A Rapid Extraction Procedure of Human Hair Proteins and Identification of Phosphorylated Species

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We developed a rapid and convenient extraction procedure of human hair proteins to examine their biochemical properties in detail. This procedure is based upon the fact that the combination of thiourea and urea in the presence of a reductant can effectively remove proteins from the cortex part of human hair. The extracted fraction mainly consisted of hard α-keratins with molecular masses of 40—60 kDa, matrix proteins with 12—18 kDa, and minor components with 110—115 kDa and 125—135 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein phosphorylation in human hair was investigated by immunoblotting with antibodies against phosphoserine, phosphothreonine and phosphotyrosine. We found serine phosphorylation in α-keratins and matrix proteins and threonine phosphorylation in α-keratins. The extraction was also found to be effective when wool, chicken feathers, rat hair and human nails were used as starting materials.

Key words human hair protein; extraction procedure; keratin; phosphorylation

The protein content in a keratinized structure, including animal hair, nail, horn and feather, is approximately 80% of the total mass.[1,2] Two large groups of human hair proteins are known. One is hard α-keratins forming microfibrous intermediate filaments and the other is matrix proteins forming a nonfilamentous matrix as intermediate filaments-associated proteins. The hard α-keratins are further resolved into two subfamilies, consisting of at least 4—9 distinct type I acidic (40—50 kDa) and 4—6 type II neutral/basic (55—65 kDa) members.[3] Matrix proteins are classified into high-sulfur proteins (10—20 kDa) and high-tyrosine proteins (6—9 kDa). N-terminal acetylations have been reported for a post-translational modification of animal hair α-keratins and the related proteins.[4,5] Little is known, however, about post-translational modifications of human hair α-keratins and the related proteins, while epithelial cytokeratins or soft α-keratins have been well studied.[6]

A number of procedures have been reported to isolate hard α-keratins and their related proteins for analyses.[7,8] It is difficult to obtain them in the native state, because the hard α-keratins are highly cross-linked with each other by disulfide bonds, enabling intermediate filaments to covalently cross-link with matrix proteins. Proteins extracted from keratinized structures are generally prepared by reduction in the presence of denaturing agent and *S*-alkylation under extremely low or high pH conditions. Using a combination of these reagents, protein yields were not uniform and protein hydrolysis was liable to occur.[9] Other denaturing agents, such as anionic detergents and guanidine hydrochloride, are also used for the extraction.[10,11] However, the detergents in protein solution and the *S*-alkylation of reduced cysteine interfered with the chemical and physical analyses and their complete removal was difficult.[12,13] To avoid the above mentioned problems, another approach is protein extraction from the cells of the lower part of hair follicles as pre-keratinized material.[2,14] However, using this method it was difficult to obtain an amount of protein sufficient for analysis and, furthermore, the obtained proteins were not originated from the full-keratinized matured structure.

In this paper, we describe a new and convenient extraction procedure of proteins containing keratins from human hair in the absence of detergent called the 'Shindai method,' because the procedure was developed in Shinshu University. It is also effective for extraction of protein components from wool, chicken feathers, rat hair and human nails. Moreover, we present evidence for the first time that phosphorylated species are contained in human hair hard α-keratin and matrix proteins.

MATERIALS AND METHODS

Extraction of Hair Proteins Human hair (of a Japanese woman) was washed with ethanol; external lipids were removed using a mixture of chloroform/methanol (2 : 1, v/v) for 24 h. The delipidized hair (20 mg) was mixed with a solution (5 ml) containing 25 mM Tris–HCl, pH 8.5, 2.6 M thiourea, 5 M urea and 5% 2-mercaptoethanol (2-ME) (Shindai method) or 25 mM Tris–HCl, pH 9.5, 8 M urea and 5% 2-ME (conventional method) at 50 °C for 1—3 d. The mixture was filtered and centrifuged at 15000×*g* for 20 min at room temperature. The obtained supernatant was used as a hair protein fraction. The pellet was recovered, washed with distilled water and used as an extracted hair sample.

Determination of Extracted Proteins Dry Weight Method: A hair protein fraction was dialyzed against 2 l of distilled water with 5—7 changes, lyophilized, and then dried sufficiently in a silicagel box. Alternatively, an extracted hair sample (residue fraction) was washed with distilled water and then dried in a silicagel box. The amounts of a hair protein fraction and of an extracted hair sample (residue fraction) were obtained by weighing the dried samples using an electronic balance.

