we measured the extent of degradation against treatment time. A typical degradation profile is

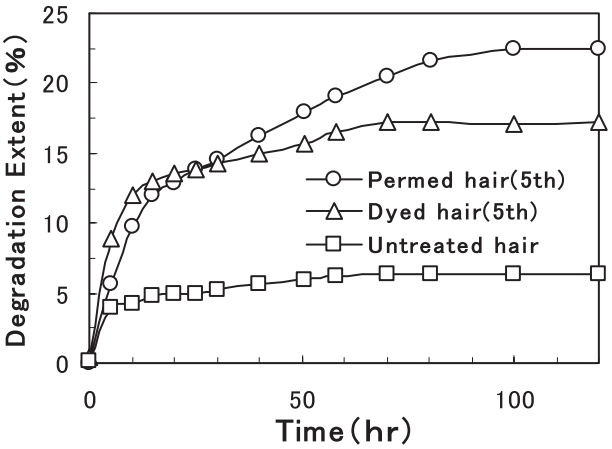

Fig. 1. The Time Course of Degradation of Hair with Pronese E

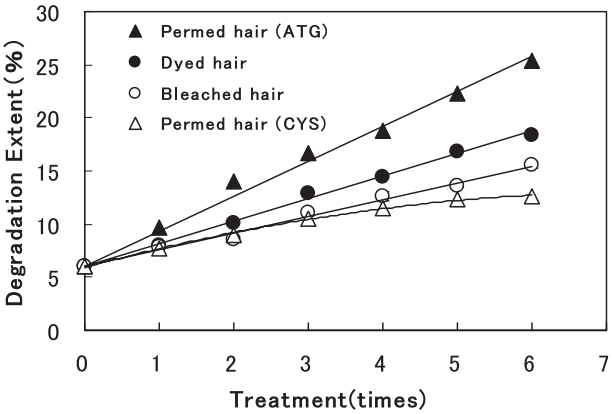

Fig. 2. Correlation between Frequency of Treatments and Degradation Extent
Each value represents the average (n = 3).

shown in Fig. 1.

In untreated hair, the rate of degradation was greatest in the first 5 hr, showing about 4.0% degradation during that time period. Beyond 15 hr, only little degradation was observed. It has been known that a cuticle component of untreated hair is degradable with another protease treatment.[14] Therefore we must presume that the specific region of hair surface was degraded.

On the other hand, degradation of hair permed 5 times with ATG and hair dyed 5 times was exceedingly rapid for the first 10 hr, slowing down thereafter and very little degradation occurred after 80 hr. It has been known that a protease penetrates from cuticle to cortex through the cell membrane complex (CMC) within 50 hr due to its large molecule.[15] Therefore, it appears that the surface region of hair which degrades rapidly whereas the inner region degrades more slowly in permed and dyed hair. From our findings, we set the standard protease treatment time at 100 hr.

The degradation of hair with different frequency of chemical treatment is shown in Fig. 2. Degradation increased linearly with an increase in the frequency of treatments for each chemical treatment except permed hair with CYS. This finding indicates that there is a high correlation between the degree of degradation and hair damage. Previously we reported that the degradation of hair permed *in vitro* accelerated with an increase in the frequency of treatments using Protenase K which has high keratin activity.[12] Additionally we showed that the accelerating factor is closely related to the denaturation of α-helical microfibril protein of hair. Therefore, the increased degradation with Pronase E in

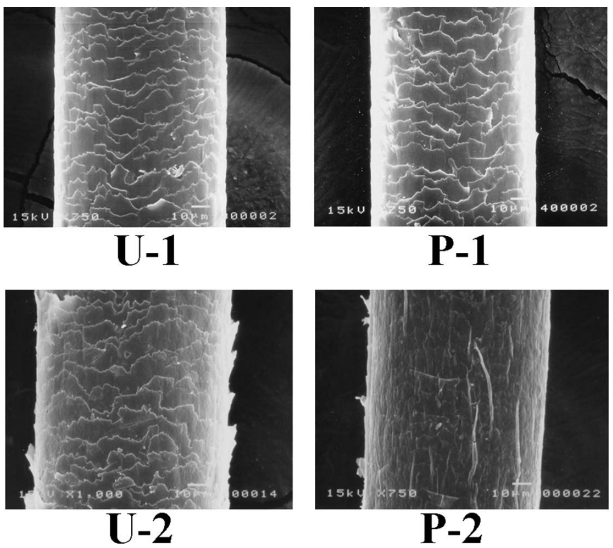

Fig. 3. SEM Photographs of Hair before and after Pronase E treatment
U-1, Untreated hair; P-1, Permed hair (5th); U-2, Untreated hair after pronase E treatment; P-2, Permed hair (5th) after pronase E treatment.

this study also suggests that the effect is closely related to the denaturation of microfibril protein not only in permed hair but also dyed and bleached hair.

