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Analysis of the damaged components of permed hair using biochemical technique

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Synopsis

We have developed a new method of fractionating hair components in order to analyze the damaged components and the degree of damage due to perming. We found that the amount of constituent proteins extracted by an anionic surfactant with reductant was influenced by the concentration of the reductant. Using this method, the matrix and the microfibril protein could be easily separated and quantified. Applying this method to the analysis of individual hairs, we found a significant decrease in the "intact" microfibril protein on the tip end of permed hair.

INTRODUCTION

Many cosmetic investigators are studying ways to evaluate the degree of hair damage or to develop anti-damaging products, but hair is a complex organization and the damage can first be seen at the hair's tip, which may have been on the head 2–3 years and damaged by cumulative cosmetic behavior. Therefore, efforts to confirm the correlation between damage and cause are very difficult. It has been reported that hair is damaged by various causes such as sunlight (1), grooming (shampooing, drying, brushing, and combing) (2), and cosmetic treatments (perming, dyeing, and bleaching) (3). Robbins concluded that the cause of hair damage is the sum of all grooming practices (rubbing, stretching, and washing), while sunlight or chemical processing treatments make the hair more susceptible to such damaging actions (4).

However, these studies mainly dealt with mechanical properties or morphological changes such as decreasing cuticle layers, scale lift, and split ends, examined by electron microscopy. A few investigators have studied the alteration of the total amino acids or lipid compositions (5,6).

The proteins of hair constitute 80% or more of the total mass, but studying these proteins is difficult because of their insolubility due to the disulfide-bonded polymeric structure. Consequently, a few investigations were reported (7,8), but it is still unknown

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how the hair components, especially proteinous molecules, change due to perming. Generally, the hair proteins are extracted by reductants and denaturing agents such as urea or guanidine. Further isolation of the main classes of hair proteins (the matrix and the microfibril) requires an isoelectric precipitation step or electrophoretic method (9). This method is not of practical use for the analysis of individual hairs because it requires many hairs and is a complicated method.

Thus, we attempted to develop a simple and easy extraction method for quantifying hair components using a reductant with an anionic surfactant. The goal of this study is the analysis of damage to an individual hair due to perming.

MATERIALS AND METHODS

CHEMICALS

Analytical grade 2-mercaptoethanol (2-ME), dithiothreitol (DTT), tricine, and SDS were supplied by Nacalai Tesque, Inc. (Kyoto, Japan). 4-Bromomethyl-7-methoxycoumarin was purchased from Dojindo Laboratories, Inc. (Kumamoto, Japan). γ-Glutamyl-ε-lysine (isopeptide), pronase, leu-aminopeptidase, prolidase, and carboxypeptidases were purchased from the Sigma Chemical Co. (St. Louis, MO).

HAIRS

For the development of the methods, we collected the root end hair from ten Japanese women who had not been exposed to chemical treatments such as perming or dyeing. Hair was washed using 1% (w/w) SDS and then thoroughly rinsed with tap water. The hair was dried, and external lipids were extracted for 16 hours at room temperature in a 100-fold solution of chloroform/methanol (2:1 v/v). The delipidized hair was cut into 10-mm sections. For the analysis of an individual hair, we collected 100 fibers of permed hair from two Japanese women who had permed every two or three months. One hundred hairs that had not been exposed to cosmetic treatments such as perming or dyeing were also collected from one Japanese woman. The hair was cut into 100-mm lengths from the root end to the tip end and then treated as described.

METHODS

Extraction of hair proteins. The hair proteins were extracted using an anionic surfactant with reductant. In detail, a 10-mg sample of delipidized hair was immersed in 1 ml of 25 mM Tris-HCl buffer (pH 8.3) that contained 1% SDS and various concentrations of 2-ME. The protein was extracted for three days at 50°C. The extracted protein was analyzed using Tricine-SDS-PAGE (10).