Bradford Method: Protein amounts were determined by the colorimetric method of Bradford[15] using the Bio-Rad protein assay (Bio-Rad).

Gel Electrophoresis/Two Dimensional Electrophoresis (2DE-EP) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[16] with an 8—18% slab gel. Proteins in the gel were stained with 0.1% Coomassie brilliant blue R-

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250, 10% acetic acid and 40% ethanol for 1 h and destained in 10% acetic acid and 40% ethanol. 2DE was performed according to the method developed by O'Farrell[17] using 2% ampholine (pI 3.5—10).

Western Blot Analysis Proteins separated on SDS-polyacrylamide gel were transblotted onto a nitrocellulose membrane in a solution containing 20 mM Tris–glycine, pH 8.3 and 10% methanol. The membrane was blocked with 5% BSA in TBS-T (25 mM Tris–HCl, pH 7.2, 50 mM NaCl and 0.5% Tween-20) at room temperature and reacted with 1 : 1000 phosphotyrosine (Wako), 1 : 1500 phosphoserine (Affiniti), and 1 : 2000 phosphothreonine (Sigma) antibodies for 1 h at room temperature. After extensive washing in TBS-T, the membrane was incubated with 1 : 30000 peroxidase-conjugated secondary antibodies against rabbit immunoglobulin in TBS-T for 1 h at room temperature. The blots were visualized with the Super Signal CL-HRP Substrate System (Pierce).

Light Microscopy Morphological changes of the hair surface were examined using a light microscope (Nikon, Labophot-2).

RESULTS AND DISCUSSION

Effects of Thiourea, Urea and Reductant on Protein Extraction from Human Hair We examined a combination of thiourea and urea for extraction of human hair proteins (Table 1). When the hair was incubated with 2—8 M urea in buffer containing 25 mM Tris–HCl, pH 8.5 and 5% 2-ME, the protein amounts recovered in the supernatant were 8—27% of the original level. Addition of thiourea to the buffer containing 5 M urea increased the extracted protein amounts, although this effective action was not found in the absence of urea. The maximal yield was more than 65%. When 2-ME was removed from the extraction buffer, the protein amount recovered was decreased to about 5%, indicating that the presence of reductant was also essential for this effective extraction. Similar results were obtained when dithiothreitol (0.5—15%) or thioglycolic acid (1—20%) was used in place of 2-ME. Therefore, three kinds of reagents consisting of thiourea, urea and reductant are necessary for the effective extraction from human hair.

The time course of the protein extraction by the Shindai method was compared with that by the conventional method (Fig. 1). The extraction velocity in the presence of thiourea, especially for the initial 3 h, was much faster (3—5 fold) than that in its absence at 50 °C. The obtained proteins were analyzed by SDS-PAGE (Fig. 2A). The protein fraction extracted by the Shindai method was composed of microfibril keratins with a molecular mass of 40—60 kDa, matrix with a molecular mass of 10—20 kDa and high molecular weight minor components (110—115 and 125—135 kDa), while the fraction produced by the conventional method was mainly composed of two protein doublets of keratins with a molecular mass of 40—60 kDa. These observations strongly indicate that the presence of thiourea caused an effective dissociation between keratin proteins and their associated proteins. Since no detergent was contained in the extraction buffer, we further examined the protein composition by 2DE-EP (Fig. 2B). 2DE-EP showed that type I keratin resolved into at least two spots in the more acidic region, while type II keratin was distributed in a streak from acidic to basic regions. Matrix proteins also resolved into at least three spots in a streak from acidic to neutral regions.

Table 1. Effect of Urea, Thiourea and 2-ME on the Protein Extraction from Human Hair

Reagent	Yield (%)[a]	Yield (%)[b]
2 M urea + 5% 2-ME	10	8
5 M urea + 5% 2-ME	23	22
8 M urea + 5% 2-ME	27	24
0.6 M thiourea + 5 M urea + 5% 2-ME	31	25
1.2 M thiourea + 5 M urea + 5% 2-ME	43	49
1.8 M thiourea + 5 M urea + 5% 2-ME	59	60
2.4 M thiourea + 5 M urea + 5% 2-ME	61	65
3 M thiourea + 5 M urea + 5% 2-ME	60	67
2.4 M thiourea + 5% 2-ME	19	18
2.4 M thiourea + 5 M urea	4	5
2.4 M thiourea + 5 M urea + 5% DTT	67	70

The protein was extracted from human delipidized hair at 50 °C for 2 d in 25 mM Tris–HCl, pH 8.5 containing the reagents as indicated. *a*) Dry weight method. *b*) Bradford method.

