Raman Spectroscopic Analysis of L-Phenylalanine and Hydrolyzed Eggwhite Protein Penetration into Keratin Fibers

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ABSTRACT: To investigate the penetration of amino acids and hydrolyzed protein derived from eggwhites (HEWP) into keratin fibers, the structure of cross-sectional samples at various depths of virgin white human hair treated with L-phenylalanine (Phe) and bleached black human hair treated with HEWP was directly analyzed without isolating the cuticle and cortex using Raman spectroscopy. The hydrophobic amino acids clearly penetrated into the virgin white human hair, while hydrophilic amino acids did not penetrate at all. The Phe hydrophobic amino acid content at various depths of the virgin white hair increased by performing the Phe treatment (at 50°C for 16 h), indicating that Phe deeply penetrated into the virgin human hair. Also, the disulfide (—SS—) and random coil contents at various depths of the bleached human hair increased by performing the HEWP treatment, indicating that HEWP also deeply penetrated into the bleached human hair. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 122: 2680–2689, 2011

Key words: hair keratin fibers; penetration; Raman spectroscopy; amino acid; hydrolyzed protein

INTRODUCTION

Hydrolyzed proteins, which are derived from eggwhites, keratin, silk fibroin, soybean, and wheat are widely used as an effective ingredient in hair care products, such as shampoo and conditioner to improve the moisturizing effect, cuticle lift resistance, as well as contributing to an increase in tensile strength and waving efficiency. Amino acids are also widely used as essential components in hair care product. Therefore, it is important to investigate how amino acids, peptides, and hydrolyzed proteins diffuse into keratin fibers and how the structural stability of keratin fibers is influenced. However, studies on the penetration of hydrolyzed proteins and amino acids into keratin fibers are still lacking comprehensiveness. Especially, it is difficult to detect whether amino acids penetrated into keratin fibers since amino acids are an essential element of keratin fibers.

Swift[1] evaluated the penetration of hydrolyzed wheat proteins into human hair by observing the cross-sectional hair samples treated with fluorescein isothiocyanate (FITC)-labeled hydrolyzed wheat protein with a confocal laser scanning fluorescence microscope. The FITC-labeled hydrolyzed protein penetrates deeper than the untreated hydrolyzed protein into human hair, because the FITC labeled hydrolyzed protein has a higher hydrophobicity than the untreated hydrolyzed protein. Thus, this method cannot obtain accurate information about the penetration of untreated hydrolyzed protein into human hair.

The direct characterization of keratin fibers has been performed using X-ray diffraction,[2–8] solid-state NMR,[4,9] Raman,[10–25] and infrared spectroscopy.[10,12,13] Information about the amorphous region (the cuticle and matrix) of keratin fibers cannot be obtained from X-ray diffraction, since the information obtained from X-ray diffraction only reflects the state of the high crystalline structure in keratin fibers. Solid-state NMR has a low sensitivity, uses a lot of sample volumes, and it is not possible to obtain separate information about the cuticle and cortex structure of keratin fibers, which have a hierarchical structure. On the other hand, when directly characterizing the cuticle and cortex structure of a single keratin fiber, the analytical technique using a Raman microscope is effective, since it can be measured at a spot diameter of 1 μm.[17–23] Most importantly, by using this method, information about the crystalline fibrous (microfibril) and amorphous (matrix) structures existing in the cortex region can be obtained.

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The advantage of Raman spectroscopy for studying keratin fibers is that it is nondestructive, requires no sample extraction or purification, and provides information about —SS— groups through reduction and oxidation, which is impossible to record using infrared spectroscopy, since bands can be assigned to S—S and C—S vibrations of cystine. Also, structural information is provided by amide I and amide III vibrations, and the skeletal C—C stretch (α), which is only weakly active in the infrared absorption spectrum of keratin fibers.

In this study, to investigate the penetration of amino acids, peptides, and hydrolyzed proteins into keratin fibers, cross-sectional samples of virgin white hair treated with each were prepared. The amino acids, peptides, and hydrolyzed proteins in the cross-sectional samples were dyed with methylene blue, and finally the penetration of each was investigated with optical microscopy. Next, the structure of cross sections at various depths of white human hair treated with L-phenylalanine (Phe) was directly analyzed using a Raman microscope. Also, the structure of cross sections at various depths of bleached black human hair treated with hydrolyzed protein derived from eggwhites (HEWP) was directly analyzed using a Raman microscope.

EXPERIMENTAL

Materials

Virgin black and white Chinese hair bundles (average fiber diameter: 74 μm) to be used as keratin fibers were purchased from Beaulax Co. (Tokyo, Japan).

Hydrolyzed protein derived from eggwhites (HEWP) (average of Mw: 1000) was supplied by Pharma Foods International Co., (Kyoto, Japan). Hydrolyzed protein derived from collagen (Type 30: containing 30% tripeptide) was supplied by Jellice Co., (Miyagi, Japan). A bleaching cream (Product name: Gatsby Ex Hi-Bleach, Mandom Corp., Osaka, Japan) as an oxidizing agent (a bleaching agent) was used. The bleaching cream consists of three components and becomes 5.9 wt % hydrogen peroxide concentration and pH 10.3 when the three components are mixed. Also, other active ingredients, in the bleaching cream that aid in bleaching are potassium persulfate, ammonium persulfate, and sodium persulfate. The amino acids: L-alanine (Ala), L-aspartic acid (Asp), L-cysteine (Cys), glycine (Gly), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-Phenylalanine (Phe), L-proline (Pro), L-Valine (Val), the peptides: glycylglycine (GlyGly), glycylglycylglycine (GlyGlyGly), and finally methylene blue used as a basic dye and sodium laurylsulfate used as a washing agent were purchased from Wako Pure Chemical Industries (Osaka, Japan). Also, D, L-alanylglycine (AlaGly) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan).