Fractionation of hair components. The hair components were fractionated according to the scheme illustrated in Figure 1. A 10-mg sample of delipidized hair was immersed in a 1-ml solution of 25 mM Tris-HCl buffer (pH 8.3) that contained 1% SDS and 2 M 2-ME. The matrix protein was extracted for three days at 50°C. The hair was washed with 25 mM Tris-HCl buffer, then immersed in a 1-ml solution of 25 mM Tris-HCl buffer (pH 8.3) that contained 1% SDS and 0.4 M 2-ME. The microfibril protein was

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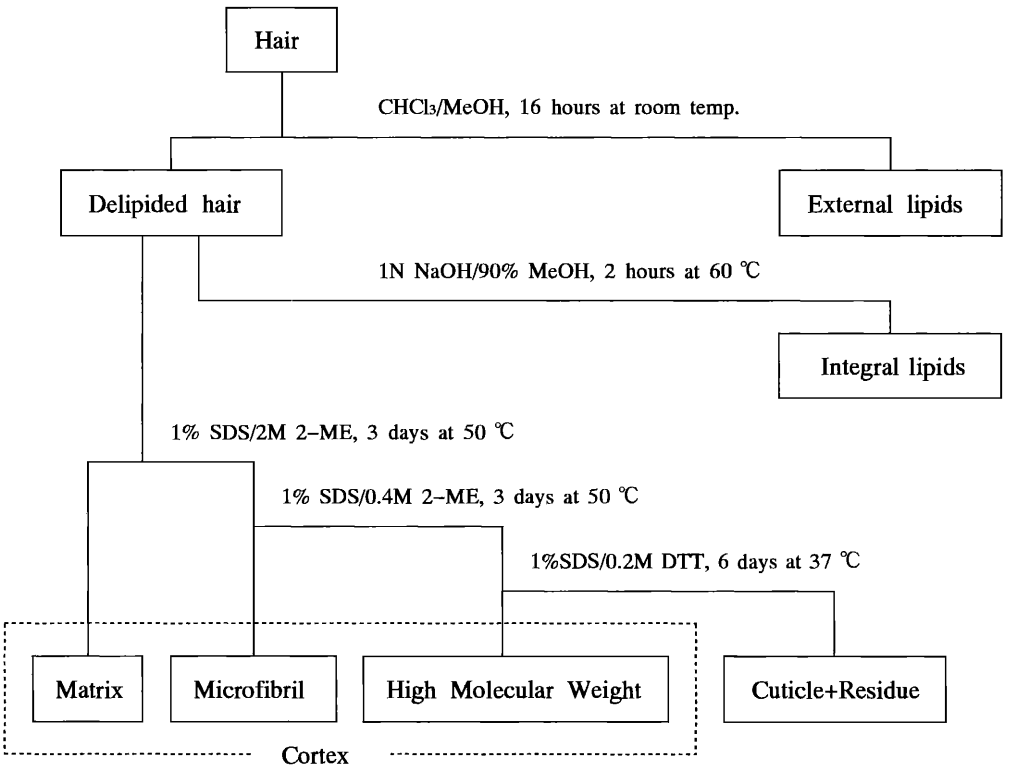

Figure 1. Scheme for fractionation of hair components. A 10-mg hair was used in this work, with liquor ratio at 100. See Materials and Methods for details.

extracted for three days at 50°C. The residue was removed and washed with water, then lyophilized and weighed. Furthermore, the residue was extracted with a 1-ml solution of 25 mM Tris-HCl buffer (pH 8.3) that contained 1% SDS and 0.2 M DTT for six days at 37°C to extract the high-molecular-weight protein. After washing and lyophilization, the residue was weighed again. The loss in weight was regarded as the high-molecular-weight protein. The residue included the cuticle. The amounts of the matrix and microfibril protein were determined using the Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA) and compared with a protein calibration curve of BSA in the extraction buffer.

Characterization of the extracted fractions. Each extracted fraction was confirmed by amino acid composition and molecular weight. The molecular weight of the extracted protein was determined using Tricine-SDS-PAGE (10). The amino acid analysis was performed according to the Pico-Tag® method supplied by Millipore Co. (Milford, MA). The half-cystine content was estimated as cysteic acid converted by performic acid.

Determination of the isopeptide (IP). This method was previously published by Adamski (11). Isopeptide was determined by amino acid analysis after successive peptidase digestion.

Determination of 18-methyl-eicosanoic acid (MEA). 18-Methyl-eicosanoic acid, which exists only in the cuticle, was analyzed by the method of Wertz (12) with slight modification. Briefly, 10 mg of delipidized hair was hydrolyzed with 1 N sodium hydroxide/90%

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methanol at 60°C for two hours. The fatty acids liberated with solvent were analyzed by HPLC after being labeled with 4-bromomethyl-7-methoxy-coumarin (13).