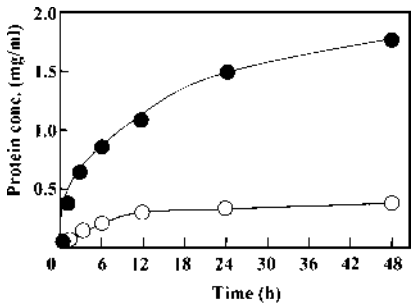

Fig. 1. Time Course of the Extraction of Protein from Human Hair in the Presence or Absence of Thiourea

The protein was extracted from human delipidized hair at 50 °C using Shindai method (●) or conventional method (○). Aliquots were measured for protein concentration.[15]

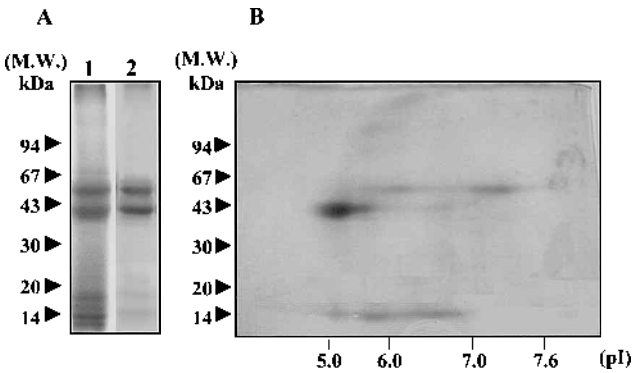

Fig. 2. SDS-PAGE and 2DE of Hair Proteins

(A) The hair proteins extracted at 50 °C for 2 d were subjected to 8—18% gradient gel. Lane 1, Shindai method; lane 2, conventional method. (B) 2DE of hair proteins prepared by Shindai method. Molecular weight standards in kDa and pI values are shown on the left and below, respectively.

Visualization of Hair Surface by Light Microscopy Figure 3 shows the morphological image of hairs by light microscopy after protein extraction with the Shindai method. After 48 h of incubation at 50 °C, cuticle layers were clearly observed because of almost perfect removal of melanin and protein components. Melanin had seemed to prevent the ob-

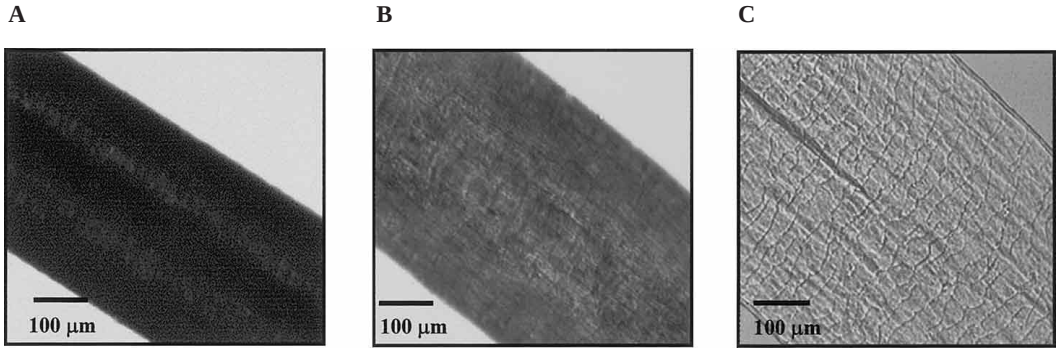

Fig. 3. Morphological Observation of Human Hair after Protein Extraction

Human hair was incubated at 50 °C with the solution of 25 mM Tris–HCl, pH 8.5, 2.6 M thiourea, 5 M urea and 5% 2-ME. (A) Control; (B) after 6 h of incubation; (C) after 2 d of incubation.

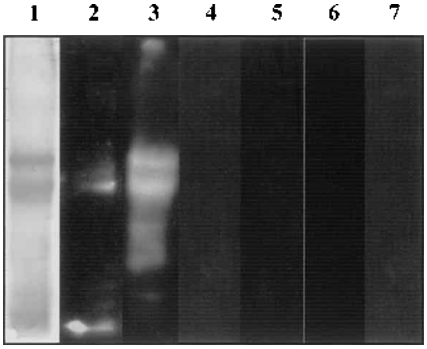

Fig. 4. Western Blot Analysis of Human Hair Proteins Using Antibodies against Phosphoamino Acids

Human hair proteins separated by SDS-PAGE (8—18% gel) were transferred to nitrocellulose membrane and stained with amide black (lane 1) or immunostained with antibodies against phosphoamino acid (lanes 2—7). Lane 2, phosphoserine antibody; lane 3, phosphothreonine antibody; lane 4, phosphotyrosine antibody; lane 5, second antibody only; lane 6, 0.2 mM phosphoserine+phosphoserine antibody; lane 7, 0.2 mM phosphothreonine+phosphothreonine antibody.

servation of cuticle layers after 6 h of incubation. These results suggest that our method is also effective for observation of the surface of hairs.

