

Effects of oxidative treatments on human hair keratin films

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Synopsis

The effects of hydrogen peroxide and commercial bleach on hair and human hair keratin films were examined by protein solubility, scanning electron microscopy (SEM), immunofluorescence microscopy, immunoblotting, and Fourier-transform infrared spectroscopy. Protein solubility in solutions containing urea decreased when the keratin films were treated with hydrogen peroxide or bleach. Oxidative treatments promoted the urea-dependent morphological change by turning films from opaque to transparent in appearance. Immunofluorescence microscopy and immunoblotting showed that the oxidation of amino acids and proteins occurred due to the oxidative treatments, and such occurrence was more evident in the bleach-treated films than in the hydrogen peroxide-treated films. Compared with hair samples, the formation of cysteic acid was more clearly observed in the keratin films after the oxidative treatments.

INTRODUCTION

Human hair is generally classified into three layers. From the outside inward, it is comprised of cuticle, cortex, and medulla. The cortex, which occupies 80% or more of the total mass, is constructed of a hierarchical fibrous structure mainly consisting of keratin filaments and keratin-associated proteins (KAPs). Since the cysteine contents in keratin and KAPs are considerably higher than in other proteins (1,2), the cysteine residues of these proteins can easily crosslink and form intermolecular and intramolecular covalent bonds. Thus, keratin and KAPs are considered to be closely related to the physical and mechanical properties of the hair.

In order to create straight, curly, or colored hair styles, human hair usually goes through chemical treatments and heating. During these processes, the disulfide crosslinks among the fibrous structures of keratin and KAPs are occasionally cleaved and recombined at different sites. Such oxidation and reduction of hair and its proteins is responsible for hair damage (2,3). Amino acid analysis showed that the cystine, methionine, and tyrosine contents of human hair decreased after bleach treatment, while the amount of cysteic acid increased (4,5). Nagai *et al.* (6) indicated a possibility that the isoelectric points of human hair proteins are changed when hair is treated by bleaching, dyeing, or perming. Recently, Plowman *et al.* (7) reported that oxidative treatment of wool had little effect on the

isoelectric points of keratin type I and II components measured by two-dimensional gel electrophoresis, though the isoelectric points and spots of KAP components consisting of high glycine-tyrosine proteins and high-sulfur proteins were changed by the treatment. FT-IR and Raman analyses indicated that keratin fibers in hair were disordered by oxidative and reductive treatments (8,9). These results suggest that these treatments on human hair will affect the higher order structure of hair through conformational changes in the fibrous proteins.

We developed novel procedures for preparing human hair protein solutions and the protein films from them using the self-assembly of α -keratin (10–12). The conversion ratios from the solutions to the films were 60–80%, and the films mainly consisted of α -keratin types I and II and KAPs. Any significant degradation was not observed during the preparation process and after storage for several months. SEM observations showed that the protein films consisted of particles and filamentous structures (11,12). However, regular filamentous architectures like the hair cortex were not observed. Recently, we developed a sensitive method to detect UV-induced photodegradation in hair proteins using fluorescent microscopy (13). The sensitivity was significantly higher when hair keratin film prepared by the pre-cast method was used instead of hair samples. This indicates that hair keratin films can serve as an alternative cortex filament to evaluate hair damage caused by protein degradation. The effect of oxidative treatments on hair keratin films have not been reported.

In the present study, we report the effects of hydrogen peroxide and commercial bleach on hair keratin films. The morphological and biochemical properties of the keratin film and its proteins were examined in detail.