Preparation of human hair treated with amino acids, peptides, and hydrolyzed proteins

Virgin white Chinese hair bundles were immersed in a solution of 0.5 wt % sodium laurylsulfate at a ratio of hair to solution of 1 : 60. The hair bundles were soaked for 60 min at 50°C. Next, the hair bundles were washed in distilled water and then dried in air (SLS treatment).

As a control, two hair bundles were treated with a phosphate buffer (PB) according to the following procedure. The hair bundles were immersed in a solution of 0.5*M* phosphate buffer (pH 7.0) at a ratio of hair : solution = 1 : 250. The hair samples were soaked at 50°C for 1 and 16 h. After washing in distilled water for 1 min, the hair samples were then dried at room temperature (PB treatment procedure).

In addition, twenty-three human hair bundles were immersed in a solution of 1.0 wt % amino acids and peptides/0.5*M* phosphate buffer (pH 7.0) at a ratio of hair : solution = 1 : 250. Finally, four human hair bundles were immersed in solution of 5.0 wt % hydrolyzed proteins at a ratio of hair : solution = 1 : 250. The hair samples were soaked at 50°C for 1 and 16 h. After washing in distilled water for 1 min, all samples were dried at room temperature.

Preparation of human hair treated with Phe for analysis using Raman spectroscopy

The SLS treatment procedure was performed on another virgin white Chinese hair bundle.

Sample 1 (Control)

As a control, half of a single white human hair fiber (fiber diameter: 85 μm) was prepared by the following procedures. The hair sample was immersed in a solution of 0.5*M* phosphate buffer (pH 7.0) at a ratio of hair : solution = 1 : 250. The hair sample was soaked at 50°C for 16 h. After washing in distilled water for 1 min, the hair sample was dried at room temperature.

Sample 2 (Phe)

The other half of the above single human hair fiber was prepared by the following procedures. The hair sample was immersed in a solution of 1% Phe/0.5*M* phosphate buffer (pH 7.0) at a ratio of hair : solution = 1 : 250. The hair sample was soaked at 50°C for

16 h. After washing in distilled water for 1 min, the hair sample was dried at room temperature.

Preparation of human hair treated with HEWP for analysis using Raman spectroscopy

The SLS treatment procedure was performed on a virgin black Chinese hair bundle. Next, the bleached virgin black Chinese hair bundle was prepared by the following procedures. The above hair bundle was treated with a bleaching cream at 25°C for 30 min at a ratio of hair : cream = 1 : 2 and then washed in distilled water for 1 min. The same procedure was repeated five times (bleaching treatment). Finally, the hair bundle treated with the bleaching cream was washed in distilled water for 1 min and then dried at room temperature.

Sample 3 (Control)

As a control, half of a single bleached black human hair fiber (fiber diameter: 85 μm) was prepared by the following procedures. The hair sample was immersed in distilled water at a ratio of hair : solution = 1 : 250. The hair sample was soaked at 50°C for 16 h and then dried with a hair dryer for 1 min.

Sample 4 (HEWP)

The other half of the above single human hair fiber was prepared by the following procedures. The hair sample was immersed in a solution of 5% HEWP (pH 6.6) at a ratio of hair : solution = 1 : 250. The hair sample was soaked at 50°C for 60 min and then dried with a hair dryer for 1 min.

Indirect evaluation of the penetration of amino acids, peptides, and hydrolyzed proteins into human hair

White human hair fibers treated with amino acids, peptides, and hydrolyzed proteins as described in the previous section were embedded in a resin (Tissue-Tek O.C.T.4583 Compound) and frozen. The frozen blocks were microtomed on a Leica CM1800 (Leica Instruments GmbH, Heidelberger, Germany) to 10-μm thickness and mounted on a slide glass. Next, the amino acid, peptide, and hydrolyzed protein penetrated parts of the cross-sectional samples were dyed with a solution of 0.001 wt % methylene blue using a syringe at room temperature. Finally, the penetration of the amino acids, peptides, and hydrolyzed proteins into the cross-sectional samples was indirectly observed by optical microscopy.

Raman spectra

All Raman spectra were recorded on a Ramanor T-64000 Raman microscope system (Jobin Yvon, Longjumeau, France), which comprised an optical microscope adapted to a single grating spectrograph and a charge-coupled device (CCD) array detector. The laser excitation was provided by an argon ion laser operating at 50 mW of 514.5-nm output. The laser beam on the sample was focused to a spot diameter of 1 μm using a 100× microscope objective. Spectra were recorded by scanning the 200–2000 cm^{-1} region with a total acquisition time of 1000 s. One scan with a 1000-s laser exposure was taken to obtain a good signal/noise (S/N) ratio. A spectra resolution of 2.3 cm^{-1} was used. By collecting three spectra from the samples, and taking an average of these, it was possible to ensure no sample degradation occurred, and that the spectrum obtained was quite reproducible. Furthermore, the cosmic ray was removed.