Analysis of hair from individuals. Hair components were fractionated according to the scheme illustrated in Figure 1 using 10 mg of hair from each of three women. The contents of fractionated components were determined. IP in the residue fraction and the contents of half-cystine and cysteic acid in each fraction were also determined using amino acid analysis. MEA was determined using another 10 mg of hair.

Scanning electron microscopy (SEM). Morphological changes were examined using a Hitachi-S520 scanning electron microscope after sputtering with gold.

RESULTS AND DISCUSSION

FRACTIONATION OF HAIR COMPONENTS

The main classes of hair proteins can be discriminated by their molecular weight. The molecular weight of the matrix protein is in the range from 10 to 30 kDa, and that of the microfibril protein is 45–55 kDa (9). During the extraction of hair constituent protein using an anionic surfactant with reductant, we found that the amounts of extracted proteins were influenced by the concentration of reductant. Figure 2 shows the effect of the concentration of 2-ME on the extraction of the cortical protein. The extraction was maximized in a range between 0.4 M and 0.8 M 2-ME. When the 2-ME concentration was higher, the extracted amount of the microfibril protein was significantly decreased, and it could not be extracted at the 2M 2-ME level. However, that of the matrix protein did not change very much. Therefore, only the matrix protein was first extracted using 2M 2-ME, and then the microfibril protein could be extracted using 0.4 M 2-ME after the matrix protein had been extracted. The mass of these cortical proteins was about 70% of the hair. We could also extract a small additional amount of protein using DTT, a stronger reductant than 2-ME. The molecular weight of this

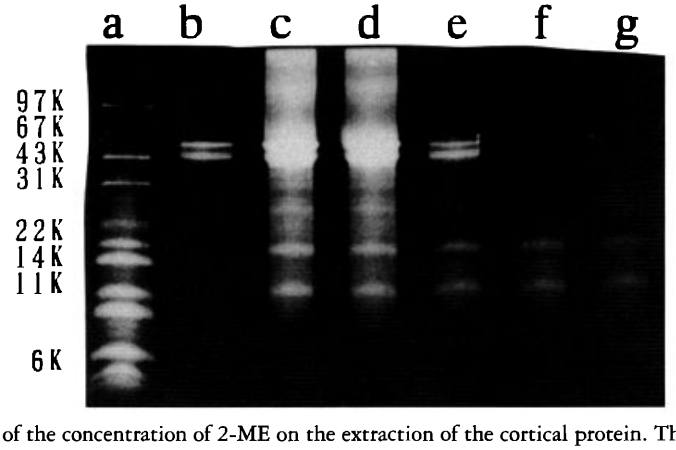

Figure 2. Effect of the concentration of 2-ME on the extraction of the cortical protein. The cortical protein was extracted for three days at 50°C in 25 mM Tris-HCl buffer (pH 8.3) with 1% SDS and 2-ME. 2-ME concentrations were (b) 0.2 M, (c) 0.4 M, (d) 0.8 M, (e) 1.2 M, (f) 1.5 M, and (g) 2.0 M, respectively. Aliquots were submitted to Tricine-SDS-PAGE. (a) Molecular weight standards.

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protein is more than 100 kDa (Figure 3), and so we called this protein the *high-molecular-weight protein (HMW).*

We confirmed the purity and identity of extracted fractions not only by molecular weight but also by amino acid composition. The amino acid composition of each fraction from the root end hair is summarized in Table I. The extracted protein using 2M 2-ME was in good agreement with the matrix protein, and the 0.4 M extract was the microfibril protein when prepared by reduction following alkylation (9). The amino acid composition of HMW, which increased toward the tip end, as discussed later, was similar to that of microfibril protein. The residue was almost the same as the amino acid composition of the cuticle prepared by physical isolation (14,15) in the root end. Figure 3 shows the Tricine-SDS-PAGE of each fraction except the residue. The molecular weight of each fraction was also confirmed, and they showed good purity.

When viewed by SEM, the hair from which only matrix protein had been extracted was shriveled but still had a fibrous shape, as seen in Figure 4a. On the other hand, when both the matrix and the microfibril protein were extracted, the hair was no longer fibrous and only the cuticle layers remained just like the sheath (Figure 4b). Consequently, we have established a method of fractionating the hair components, as shown in Figure 1.