EXPERIMENTAL

PREPARATION OF HUMAN HAIR KERATIN FILMS

Human hair protein solution was prepared according to the “Shindai method,” which has been previously described by us (10). Briefly, ethanol-treated hair samples that had not undergone chemical treatments such as bleaching or permanent waving were extracted using a solution containing 20 mM Tris-HCl (pH 8.5), 2.6 M thiourea, 5 M urea, and 150 mM dithiothreitol (DTT) at 50°C for 24 hr. This solution was added to hair at 60 mg/ml. After filtration and centrifugation, about 70% of the solution was recovered as extracted protein solution, which was then used for preparation of the pre-cast film (11). The hair protein solution was mixed with acetic acid to a final concentration of 20 mM and then poured into tissue culture dishes (diameter 40 mm) containing distilled water. A membrane-like aggregate was formed that spread out on the bottom of the dishes. After being washed with tap water for 36 hr and distilled water for 6 hr, the human hair keratin films were recovered after drying at room temperature.

OXIDATIVE TREATMENTS OF THE HAIR KERATIN FILMS AND PROTEIN SOLUBILIZATION

The oxidative treatments of the hair keratin films were done with solutions containing 0–10% hydrogen peroxide and 50 mM Tris-HCl (pH 8.5) at 25°C. The average weight

of the films used was 18 mg/dish, and the solution was added at 2.5 ml/dish. After incubation for 0–10 min, the solutions were discarded and the films were washed with tap water for 10 min and then with distilled water for 6 min. The films were dried at room temperature and used. Additional hair protein films were bleached using a commercial bleaching agent (UNO hard bleach, Shiseido, Japan), following the manufacturer's instructions, at 25°C for 10 min. The bleaching agent contained 2% ammonium hydroxide, 1.5% monoethanolamine (pH 10.1), 3% H₂O₂, and potassium persulfate. The films were then similarly rinsed and dried. Then, the films were collected, ground to powders, and used in the following experiments.

Solubilization of proteins from the untreated, oxidized, or bleached keratin films was determined using solution A (50 mM Tris-HCl, pH 8.5), solution B (50 mM Tris-HCl, pH 8.5, containing 8 M urea), solution C (50 mM Tris-HCl, pH 8.5, containing 5 mM DTT), and solution D (50 mM Tris-HCl, pH 8.5, containing 8 M urea and 5 mM DTT). The powdered films were mixed with solutions A, B, C, or D at 5 mg/ml and incubated at 50°C for 3 hr. After centrifugation at 12,000g for 5 min, the supernatants were recovered in test tubes and used for measurement of the protein concentration and for electrophoresis. The protein concentrations were determined according to Bradford, using bovine serum albumin as the standard (14).

SEM OBSERVATION

The hair keratin films before and after the treatments with hydrogen peroxide, the bleaching agent, or solution B were sputtered with platinum, and the fine structures of the films was observed by scanning electron microscopy (Neoscope JCM-5000, JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV.

FLUORESCENCE MICROSCOPY

The hair keratin films were treated with distilled water, 1% hydrogen peroxide, or the bleaching agent at 25°C for 10 min. Then the films were incubated with 3 ml of the staining solution, which consisted of 20 μ M fluorescein-5-thiosemicarbazide (5-FTSC) in 100 mM 2-morpholinoethane sulfonic acid-NaOH (pH 5.5) at 25°C for 2 hr (15). To remove non-reacting 5-FTSC, the films were rinsed with 0.1% SDS, 300 mM NaCl, and 30 mM sodium citrate buffer (pH 7.0) for 30 min at 50°C, then in 0.1% SDS, 30 mM NaCl, and 3 mM sodium citrate buffer (pH 7.0) for 30 min at 25°C, and finally in distilled water for 10 min at 25°C. After rinsing, the films were dried at room temperature. All procedures were carried out in a dark room. Afterward, the films were observed and photographed using fluorescence microscopy (VB-G25, Keyence, Japan).

GEL ELECTROPHORESIS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (16) using a 5–20% gradient polyacrylamide gel. After the electrophoresis, proteins in the gel were stained with 0.1% Coomassie brilliant

blue R-250, 10% acetic acid, and 40% ethanol for 1–2 hr. Destaining was carried out in 10% acetic acid and 40% ethanol.