Normalization of Raman spectra of keratin fibers was carried out based on the C—H band at 1450 cm^{-1}, in which the peak area is large and is not influenced by the reduction treatment.[17,18,20–23] The disulfide (—SS—) content of the hair samples was compared by estimating the ratio of the peak area of the S—S band (calculated from the peak to a baseline, which was drawn between 470 and 560 cm^{-1}) divided by the peak area of the C—H band (calculated from the peak to a baseline, which was drawn between 1375 and 1500 cm^{-1}). The cysteic acid content of the hair samples was compared by estimating the ratio of the peak area of the S—O band (calculated from the peak to a baseline, which was drawn between 1013 and 1095 cm^{-1}) divided by the peak area of the C—H band. The random coil content of the hair samples was compared by estimating the ratio of the peak area of the amide III (unordered) band (calculated from the peak to a baseline, which was drawn between 1200 and 1288 cm^{-1}) divided by the peak area of the C—H band (amide III band analysis). The Phe content of the hair samples was compared by estimating the ratio of the peak area of the Phe peak (calculated from the peak to a baseline, which was drawn between 986 and 1020 cm^{-1}) divided by the peak area of the C—H band. Moreover, the proportion of the eight band components of the hair samples was evaluated by spectral simulation of the amide I band region, assuming Gaussian line shapes and appropriate line width (amide I band analysis).[12,19,22,23] The band frequency and line width of the eight components in the amide I band region are shown in Table I. According to Church et al.'s method,[12] the band frequency of the eight components was selected. Here, the band frequency and line width

TABLE I
Band Frequency and Line Width of the Eight Components in the Amide I Band Region

Components	Band frequency (cm^{-1})	Band line width (cm^{-1})
1[a]	1725	25
2[b]	1695	20
3[c]	1671	35
4[d]	1652	30
5	1630	20
6[e]	1616	20
7	1605	20
8	1585	10

[a] Assigned to the C=O stretching vibration of the protonated carboxylic acid groups of aspartic and glutamic acid side chains.[12,19,22,23,26,27]
[b] Assigned to the amide groups of the asparagines and glutamine side chains.[12,19,22,23,28,29]
[c] Assigned to the β-sheet and/or random coil forms.[10,12,19,22,23]
[d] Assigned to the α-helix form.[10,12,19,22,23]
[e] Assigned to tyrosine and tryptophan.[12,19,22,23]

of the eight components of all hair samples were tentatively fixed, as the band intensity of all hair samples was changed. Also, the component content (β/R) at 1671 cm^{-1} assigned to the β-sheet and/or random coil forms[10,12,19,22,23] and the component content (α) at 1652 cm^{-1} assigned to the α-helix form[10,12,19,22,23] of the hair samples was compared by estimating the ratio of the peak area of each component divided by the peak area of the C—H band (calculated from the peak to a baseline, which was drawn between 1375 and 1500 cm^{-1}). The mean and standard deviation of —SS— content, random coil content, β/R content, and α content in the cortex region of hair samples were calculated from the respective contents measured at the five analysis points (depths of 3, 5, 10, 20, and 30 μm) in the cortex region by assuming that the respective contents in the cortex region were constant.

Finally, hair samples were embedded in an epoxy resin (Refine Tec, Yokohama, Japan), and the cured blocks were microtomed on a microtome HM360 (Microm international GmbH, Walldorf, Germany) to 1-μm thickness and mounted on a slide glass. Cross-sectional samples were produced using virgin white human hair, and sections of the hair at varying depths (1, 3, 5, 10, and 20 μm) from the surface (spot diameter: 1 μm) were measured with a Raman microscope. Also, cross-sectional samples were produced using bleached black human hair, and sections of the hair at varying depths (3, 5, 10, 20, and 30 μm) from the surface (spot diameter: 1 μm) were measured with a Raman microscope.

RESULTS AND DISCUSSION

Penetration of amino acids, peptides, and hydrolyzed proteins into virgin human hair

Amino acids, peptides, and hydrolyzed proteins have anionic charges above the isoelectric point because of a carboxyl group in the molecule. So, the penetration of amino acids, peptides, and hydrolyzed proteins into the human hair can be theoretically observed by dyeing amino acid, peptide, and hydrolyzed protein penetrated parts with a basic dye (methylene blue).

Here, we prepared cross-sectional samples of human hair treated with amino acids, peptides, or hydrolyzed proteins. Next, the penetration of the amino acids, peptides, or hydrolyzed proteins of the cross-sectional samples dyed with methylene blue was indirectly observed by optical microscopy. The photomicrograph of the white human hair only treated with PB (PB treatment procedure) at 50°C and pH 7.0 for 1 h, then cross-sectioned, and finally dyed with methylene blue is shown in Figure 1. The cuticle and the cortex of the untreated white human hair sample did not adsorb the methylene blue. However, the medulla, which exists in the center of hair fibers, adsorbed the methylene blue, since the medulla is rich in glutamic acid and consists of porous proteins. The photomicrographs of the white human hair treated with 1.0 wt % Phe and Gly at 50°C and pH 7.0 for 1 h, then cross-sectioned, and finally dyed with methylene blue are shown in Figures 2 and 3, respectively. The white human hair treated with Phe at 50°C and pH 7.0 for 1 h adsorbed the methylene blue through the cuticle and partially into the cortex (Fig. 2). On the other hand, the white human hair treated with Gly at 50°C and pH 7.0 for 1 h did not adsorb the methylene blue into the cuticle and cortex (Fig. 3). This suggests that the penetration of amino acids into the human hair was influenced by the hydrophobic property of amino acids.