The increase of degradation extent of permed hair with ATG treatment is significantly greater than that of CYS treated hair. Karasawa et al. showed that the decrease on α-helical structure of hair is less for CYS perm and greater for ATG perm.[16] Therefore it is possible that the denaturation of microfibril protein is accelerated in permed hair with greater extent in ATG treatment than in CYS treatment.

Surface Morphology of Hair Chemically Treated *In Vitro* and Untreated Hair

Figure 3 shows typical SEM images of hair be-

fore and after protease treatment. Untreated hair (U-1) had clear cuticle edges, after protease treatment (U-2) exhibited cuticle lifting, suggesting the possibility of weakening of the CMC or of the cuticle related protein which participates in adhesion of cuticles.[17)]

On the other hand, hair permed 5 times *in vitro* (P-1) exhibited slightly lifting of cuticle edges at large sections, the difference was negligible between hair P-1 and hair U-1. After protease treatment of hair permed 5 times *in vitro* (P-2) exhibited the axial wrinkles of cortical cells resulting from partial degradation of the cuticle. It has been known that the cuticle layer comprising 10–15% of hair.[18)] From degradation extent of hair P-2 is about 22%, we must presume that the cortex comprising about 80% of hair was degraded.

Although only a small difference was observed between hair parmed in vitro and untreated hair in morphology, it is possible to identify the true damage caused by the chemical treatment with protease.

Amino Acid Composition of Degradable Fractions

The histological components corresponding to the degradable fractions of hair were examined by amino acid analysis. The amino acid compositions obtained in this study were compared with fractions from hair.

As can be seen in Table 1, the degradable fraction of untreated hair had a remarkably low sulfur content with a total amount of half-cystine and cysteic acid of 2.9%. This is consistent with the composition of endo-cuticle A protein from its low sulfur and acidic amino acid content.[19)]

The degradable fraction of hair permed 5 times and dyed 5 times *in vitro* also had low sulfur content where the total amount of half-cystine and cysteic acid were 4.5% and 7.8%, respectively.[20)] From cortex mainly consist of about 60% of microfibril protein with low sulfur content and about 40% of matrix protein with high sulfur content, these two components were very similar to the microfibril protein, and for the helix-favoring residues Glutamic acid and Leucine are more abundant relative to matrix protein.

These results suggest that the degradation gradually advanced from surface to microfibril of hair with increased frequency of chemical treatments and are consistent with the results of the degradation profile, SEM images described above. It has been known that the α-helix in microfibril decreased with permanent wave or dye treatment.[21)] Therefore, our results indicate that the denatured α-helical region from chemical treatment was degraded with protease.

Table 1. Amino Acid Composition of Degradable Fractions and Fractions from Hair[a)]

	Degradable Fractions			Fractions from Hair		
	Untreated hair	Permed hair (5th)	Dyed hair (5th)	Endcuticle A[20)]	Microfibril[4)]	Matrix[4)]
Aspartic acid	11.2	8.5	9.2	9.5	8.3	2.9
Threonine	8.1	7.4	8.1	5.5	6.9	10.7
Serine	9.3	10.9	10.3	8.4	8.0	12.0
Glutamic acid	10.1	13.3	12.1	13.4	17.2	8.4
Proline	5.0	6.0	5.8	6.0	5.1	12.3
Glycine	11.5	8.4	8.5	8.0	5.2	6.2
Alanine	9.1	7.4	2.4	8.1	6.3	2.0
Half-cystine +Cysteic acid	2.9	4.5	7.8	1.8	9.0	23.5
Valine	7.2	7.3	7.9	7.0	6.0	5.5
Methionine	0.5	0.7	0.3	2.3	1.1	0.0
Isoleucine	3.5	3.9	3.9	4.2	3.6	2.0
Leucine	7.0	8.1	7.8	9.1	9.2	3.3
Tyrosine	3.1	1.7	3.3	3.3	2.5	1.6
Phenylalanine	2.9	2.6	2.9	3.1	2.0	1.4
Lysine	3.5	3.3	3.8	4.3	3.5	0.6
Histidine	1.2	0.9	1.1	1.1	0.7	1.0
Arginine	4.0	5.1	4.8	4.9	5.4	6.6

[a)] Expressed as residues per 100 residues.