As we have previously described (16), we could not separately extract the matrix and the microfibril protein when the reductant is thioglycolic acid (TGA), and the extracting efficiency is not enough when the surfactant has the properties of low denaturation. We considered that the microfibril protein requires a strong denaturing surfactant for extraction since it is a tightly packed structure. Both 2-ME and DTT have alcoholic OH in their molecules, and the denaturing efficiency of the surfactant is weakened if the concentration of these reductants is too high. This is the reason why only the matrix protein was extracted using the concentrated reductant. In fact, the mild surfactants such as sodium dodecyltri (oxyethylene) sulfate could not extract the microfibril protein (16). This is a unique method of isolating the main classes of hair proteins (the matrix and the microfibril). It is an easy, high-yield method that does not need to be modified. In this

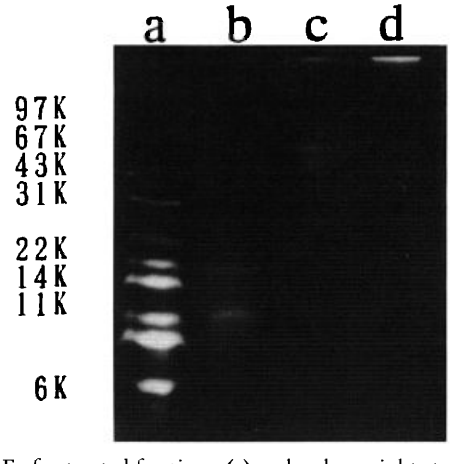

Figure 3. Tricine-SDS-PAGE of extracted fractions: (a) molecular weight standards; (b) 2 M 2-ME extract; (c) 0.4 M 2-ME extract after (b); (d) 0.2 M DTT extract after (c). Refer to Figure 1 for detail.

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Table I
Amino Acid Compositions of Extracted Fractions From the Root End[a]

	Determined in this study				Literature		
	2 M extract	0.4 M extract	HMW[b]	Residue	Matrix[c]	Microfibril[c]	Cuticle[d]
Lysine	0.6	3.5	2.0	3.1	0.6	3.5	3.4
Histidine	1.0	0.7	0.5	1.2	0.9	0.7	0.5
Arginine	6.6	5.4	7.0	4.6	5.4	7.1	2.6
Half-cystine[e]	23.5	9.0	10.1	19.1	27.2	7.6	18.1
Aspartic acid	2.9	8.3	5.9	3.1	2.5	9.3	3.2
Threonine	10.7	6.9	7.9	6.2	10.3	5.4	4.6
Serine	12.0	8.0	11.8	12.6	11.9	8.9	16.1
Glutamic acid	8.4	17.2	12.4	6.4	8.4	16.5	8.9
Proline	12.3	5.1	9.1	7.5	12.7	3.8	10.5
Glycine	6.2	5.2	6.3	11.4	6.1	5.1	8.8
Alanine	2.0	6.3	5.3	4.6	2.3	6.9	5.4
Valine	5.5	6.0	7.0	6.7	5.2	6.1	7.3
Methionine	0.0	1.1	0.2	0.9	0.0	0.4	0.5
Isoleucine	2.0	3.6	3.4	3.0	1.8	3.6	2.2
Leucine	3.3	9.2	7.1	5.5	2.2	10.2	4.5
Tyrosine	1.6	2.5	2.7	2.4	1.5	2.5	2.1
Phenylalanine	1.4	2.0	1.8	1.9	1.1	1.9	1.2

All values are the mean of three experiments. Refer to Figure 1 for experimental conditions.
[a] Expressed as residues per 100 residues.
[b] High-molecular-weight protein from the tip end of permed hair.
[c] Reduction following alkylation and isoelectrical precipitation method (9).
[d] Physical isolation method (15).
[e] Estimated as cysteic acid converted by performic acid.

study, we applied this method to the analysis of hair components, but it could also be applied to the reconstitution of the intermediate filament because the disulfide bond easily reforms using only dialysis.