The penetration of amino acids, peptides, and hydrolyzed proteins into virgin human hair is shown in Table II. In the case of performing the amino acid treatment at 50°C and pH 7.0 for 1 h, the hydrophobic amino acids, especially Phe and Trp, which have a aromatic ring, most deeply penetrated into the virgin white human hair. Also, the penetration of the hydrophobic amino acids increased by increasing the methylene groups in the side chain of the hydrophobic amino acids (Ala<Val <Leu). On the other hand, the hydrophilic amino acids (Asp, Cys, Gly, and Lys) and peptides (GlyGly, AlaGly, and GlyGlyGly) did not penetrate at all. In the case of performing the amino acid treatment at 50°C and pH 7.0 for 16 h, the amino acids (Gly, Ala, and Met) and peptides (GlyGly and AlaGly) penetration, except for Pro, did not change at all.

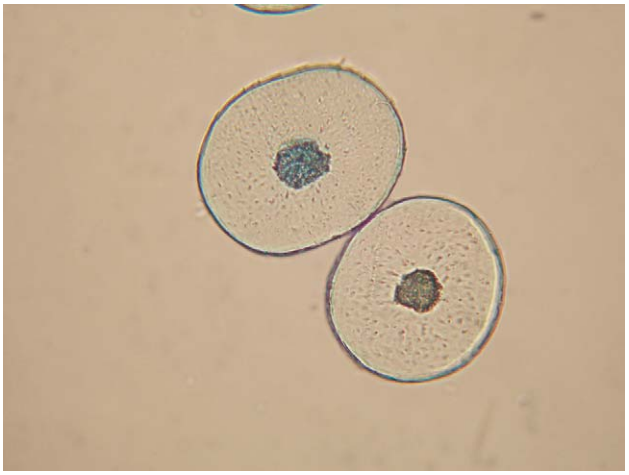

Figure 1 Photomicrograph of the white human hair only treated with PB (PB treatment procedure) at 50°C and pH 7.0 for 1 h, then cross-sectioned, and finally dyed with methylene blue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The photomicrographs of the white human hair treated with 5.0 wt % hydrolyzed protein derived from eggwhite (HEWP) and hydrolyzed protein derived from fish collagen (HCP) at 50°C for 1 h, then cross-sectioned, and finally dyed with methylene blue are shown in Figures 4 and 5, respectively. The white human hair treated with HEWP at 50°C and pH 7.0 for 1 h adsorbed the methylene blue through the cuticle and partially into the cortex (Fig. 4). On the other hand, the white human hair treated with HCP at 50°C and pH 7.0 for 1 h adsorbed the methylene blue into the cuticle but hardly into the cortex at all (Fig. 5). In the case of performing HCP treatment at 50°C and pH 7.0 for 16 h, HCP penetrated through the cuticle and partially into the cortex (shown in Table II). However, the penetration of HCP for 16 h was totally weaker than that of HEWP (shown in Figs. 4 and 5).

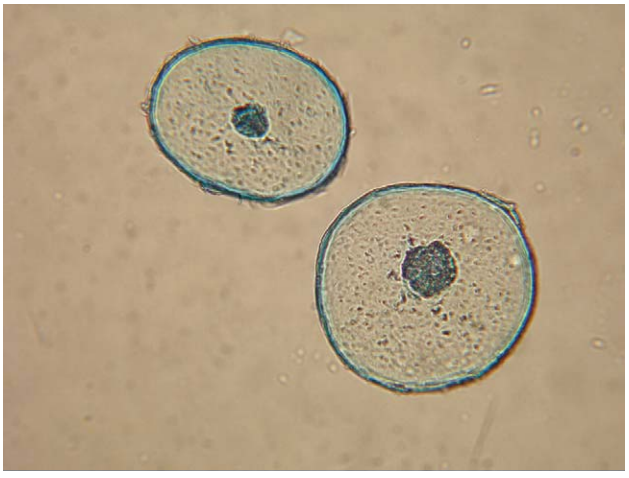

Figure 2 Photomicrograph of the white human hair treated with 1.0 wt % L-Phenylalanine (Phe) at 50°C and pH 7.0 for 1 h, then cross-sectioned, and finally dyed with methylene blue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

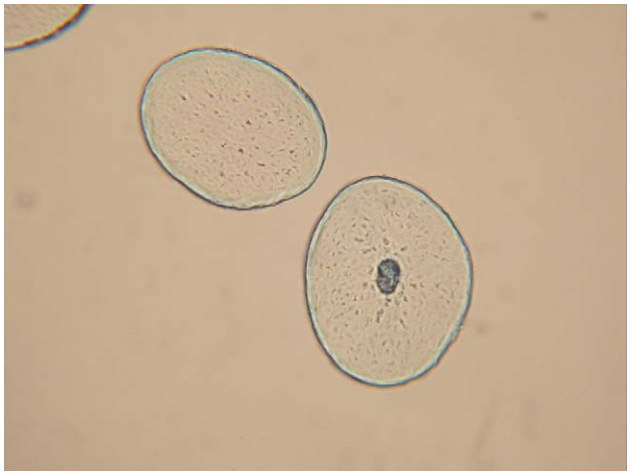

Figure 3 Photomicrograph of the white human hair treated with 1.0 wt % Glycine (Gly) at 50°C and pH 7.0 for 1 h, then cross-sectioned, and finally dyed with methylene blue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Raman spectra of hair keratin fibers treated with Phe