Identification of Phosphorylated Proteins Heid *et al.*[3] have shown the phosphorylation of pre-keratinized intermediate filaments only by labeling of proteins of the lower part of human, cow and sheep hair follicles with ^{32}P-phosphate. However, it is still not clear about post-translational modifications of hard α-keratins contained in a matured structure. Therefore, we studied whether human hair proteins contained phosphorylated species using phosphoserine, phosphothreonine and phosphotyrosine antibodies (Fig. 4). The keratins cross-reacted with phosphoserine and phosphothreonine antibodies. The reactivities of type I acidic keratins were more than those of type II keratins in both antibodies. Interestingly, matrix proteins cross-reacted with the phosphoserine antibody. The cross-reactions were not observed when phosphoserine (0.2 mM) or phosphothreonine (0.2 mM) was added to the first antigen–antibody reaction mixture, indicating the presence of phosphorylated forms of keratins and matrix proteins. The reactivity of proteins with phosphotyrosine antibody was not observed under our conditions.

The intermediate filament components desmin, vimentin, glial fibrillary acidic protein and neurofilaments have been reported to be phosphorylated by several protein kinases: cAMP-dependent protein kinase, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II.[18] The phosphorylation of these intermediate filaments can induce the depolymerization. Keratins including type I keratin 18 and type II keratin 8 from epithelial cells are also known to be phosphoproteins containing phosphoserine and phosphothreonine.[19] The phosphorylations of intermediate filaments-associated proteins, such as profilaggrin, are also known.[20] The dephosphorylation and partially proteolysis of profilaggrin to filaggrin occur during keratinization and the interaction of filaggrin with keratin filaments forms a keratin pattern in cornified epithelia. The biological function of matrix proteins whose serine contents are rich (9—23%) remains unclear during keratinization. The phosphorylation and dephosphorylation of hair keratins and matrix proteins may be involved in modulating the organization and structure of the microfibrillar layer by changing the state of keratin intermediate filaments.

Table 2. Comparison between Shindai Method and Conventional Method with Protein Extraction from Biomaterials Containing Hard α-Keratins

Sample	Yield (%)	
	Shindai method	Conventional method
Chicken feather	80	6
Rat hair	78	12
Wool	85	10
Human nail	81	5

The protein was extracted at 50 °C for 2 d using Shindai method or conventional method.

Application of the Shindai Method to Protein Extraction from Other Tissues We applied this procedure for protein extraction from chicken feathers, rat hair, wool and human nails. These samples were incubated with the solution of the Shindai method or with that of the conventional method at 50 °C for 2 d. The results of a typical experiment are summarized in Table 2. When the Shindai method was applied, more than 75% of protein was recovered in each protein fraction, while the yields were approximately 5—12% when thiourea was omitted from the extraction buffer. Figure 5 shows that these components consisted of keratins as reported previously.[11,21—23] Remarkably, protein hydrolysis did not seem to occur during these extraction procedures.

We established a rapid and simple procedure of protein ex-

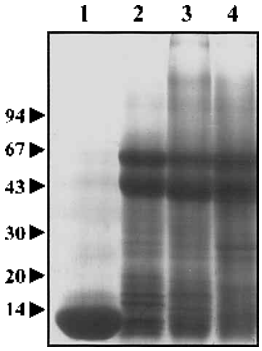

Fig. 5. SDS-PAGE of Various Keratinized Structures

The proteins extracted by the Shindai method were analyzed by 8—18% SDS-PAGE. Lane 1; chicken feather; lane 2, rat hair; lane 3, wool; lane 4, human nail. Molecular weight standards in kDa values are shown on the left.

traction from hard α-keratins containing biomaterials such as human hair and nail, feather and wool. The extracted proteins are directly analyzed using 2DE-EP, because the protein extract does not contain any ionic detergents. This novel method is available for studying not only the genetic variation and post-translational modification in the matured keratinized tissues but also for application in the textile, pelt, and cosmetic industries.

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