ANALYSIS OF THE DAMAGED COMPONENTS OF PERMED HAIR

The cortical proteins. We applied the developed method to the analysis of hairs from individual persons. The hair from three women, labeled "no treatment," was the hair that had not been exposed to any cosmetic treatment, and Type I and Type II were the hairs that had been permed every two or three months. More than 80% of the Type I hair had split ends, while the Type II hair scarcely had any split ends but contained broken hairs. The SEM micrographs of the tip ends of these hairs are shown in Figure 5. Table II shows the contents of each fraction, from the root end to the tip end. The data are the mean of three examinations. On the root end, the compositions of three hairs were very similar. The "no treatment" hair showed almost the same composition toward the tip end, but the permed hairs apparently changed. The microfibril protein significantly decreased, while the high-molecular-weight protein and the residue increased. On the other hand, the matrix protein slowly decreased whether or not the hair had been permed. As shown later, the cuticle layers actually decreased, and the increase in residue means an increase in the insoluble proteins (9). It is reported that alkaline pH will form irregular cross-linking such as lanthionine (17). We considered that the "intact" microfibril protein had partially turned into the insoluble protein and decreased.

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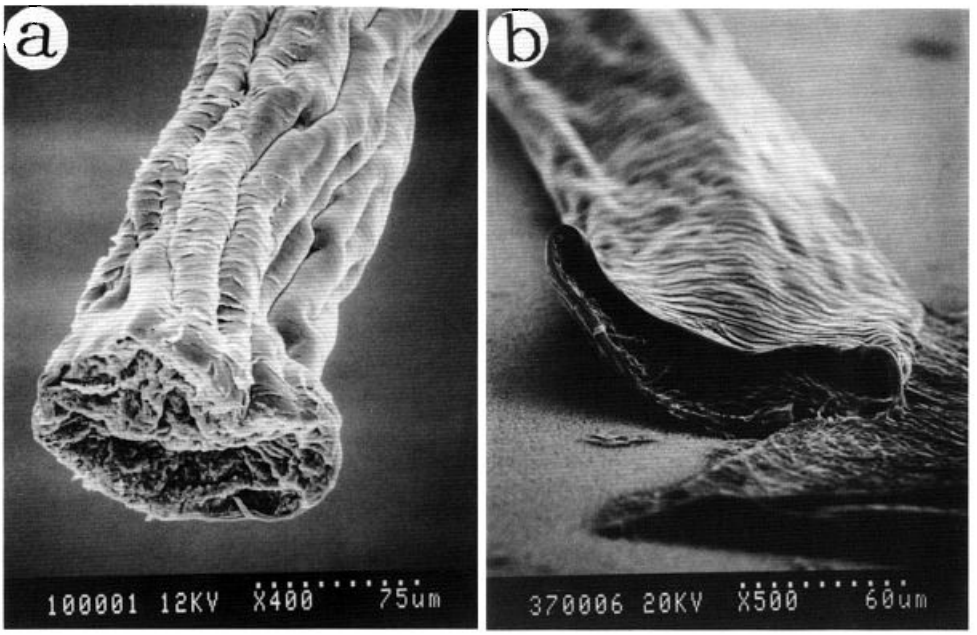

Figure 4. Scanning electron micrographs of the hairs after extraction: (a) 2 M 2-ME extraction and (b) successive extraction by 0.4 M 2-ME following (a). Refer to Figure 1 for detail.

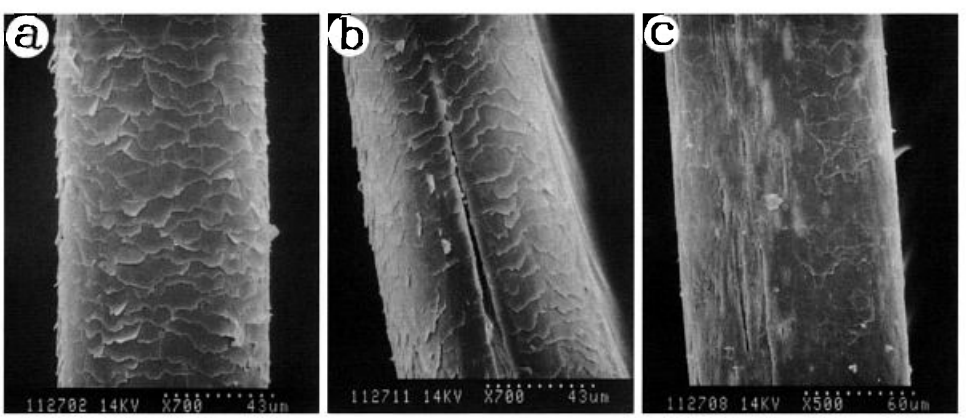

Figure 5. Scanning electron micrographs of the tip end of individual hairs: (a) "No treatment," (b) Type I, and (c) Type II. "No treatment" means the hair has not been exposed to any chemical treatment. "Type I" and "Type II" mean the hair has been permed every two or three months.