IMMUNOBLOTTING

The proteins were extracted by incubating the untreated, 1% hydrogen peroxide-, or bleach-treated keratin films and hair samples with 50 mM Tris-HCl (pH 8.5) containing 8 M urea and 5% 2-mercaptoethanol at 50°C for 20 hr. Proteins separated by 5–20% SDS-PAGE were electrophoretically transferred to nitrocellulose filters and washed with PBS (phosphate-buffered saline). The filters were reacted with dinitrophenyl (DNP) hydrazine at 25°C for 15 min (OxyBlot™ Protein Oxidation Detection Kit, Chemicon, U.S.A. and Canada). After incubation of the filters at 4°C overnight with reagent N-102 (NOF Corporation, Tokyo Japan) to block non-specific binding sites, they were incubated with 1/150 anti-DNP antibody for 1 hr at 25°C. After a rinse with PBS containing 0.1% Tween 20, the filters were reacted with 1/300 peroxidase-conjugated anti-rabbit IgG for 1 hr at 25°C. Then, the filters were washed with PBS containing 0.1% Tween 20, and the antibody binding was visualized using the Super Signal West Trial Kit (Thermo, Japan).

FOURIER-TRANSFORM INFRARED (FT-IR) MEASUREMENT

FT-IR spectra were obtained from the films and hair samples before and after treatment with 1% hydrogen peroxide or the bleaching agent. The films and hair samples were ground to fine particles with an agate mortar and pestle. The measurements were performed by attenuated total reflectance (ATR) with an IR Prestige-21 (Shimadzu, Japan), collecting 50 scans at a resolution of 4 cm⁻¹.

RESULTS AND DISCUSSION

PROTEIN ELUTION FROM THE HAIR KERATIN FILMS TREATED WITH OR WITHOUT HYDROGEN PEROXIDE AND BLEACH

When the human hair keratin films prepared by the pre-cast method were incubated with 1–5% hydrogen peroxide solution or the bleaching agent at 25°C for 10 min, the film weights after these treatments were little changed compared to those of the untreated films. These results suggest that the protein films can withstand the oxidative treatment.

Four kinds of solutions (pH 8.5) containing urea and DTT were used to examine the protein solubility from the keratin films treated with 1% hydrogen peroxide solution or bleach at 25°C for 10 min. Little protein from the untreated, hydrogen peroxide-, or bleach-treated films was dissolved by solution A (Figure 1). In solution B, containing 8 M urea, and solution C, containing 5 mM DTT, protein solubilization (5–10%) was similar for the untreated films. Interestingly, the amount of protein solubilization from the hydrogen peroxide-treated films was decreased to almost zero in solution B, while the amount was little changed in solution C. The amount of protein solubilization from bleach-treated films in solution C was higher than expected, and this is considered to be the result of a protective hardening effect by the films against oxidative treatment. When