Measurement by Raman spectroscopy becomes a beneficial means of investigating the penetration of hydrolyzed proteins and aromatic amino acids into human hair because of it being able to obtain information on the amino acids tryptophan, tyrosine, and

TABLE II
Penetration of Amino Acids, Peptides, and Hydrolyzed Proteins into Virgin Human Hair

Agents	50°C, 1 h		50°C, 16 h	
	Cuticle	Cortex	Cuticle	Cortex
Control	−	−	−	−
Ala	+	−	+	−
Val	ND[a]	ND	+	−
Leu	+	± ~ ++	+	± ~ ++
Met	+	−	+	−
Phe	+	± ~ ++	+	± ~ ++
Trp	+	± ~ ++	+	± ~ ++
Pro	+	−	+	± ~ ++
Gly	−	−	+	−
Cys	+	−	ND	ND
Lys	−	−	ND	ND
Asp	+	−	ND	ND
GlyGly	−	−	−	−
AlaGly	− ~ ±	−	+	−
GlyGlyGly	−	−	ND	ND
HCP	+	− ~ ±	+	− ~ ++
HEWP	+	± ~ ++	+	± ~ ++

−: Did not penetrate; ±: Trace; +: Penetrated; ++; Deeply penetrated into cortex region.
[a] Not done.

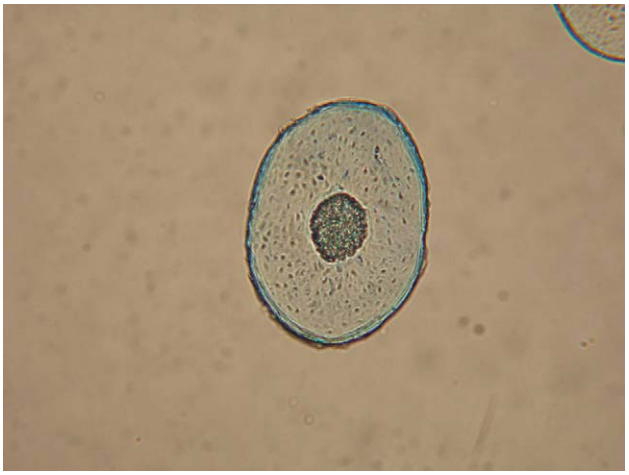

Figure 4 Photomicrograph of the white human hair treated with 5.0 wt % hydrolyzed protein derived from eggwhite (HEWP) at 50°C for 1 h, then cross-sectioned, and finally dyed with methylene blue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

phenylalanine, the S—S and C—S bonds of cystine, and the secondary structures, such as random coil, β-sheet, and α-helix in keratin fibers.

The frequencies and tentative assignments of human hair fibers at depths of 1 and 5 μm (corresponding to the cuticle and the cortex) from the fiber surface compared with that of wool are shown in Table III. The amide I peak maxima for the cortex region (depth of 5 μm from the fiber surface) was found to shift at 1666 cm^{-1}. Also, the skeletal C—C stretch (α) band at 935 cm^{-1}, assigned to the α-helical backbone, was observed in the cortex region only. This is in agreement with the findings by

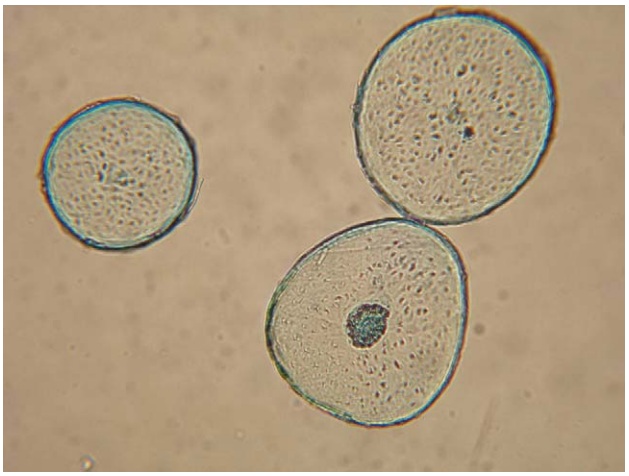

Figure 5 Photomicrograph of the white human hair treated with 5.0 wt % hydrolyzed protein derived from fish collagen (HCP) at 50°C for 1 h, then cross-sectioned, and finally dyed with methylene blue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE III
Frequencies and Tentative Assignments of Untreated Human Hair (Cuticle and Cortex Region) Compared with that of Wool

Human hair		Wool		
Cuticle[a] (cm^{-1})	Cortex[b] (cm^{-1})	Ref. 11 (cm^{-1})	Ref. 10 (cm^{-1})	Assignment
1671	1666	1658	1653	Amide I
1613	1614	1615	1614	Tyr and Trp
1553	1552	1558	1553	Trp
1448	1446	1450	1448	CH_2 bending mode
ND[c]	1336	1340	1338	CH_2 bend, Trp
ND	1315	1316	1318	Cα—H bend
1245	1243	1245	1244	Amide III (unordered)
ND	1210	1209	1207	Tyr and Phe
ND	1174	1180	1176	Tyr
1123	1123	1126	1126	C—N stretch
1040	1040	–	–	Sulfonate S—O stretch
ND	1030	1034	1031	Phe
1001	1001	1006	1002	Phe
959	ND	959	952	CH_2 rock
ND	935	935	934	Skeletal C—C stretch (α)
884	880	883	881	Trp
851	851	852	851	Tyr
ND	750	752	752	Trp
664	664	665	661	Cys C—S stretch
642	642	644	642	Tyr
505	507	512	512	Cys S—S stretch g-g

[a] Depth of 1 μm from hair surface.
[b] Depth of 5 μm from hair surface.
[c] Nondetect.