The cuticle. The abrasion of the cuticle layers by brushing, combing, etc., has been reported in the literature. We developed a quantitative analysis method using the determination of the IP, which exists only in the exo- and endo-cuticles (18), and the MEA, which exists only in the cell membrane complex (CMC) of the cuticle (19), as indicators. A summary of the results is given in Table III. The IP amount decreased toward the tip end similarly in all three hair types, while perming slightly accelerated the damage of the cuticular proteins. On the other hand, the amount of MEA decreased faster in Type II hair than in other hair types. When viewed using the SEM, the cuticle

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Table II
Changes in the Contents of Each Fraction Toward the Tip End[a]

Hair type	Length (cm)	Matrix	Microfibril	HMW	Residue + cuticle
No treatment	0–10	28.2 ± 2.3[b]	43.4 ± 3.3	6.9 ± 1.3	21.5 ± 1.7
	10–20	26.1 ± 2.0	46.0 ± 2.8	6.6 ± 0.7	21.4 ± 1.6
	20–30	24.6 ± 1.4	45.9 ± 3.1	7.9 ± 0.5	21.6 ± 2.2
	30–40	22.6 ± 2.6	43.6 ± 4.4	8.3 ± 2.3	25.5 ± 2.9
Type I	0–10	26.1 ± 0.9	44.7 ± 3.0	6.5 ± 1.9	22.7 ± 2.0
	10–20	23.6 ± 1.3	42.4 ± 5.3	8.5 ± 2.0	25.5 ± 1.4
	20–30	23.9 ± 2.3	33.3 ± 3.3	12.3 ± 3.3	30.5 ± 1.7
	30–40	22.4 ± 1.1	24.0 ± 4.2	18.1 ± 3.9	35.5 ± 3.4
Type II	0–10	25.7 ± 1.2	46.3 ± 3.9	9.7 ± 1.9	18.3 ± 1.8
	10–20	22.9 ± 2.3	45.8 ± 2.8	10.4 ± 2.4	21.0 ± 1.4
	20–30	23.4 ± 2.5	39.2 ± 3.3	13.9 ± 3.1	23.4 ± 2.6
	30–40	21.6 ± 3.3	26.9 ± 5.3	8.2 ± 0.3	33.3 ± 2.5

All values are the mean of three experiments. Refer to Figure 1 for experimental conditions.
[a] Expressed as w/w% of the total recovery.
[b] ± SD.

Table III
Changes in the Amount of IP and MEA Toward the Tip End

Hair type	Length (cm)	IP[a]	MEA[b]
No treatment	0–10	1.1 ± 0.18[c]	56 ± 3.9
	10–20	1.0 ± 0.12	42 ± 4.5
	20–30	1.0 ± 0.12	38 ± 4.4
	30–40	0.9 ± 0.23	36 ± 5.6
Type I	0–10	1.2 ± 0.02	52 ± 1.9
	10–20	0.8 ± 0.14	42 ± 3.3
	20–30	0.7 ± 0.19	41 ± 5.2
	30–40	0.4 ± 0.15	31 ± 3.4
Type II	0–10	0.7 ± 0.22	56 ± 3.8
	10–20	0.7 ± 0.13	29 ± 3.3
	20–30	0.5 ± 0.19	17 ± 1.7
	30–40	0.2 ± 0.19	12 ± 2.2

All values are the mean of three experiments. Refer to Materials and Methods for experimental conditions.
[a] Isopeptide is expressed as residues/1000 amino acids of hair.
[b] 18-methyl-eicosanoic acid is expressed as micrograms/100 mg hair.
[c] ± SD.

layers of the Type II hair were significantly abraded (Figure 5c). It was reported that the CMC plays a role in intercellular adhesion. The split-end hair was not so damaged on the CMC, and the cuticle layers may remain (Figure 5b).