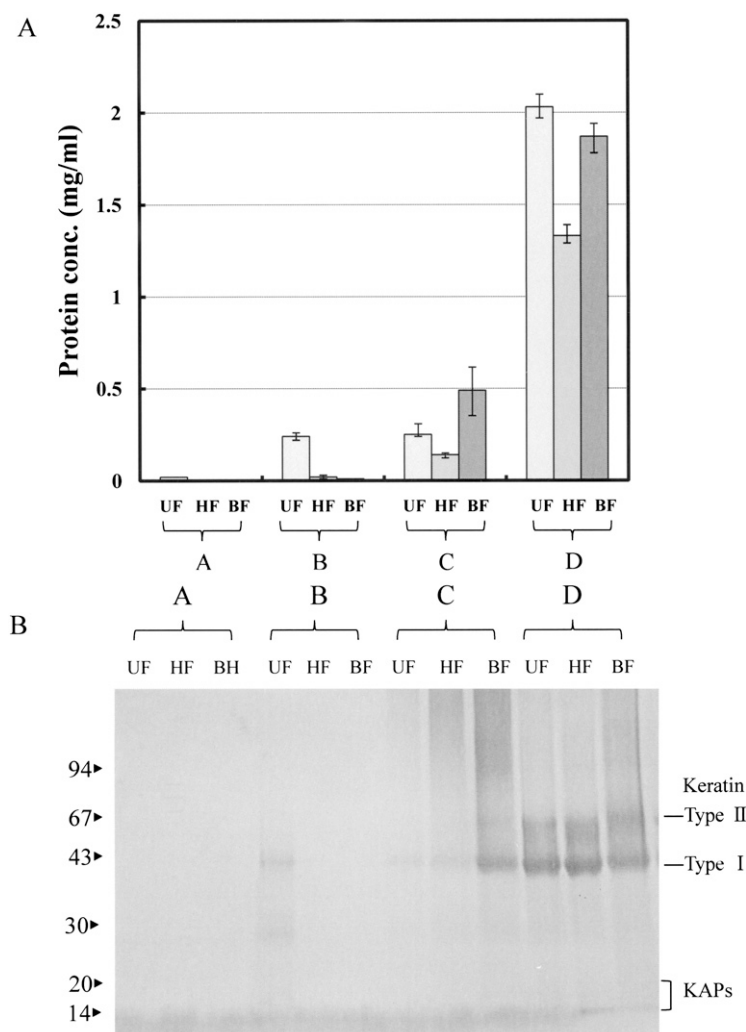


Figure 1. Solubility from untreated, hydrogen peroxide-treated, or bleach-treated hair keratin films by various solutions and SDS-PAGE analysis. Upper panel (A): Hair keratin films were incubated with distilled water (UF), 1% hydrogen peroxide solution (HF), and commercial bleach mixture (BF). After washing and drying, the films were incubated with various solutions at 50°C for 3 hr for protein solubility. A: 50 mM Tris-HCl (pH 8.5) (solution A). B: 50 mM Tris-HCl (pH 8.5) containing 8 M urea (solution B). C: 50 mM Tris-HCl (pH 8.5) containing 5 mM DTT (solution C). D: 50 mM Tris-HCl (pH 8.5) containing 8 M urea and 5 mM DTT (solution D). Lower panel (B): The protein components were analyzed by 5–20% SDS-PAGE.

films were incubated with solution D, containing urea and DTT, most of the proteins dissolved and the insolubility of the hydrogen peroxide-treated films decreased.

CHARACTERIZATION OF HYDROGEN PEROXIDE-TREATED KERATIN FILMS

Urea is known as a typical denaturant that can destroy protein structures. In the absence of DTT, the protein dissolution from the untreated film was dependent on the concentration

of urea, while the protein dissolution from the hydrogen peroxide-treated films increased only slightly as urea increased from 0 to 8 M (Figure 2A).

Next, we examined the effects of hydrogen peroxide concentration on the protein dissolution from the keratin films (Figure 2B). The amount of proteins dissolved in solution B began to decrease when the concentration of hydrogen peroxide was more than 0.001%. Dissolved proteins from the films decreased linearly when the films were treated with increasing concentrations of hydrogen peroxide. The half-value was estimated to be *ca.* the 0.01% hydrogen peroxide solution, indicating that the hair protein film was highly sensitive to the oxidative processing.