Fraser et al., in which the microfibril that exists in the cortex region is mainly composed of α-helical protein.[3,30] On the other hand, the amide I peak maxima for the cuticle region (depth of 1 μm from the fiber surface) was found to be at 1671 cm^{-1}, assigned to the β-sheet and/or random coil forms, but the skeletal C—C stretch (α) band, assigned to the α-helical backbone, did not appear. Also, the amide III (unordered) band intensity, assigned to the random coil form, at 1245 cm^{-1} for the cuticle region was higher than that for the cortex region. These results indicate that the α-helix form does not exist in the hair cuticle, and that the cuticle has a more amorphous structure. Moreover, the S—O band intensity, assigned to cysteic acid, at 1040 cm^{-1} for the cuticle was low, whereas the S—O band for the cortex region was very weak.

In this section, the white human hair samples treated with a solution of 1.0 wt % Phe (pH = 7.0) at 50°C for 16 h were analyzed at various depths using a Raman microscope. The cuticle Raman spectra (depth of 1 μm from the fiber surface) of the virgin white human hair (Sample 1) and the virgin white human hair treated with Phe (Sample 2) is shown in Figure 6. As is shown, the band shape as well as peak maximum frequency existing in the cuticle region of the virgin white human hair were almost unchanged by performing the Phe treatment.

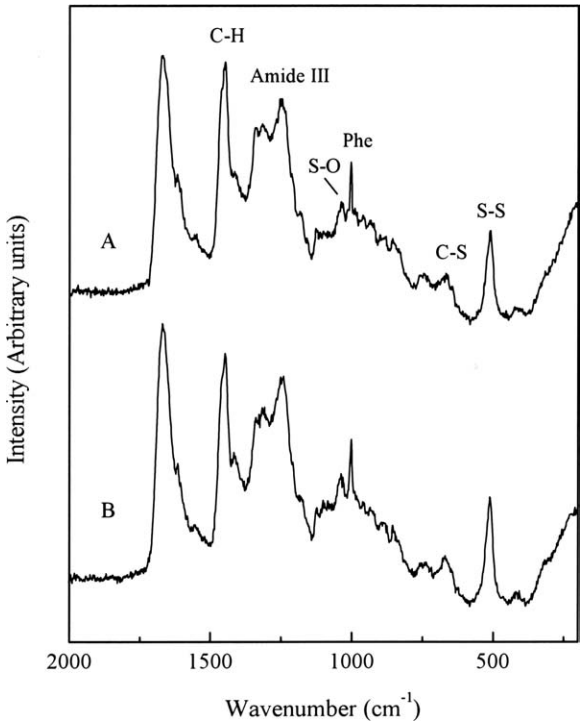

Figure 6 Cuticle Raman spectra (depth of 1 μm from the fiber surface) of the virgin white human hair (Sample 1) and the virgin white human hair treated with Phe (Sample 2): (A) Sample 1 and (B) Sample 2.

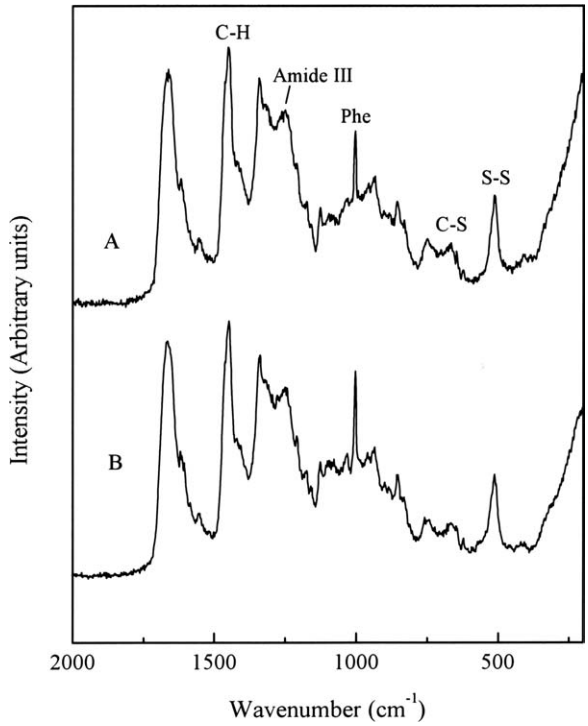

Figure 7 Representative cortex Raman spectra (depth of 5 μm from the fiber surface) of the virgin white human hair (Sample 1) and the virgin white human hair treated with Phe (Sample 2): (A) Sample 1 and (B) Sample 2.