Degradation Study Using Hair Samples from Live Subjects

The results of protease degradation study using hair samples taken from live subjects are shown in Fig. 4. The data from dyed hair of 20's female, the degradation showed an increase from root section to tip section and the rate of the increase was greater compared to other hair samples. Additionally, the tip section of dyed hair showed extremely higher degradation than that of root section indicated that the excessive denaturation of hair protein induced.

In permed hair of 20's female with CYS treatment, the degradation also increased from root section to tip section. However, the increase was smaller than that of dyed hair, it is considered that the denaturation of hair protein may not advanced due to the weak denaturing efficiency of CYS.[16)]

In untreated hair of 20's female, the predominant degradation was not observed with different hair sections. Furthermore, in the case of another two untreated hair samples from a five year old child and a 40 year old female, the predominant degradation was also not observed. A damage can be induced even in untreated hair from washing, friction of brushing, heat drying, irradiation of sunlight. As a result, the damage can be detected at the tip section of untreated hair using conventional chemical and physical method.[8)] However, these types of damage do not appear to denature the microfibril protein.[4)] Therefore in our experiments, the protease degradation did not take place.

Figure 5 shows typical SEM images of 3 indi-

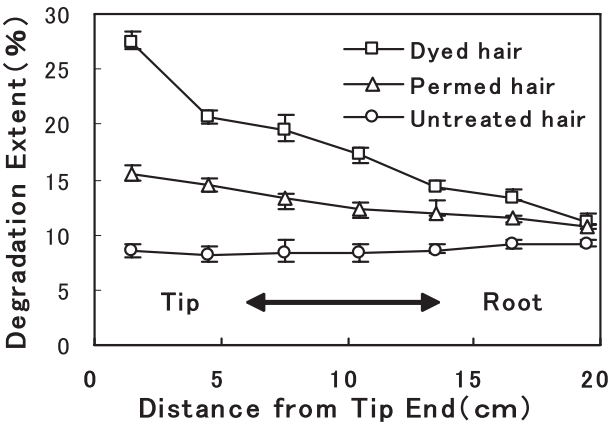

Fig. 4. Degradation Extent from Tip to Root Section
Each value represents the means ± S.D. (n = 3).