MORPHOLOGY OF THE KERATIN FILMS WITH VARIOUS TREATMENTS

The keratin films were incubated with 1% hydrogen peroxide or the bleaching agent at 25°C for 10 min. The films remained opaque irrespective of the hydrogen peroxide and the bleaching treatments (Figure 3A, panels a, b, c). However, the color was changed to the naked eye, from light brown to white, by the bleaching treatment, while the hydrogen

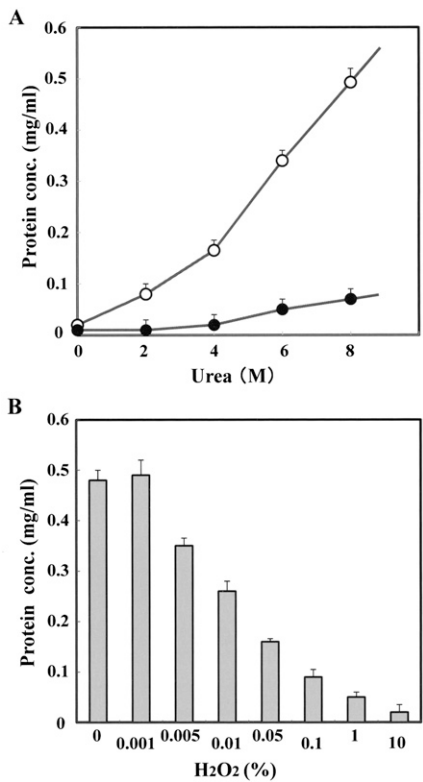


Figure 2. Effects of urea and hydrogen peroxide on protein solubility from the hair keratin films. (A) The keratin films treated with distilled water (○) and 1% hydrogen peroxide (●) were incubated with solution A containing 0–8 M urea. The protein concentrations of the supernatants were measured. (B) The keratin films were treated with 0.001–10% hydrogen peroxide and the solubility of solution B was examined.

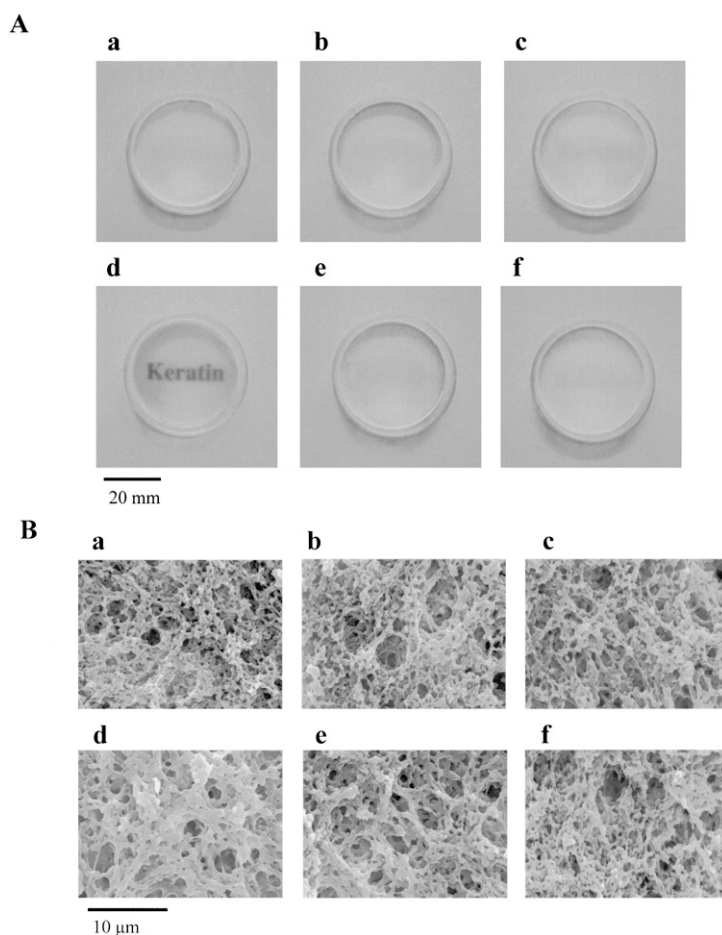


Figure 3. Changes in appearances of the hair keratin films after various treatments and SEM observations. (A) The hair keratin films were incubated with distilled water (a,d), 1% hydrogen peroxide (b,e), and bleach solution (c,f) at 25°C for 10 min. The films (d–f) were further incubated with solution B at 50°C for 3 hr. (B) After washing and drying, the films were used for morphological observations. Bars: 20 mm (A); 10 μm (B).