The representative cortex Raman spectra (depth of 5 μm from the fiber surface) of the virgin white human hair (Sample 1) and the virgin white human hair treated with Phe (Sample 2) are shown in Figure 7. As is shown, the band shape as well as peak maximum frequency existing in the cortex region of the virgin white human hair were almost unchanged by performing the Phe treatment. Also, it is shown that the band shape as well as peak maximum frequency of the cuticle region of the human hair fiber were significantly different from those of the cortex region of the human hair fiber.

Next, to investigate the influence of the Phe treatment on the cuticle and cortex of human hair, the Phe content at various depths of the hair fibers untreated and treated with Phe was compared by Raman spectroscopic analysis. Normalization of Raman spectra of keratin fibers is often carried out based on the C—H band at 1446 cm^{-1},[17,18,20–23] amide I band at 1666 cm^{-1},[11] and the Phe peak at 1001 cm^{-1}.[14–16,19] Here, we chose the C—H band, which is assigned at 1446 cm^{-1}, for normalization, because the peak area is large and is not influenced by the Phe treatment.

TABLE IV
Phe Content in Hair Samples (Samples 1 and 2) at Depths of 1, 3, 5, 10, and 20 μm from the Fiber Surface

Sample	Distance from fiber surface (μm)				
	1	3	5	10	20
1 (Control)	0.05	0.07	0.06	0.07	0.06
	0.04	0.06	0.06	0.06	0.07
	0.05	0.07	0.06	0.07	0.07
	0.05	0.06	0.06	0.06	0.06
	0.04	0.07	–	–	–
Mean ± SD	0.046 ± 0.005	0.066 ± 0.005	0.060 ± 0.000	0.065 ± 0.005	0.065 ± 0.005
2 (Phe)	0.06	0.09	0.09	0.08	0.08
	0.06	0.09	0.08	0.08	0.08
	0.06	0.09	0.08	0.08	0.09
	0.06	0.09	0.07	–	0.08
	0.06	0.09	–	–	–
Mean ± SD	0.060 ± 0.000	0.09 ± 0.000	0.080 ± 0.007	0.080 ± 0.000	0.083 ± 0.004

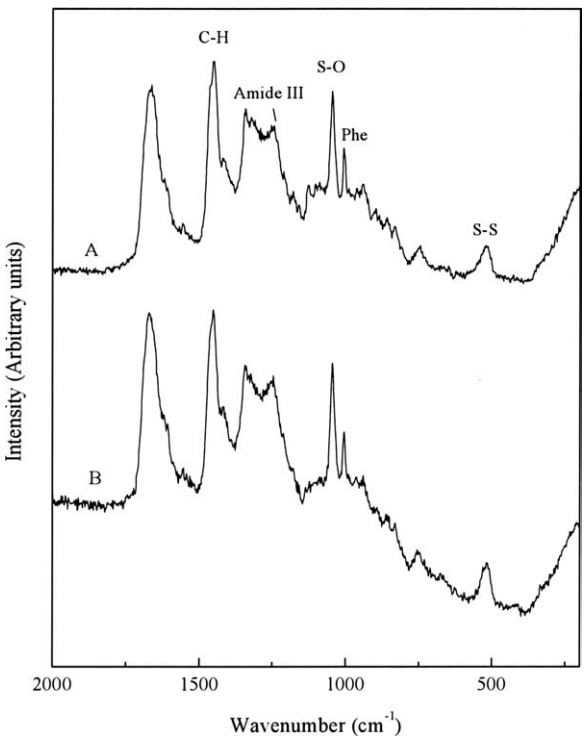

Figure 8 Representative cortex Raman spectra (depth of 5 μm from the fiber surface) of the bleached black human hair fiber (Sample 3: Control) and the bleached black human hair treated with HEWP (Sample 4: HEWP): (A) Sample 3 and (B) Sample 4.

The Phe content in hair samples (Samples 1 and 2) at depths of 1, 3, 5, 10, and 20 μm from the fiber surface are shown in Table IV. The Phe content (0.066) of virgin white human hair (Sample 1: Control) for the cortex region (depth of 3 μm from the fiber surface) increased compared with that (0.046) of the cuticle region (depth of 1 μm from the fiber surface) ($P < 0.001$). The ratio of Phe content in the cuticle and cortex region (Phe content of cuticle/Phe content of cortex = 0.046/0.066) was 0.70. This ratio is in excellent agreement with that obtained from the amino acid analysis (11.5/16.5 = 0.070), namely, the Phe content of the cuticle and cortex were 11.5 mol/1000 mol total amino acids and 16.5 mol/1000 mol total amino acids, respectively.[31] This result indicates that the Phe content of the cuticle and cortex can be reliably estimated using Raman spectroscopic analysis.

The Phe content of Sample 2 (the Phe treatment) at the distance of 1, 3, 5, 10, and 20 μm from the fiber surface were clearly higher than that of Sample 1 (Control), indicating that the Phe penetrated into virgin white human hair by performing the Phe treatment.

From these experiments, Phe was found to have more deeply penetrated the virgin white human hair samples by performing the Phe treatment at pH 7.0 for 16 h.

Raman spectra of bleached hair keratin fibers treated with HEWP

In this section, the bleached black human hair sample treated with a solution of 5.0 wt % HEWP at 50°C for 16 h was analyzed at various depths using a Raman microscope. The representative cortex Raman spectra (depth of 5 μm from the fiber surface) of the bleached black human hair fiber (Sample 3: Control) and the bleached black human hair treated with HEWP (Sample 4: HEWP) are shown in Figure 8. As is shown, the band shape as well as peak maximum frequency existing in the cortex region of the bleached black human hair were almost unchanged by performing the HEWP treatment. However, the amide III (unordered) and —SS— band intensities slightly increased by performing the HEWP treatment.