The oxidation of each component. Cysteic acid is the major product of cystine oxidation due to various causes, and many investigators have studied the changes in cysteic acid content. Our method permitted the analysis of the oxidation of each component. Tables IV and V show the decrease in cystine and the increase in cysteic acid. It has been

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Table IV
Changes in the Half-Cystine Contents of Each Fraction Toward the Tip End[a]

Hair type	Length (cm)	Matrix	Microfibril	Residue + cuticle
No treatment	0–10	25.0 ± 4.5[b]	9.2 ± 3.2	16.4 ± 3.3
	10–20	23.6 ± 2.9	9.2 ± 2.9	15.6 ± 4.2
	20–30	22.4 ± 3.1	10.0 ± 3.0	15.2 ± 5.8
	30–40	22.4 ± 3.3	9.7 ± 1.8	15.1 ± 4.9
Type I	0–10	25.4 ± 5.5	10.8 ± 2.3	15.8 ± 3.8
	10–20	22.4 ± 5.5	7.2 ± 4.5	15.5 ± 2.9
	20–30	21.2 ± 6.0	6.6 ± 5.1	12.7 ± 5.3
	30–40	20.1 ± 2.5	6.0 ± 6.2	10.0 ± 4.7
Type II	0–10	20.2 ± 4.9	11.9 ± 3.7	19.5 ± 4.1
	10–20	19.8 ± 4.1	11.6 ± 2.7	16.8 ± 4.4
	20–30	18.9 ± 3.9	11.4 ± 4.2	15.8 ± 6.7
	30–40	18.9 ± 3.6	11.5 ± 5.7	10.4 ± 4.6

All values are the mean of three experiments. Refer to Figure 1 for experimental conditions.
[a] Estimated as cysteic acid and expressed as residues/100 amino acids.
[b] ± SD.

reported that the disulfide bond reformation with peroxide following perming is not complete and that the cysteic acid content in the permed hair is higher than that in untreated hair. Our results showed similar concerns with the Type I hair, but the Type II hair showed an interesting result: although the residue (including the cuticle) was also oxidized just as in Type I, the cortical protein was not oxidized like the untreated hair. In other words, in the split-end hair, not only the cuticle but also the cortical proteins were oxidized.

Table V
Changes in the Cysteic Acid Contents of Each Fraction Toward the Tip End[a]

Hair type	Length (cm)	Matrix	Microfibril	Residue + cuticle
No treatment	0–10	3.7 ± 0.3[b]	2.5 ± 0.1	4.2 ± 0.3
	10–20	4.5 ± 0.4	3.0 ± 0.9	4.3 ± 0.6
	20–30	5.3 ± 1.1	3.3 ± 0.6	4.9 ± 0.6
	30–40	7.2 ± 0.9	4.6 ± 0.5	6.3 ± 1.0
Type I	0–10	3.2 ± 1.2	2.6 ± 1.1	4.3 ± 0.8
	10–20	8.4 ± 2.1	6.7 ± 1.3	7.5 ± 1.2
	20–30	16.3 ± 1.3	12.3 ± 0.8	11.7 ± 0.7
	30–40	17.5 ± 0.9	14.5 ± 2.4	13.1 ± 3.4
Type II	0–10	1.6 ± 1.5	0.2 ± 0.2	4.7 ± 2.4
	10–20	2.7 ± 0.7	1.4 ± 0.8	8.8 ± 1.3
	20–30	4.5 ± 0.6	2.4 ± 0.3	12.4 ± 4.4
	30–40	6.3 ± 2.7	2.8 ± 1.1	13.0 ± 2.9

All values are the mean of three experiments. Refer to Figure 1 for experimental conditions.
[a] Expressed as residues/1000 amino acids.
[b] ± SD.

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CONCLUSION

We have developed a novel method for quantifying hair components. This method permits the detailed analysis of components damaged due to perming. In summary, we have found a significant decrease in microfibril protein and an increase in high-molecular-weight protein on the tip end of permed hair. We suggest that the "intact" microfibril protein turns into high-molecular-weight protein due to perming. Moreover, we have found that the cuticle was not so damaged, though the cortical proteins were oxidized in the split-end hair. Using this method, additional work is currently being undertaken in order to ascertain the correlation between the morphological changes and the degree of damage.

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