peroxide-treated film apparently remained unchanged. These films were incubated with solution B at 50°C for 3 hr, washed with distilled water, and dried. Proteins in the untreated film partly dissolved and became translucent, making the letters “keratin” underneath the film visible. On the other hand, proteins in the films treated with hydrogen peroxide or the bleaching agent hardly solubilized, resulting in their clearly unchanged appearances (Figure 3A, panels d, e, f).

We reported that the surfaces of hair protein films prepared by pre- and post-cast methods were composed of porous structures with fine filaments and particles (11,12). SEM observation showed that the surfaces of hair keratin films prepared by the pre-cast method are also composed of porous structures containing small particles (Figure 3B, panels a, b, c). Such small particles on the surface of the translucent films incubated with solution B disappeared, while smooth porous structures remained (Figure 3B, panel d). Urea treatment apparently could not affect the fine structures of the films, even after the oxidative treatments.

We have not measured the amounts of cysteine sulphhydryl and disulfide bonds in the hair keratin films used. While the disulfide bonds of native hair proteins are broken during the protein extraction, most of the hair proteins are recovered as sulphhydryl forms. When the hair proteins are put under the conditions of high protein concentrations, and when the denaturant and reductant are removed slowly from the solution, the disulfide bond is allowed to reform, which leads to protein aggregates such as a film. Thus, it is likely that cysteine remains in the film to some degree and that the oxidation of it decreases the sensitivity of the film to urea extraction.

OXIDATION OF PROTEIN MEASURED BY FLUORESCENT MICROSCOPY AND IMMUNOBLOTTING

When human hair and the hair keratin films were exposed to UV irradiation, the formation of oxidized protein was detected by a fluorescent microscopy (13,15) using 5-FTSC, a specific reagent which binds to carbonyl groups of the hair proteins. We noted the presence of carbonylated proteins in the films after treatments of 1% hydrogen peroxide or the bleaching agent (Figure 4A). Compared to the film treated with distilled water, a bright green color was observed in areas after the oxidative treatments. When the fluorescence intensity of the films was calculated by image analysis, the highest intensity was found in the bleach-treated film (Figure 4B).

Next, we examined oxidized proteins by immunoblot analysis (Figure 5). As shown in Figure 5A, a significant difference in the protein component, which consisted of keratin types I and II and KAPs from hair fiber and the keratin films, was not observed. The

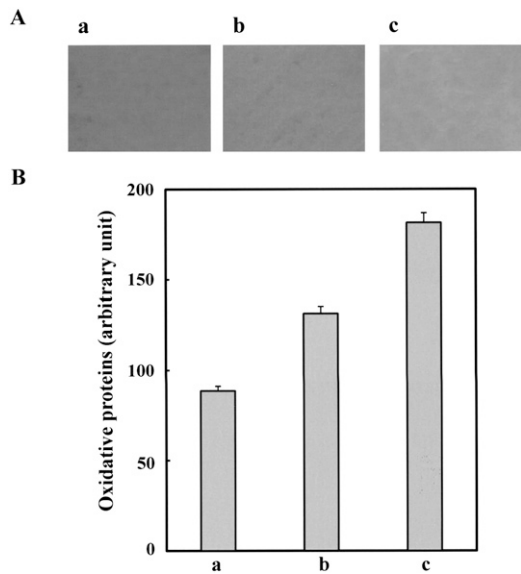


Figure 4. Fluorescence image and intensity of hair keratin films treated with hydrogen peroxide or bleaching agent. (A) After treatment with distilled water (a), 1% hydrogen peroxide (b), or bleaching agent (c) at 25°C for 10 min, the hair keratin films were reacted with 20 μ M 5-FTSC and observed using fluorescent microscopy. (B) The average of the fluorescence intensity was calculated from 40 areas per one film by image analysis.

protein samples extracted from hair fiber and the keratin films were reacted with DNP hydrazine, a specific reagent that binds to aldehydes from basic amino acids and threonine. After oxidative treatments of the films, the reactivities of KAPs and high-molecular-weight components (HMPs) with a molecular mass more than 100 kDa were higher relative to the keratin bands (Figure 5B). The reactivity of high-molecular-weight components was particularly enhanced when the films were treated with the bleach.