TABLE V
Mean and Standard Deviation (n = 5) of Disulfide Content, Cysteic Acid Content, β/R Content, α Content, and Random Coil Content in the Cortex Region (n = 5: Depths of 3, 5, 10, 20, and 30 μm from the Fiber Surface) of Hair Samples (Samples 3 and 4)

Sample	Distance from fiber surface (μm)	Disulfide content	Cysteic acid content	β/R content	α content	Random coil content
3 (Control)	3	0.16	0.32	0.69	0.34	0.21
	5	0.15	0.31	0.7	0.32	0.21
	10	0.12	0.28	0.69	0.32	0.21
	20	0.12	0.22	0.68	0.32	0.19
	30	0.13	0.24	0.67	0.33	0.21
Mean ± SD		0.14 ± 0.016	0.27 ± 0.009	0.69 ± 0.010	0.33 ± 0.007	0.21 ± 0.008
4 (HEWP)	3	0.16	0.27	0.76	0.32	0.25
	10	0.17	0.28	0.77	0.31	0.26
	15	0.17	0.27	0.79	0.32	0.22
	20	0.17	0.26	0.78	0.31	0.26
	30	0.15	0.22	0.71	0.32	0.21
Mean ± SD		0.17 ± 0.008	0.26 ± 0.009	0.76 ± 0.028	0.32 ± 0.007	0.24 ± 0.021
P		0.015	0.54	<0.001	0.076	0.016

Next, to investigate the influence of the HEWP treatment on the cortex of human hair, the —SS—, cysteic acid, and random coil contents at various depths of the hair fibers untreated and treated with HEWP were compared by Raman spectroscopic analysis. Here, we chose the C—H band, which is assigned at 1450 cm^{-1}, for normalization, because the peak area is large and is not influenced by the HEWP treatment.[17,18,20–23]

The mean and standard deviation ($n = 5$) of the disulfide (—SS—) and cysteic acid contents in the cortex region ($n = 5$: depths of 3, 5, 10, 20, and 30 μm from the fiber surface) of hair samples (Samples 3 and 4) are shown in Table V. The —SS— and cysteic acid contents in the cortex region of all hair samples were found to be almost constant. The —SS— contents of Sample 4 increased (the level of significance statistically calculated from the five measured points in the cortex region: $P = 0.015$), while the cysteic acid content did not change compared with that of Sample 3 for the cortex region (depths of 3–30 μm from the fiber surface). This result indicates that the HEWP, which is rich in —SS— groups, penetrated into the cortex region by performing the HEWP treatment.

Moreover, the secondary structure at various depths of the hair fibers was estimated by amide I band analysis.[12,19,22,23] The mean and standard deviation ($n = 5$) of the β-sheet and/or random coil content (β/R) and the α-helix (α) content in the cortex region ($n = 5$: depths of 3, 5, 10, 20, and 30 μm from the fiber surface) of hair samples (Samples 3 and 4) are shown in Table V.

The β/R and α contents in the cortex region of all hair samples were found to be almost constant. The β/R content in the cortex region of Sample 4 increased (the level of significance statistically calculated from the five measured points in the cortex region: $P = 0.0009$), while the α content in the cortex region did not change compared with that of Sample 3 (the level of significance statistically calculated from the five measured points in the cortex region: $P = 0.076$). This result indicates that the HEWP has a rich random coil content penetrated into the cortex region by performing the HEWP treatment.

Furthermore, the random coil content at various depths of the hair samples was estimated by amide III band analysis. The random coil content in the cortex region of all hair samples was found to be almost constant. The mean and standard deviation ($n = 5$) of the random coil content in the cortex region ($n = 5$: depths of 3, 5, 10, 20, and 30 μm) of hair samples (Samples 3 and 4) are shown in Table V. The random coil content of Sample 4 for the cortex region (depths of 3–30 μm from the fiber surface) increased (the level of significance statistically calculated from the five measured points in the cortex region: $P = 0.016$) compared with that of Sample 3. This result was in an excellent agreement with the results (β/R content) of the previous amide I band analysis.

The results from amide I band analysis and amide III band analysis suggests that the random coil structure of some of the proteins existing throughout the cortex region of the bleached white human hair increased rather than the β-sheet structure by performing the HEWP treatment.

CONCLUSIONS

We have developed a new method for investigating the penetration of amino acids, peptides, and hydrolyzed proteins into human hair. Using this method, the hydrophobic amino acids, especially aromatic amino acids, deeply penetrated into human hair. It was revealed that the penetration of Phe into human hair could be directly evaluated by Raman spectroscopy. In particular, we revealed that the penetration of hydrolyzed protein into human hair could be directly evaluated by measuring the random coil content using the amide I band analysis and amide III band analysis. From these experiments, we have demonstrated that the Phe deeply penetrated into the virgin white hair, and the HEWP penetrated into bleached black hair by performing the Phe and HEWP treatments at pH 7.0 for 16 h using Raman spectroscopy. Considering that only the penetration of the amino acid Phe can be evaluated using Raman spectroscopy, our proposed dyeing method with methylene blue would be an effective and convenient screening method to investigate the penetration of other amino acids, peptides, and hydrolyzed proteins.

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