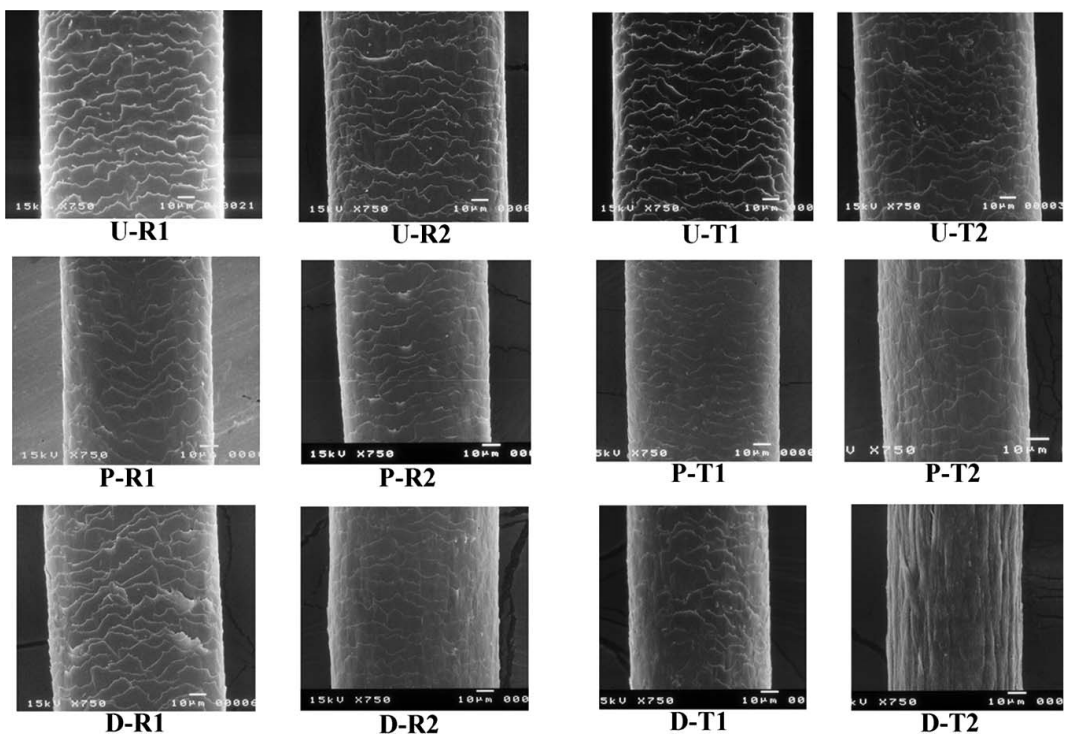

Fig. 5. SEM Photographs of Hair before and after Pronase E treatment of Different Individuals

U-R1, Root of untreated hair; U-R2, Root of untreated hair after pronase E treatment; U-T1, Tip of untreated hair; U-T2, Tip of untreated hair after pronase E treatment; P-R1, Root of permed hair; P-R2, Root of permed hair after pronase E treatment; P-T1, Tip of permed hair; P-T2, Tip of permed hair after pronase E treatment; D-R1, Root of dyed hair; D-R2, Root of dyed hair after pronase E treatment; D-T1, Tip of dyed hair; D-T2, Tip of dyed hair after pronase E treatment.

vidual hair samples with different chemical treatment described above.

Untreated hair: The root section of the hair (U-R1) had clear cuticle edges, after protease treatment (U-R2) exhibited slightly smooth cuticle edges. On the other hand, the tip section of the hair (U-T1) had also clear cuticle edges, after protease treatment (U-T2) exhibited slightly smooth surface. However, only negligible difference was observed between the root section U-R2 and the tip section U-T2 after protease treatment.

Permed hair: The root section of the hair (P-R1) had slightly unclear cuticle edges, after protease treatment (P-R2) exhibited slightly smooth cuticle edges. On the other hand, the tip section of the hair (P-T1) had little unclear cuticle edges, after protease treatment (P-T2) exhibited irregular shape of cuticle edges resulting from the degradation and axial wrinkle like appearance was observed. Thus, it is clear from our protease study that the degradation in permed hair P-T2 was greater than the untreated hair U-T2.

Dyed hair: The root section of the hair (D-R1) exhibited partial cuticle lifting, after protease treatment (D-R2) exhibited smooth and unclear cuticle edges resulting from degradation of the cuticle surface. On the other hand, the tip section of the hair (D-T1) showed irregular shape of cuticle edges due to cuticle shaving, after protease treatment (D-T2) exhibited many axial gap of cortical cells resulting from degradation of the entire cuticle.

From SEM study, it is clear that the protease degradation advanced with increase of the chemical damage in hair. Though, only a small difference in morphology can be observed among untreated hair and perm, dyed hair, it is possible to observe the true damage by using our protease treatment method in chemical treatment of the hair. Especially, it became clear that the difference was remarkable in the tip section of hair.

In conclusion, a new method for identifying chemical treatment on hair was investigated by using an enzymatic method. Using this method, we identified the damage resulting from permanent wave and dye treatment on hair samples from live subjects. The identification of chemical treatment on hair samples provides useful information. Hereafter, it is necessary to study furthermore the effect of the protease in different chemically treated hair. The method does not need an expensive apparatus, is simple and chemical treatment on hair can be identified both visually and through gravimetric analysis. The following findings are obtained in this study: there is a high correlation between the extents of degradation with damage of hair cosmetically treated *in vitro*; from degradation profile, SEM appearances and amino acid analysis, it was found that the degradation gradually advanced from surface to inner hair fiber with increase in hair damage due to chemical treatment; the α-helical microfibril protein appears to gradually degrade with an increase in the frequency of chemical treatment. Therefore, it was found that the denatured α-helical region was degraded with protease; and in practical cases from live subjects, the predominant difference was not observed on untreated hair with different hair section, the tip section of the chemically treated hair showed much higher degradation than that of root section.

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