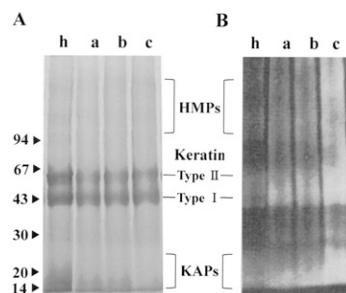


Figure 5. Analysis of solubilized oxidative proteins from hair samples and keratin films by immunoblotting. (A) CBB staining. (B) Immunostaining using anti-DNP antibody. (h, hair; a, untreated film; b, H_2O_2 -treated film; c, bleach-treated film.)

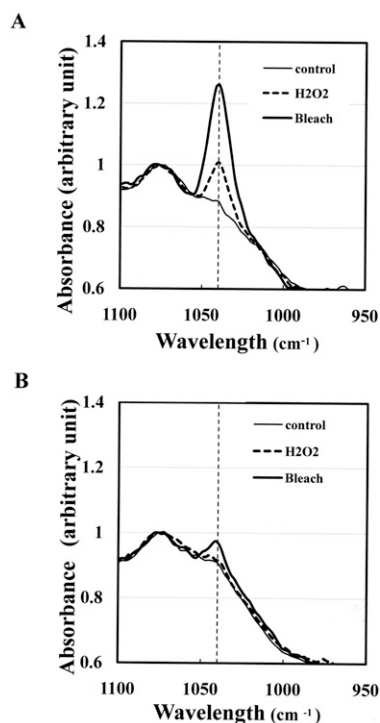


Figure 6. FT-IR spectra of keratin films and hair samples with or without oxidative treatments. After treatment with distilled water (—), 1% hydrogen peroxide (---), or the bleaching agent (—), the hair keratin films (A) and hair samples (B) were recovered. Absorbance was normalized as 1 at the peak of 1076 cm^{-1} to compare the relative amounts of $-\text{SO}_3\text{H}$ observed at 1041 cm^{-1} , which appeared prominently when using the keratin films as an oxidized form of cysteine.

FT-IR ANALYSIS

Bleaching human hair causes a change in amino acid composition in the protein; that is to say, as cysteine decreases, cysteic acid increases (5,17). The relative amounts of possible oxidation products of the S–S bond after oxidative treatments can be also determined by FT-IR measurement. In fact, the formation of cysteic acid was confirmed in bleached human hair (18,19). We examined by FT-IR measurement the formation of cysteic acid in hair samples and the keratin films after oxidative treatments (Figure 6A,B). The absorbance at the $\text{-SO}_3\text{H}$ (1041 cm^{-1}) was compared, and after normalization of the absorbance at 1076 cm^{-1} , in the untreated hair and films, the peak was hardly detected. Upon comparison of the hydrogen peroxide-treated hair and films, the peak at 1041 cm^{-1} was scarcely detected in the hair, while it was clearly evident in the films. The peaks were detected in the bleach-treated films and hair; however, the absorbance was five to ten times higher in the bleached films compared with that of bleached hair. These results suggest that an oxidized form of cysteine residues is easily identified when hair keratin films are used.

CONCLUSION

Human hair keratin film does not have features such as trace metals and diffusion-modulating morphology that are present in hair fibers. However, its use as an alternative cortex filament can be promising for the evaluation of hair damage by oxidative treatments containing bleach.

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