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A novel purification procedure for keratin-associated proteins and keratin from human hair

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The proteins in human hair consist primarily of microfibrillar keratins with a molecular mass of 40–65 kDa and keratin-associated proteins (KAPs) with a molecular mass of 6–30 kDa, according to the results obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Because an effective purification procedure of KAPs has not been established, little is known about the protein chemistry of KAPs as compared with that of keratin. When hair samples were incubated in the Shindai solution containing alcohols such as methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, and 2-methyl-1-propanol, the extraction of KAPs was enhanced, while extraction of keratin was suppressed. Using ethanol, we established a selective purification procedure for KAPs and keratin. According to Tricine/SDS-PAGE, the KAPs fraction contained six polypeptides with molecular masses of 3.5, 4.4, 5.2, 7.8, 15, and 28 kDa. The keratin fraction contained two polypeptides with molecular masses of 45 and 67 kDa and was free of low-molecular-weight components. The amino acid compositions of the KAPs and keratin fractions were mostly in agreement with the values found in the literature. The recoveries of the KAPs and keratin fractions from the hair samples were approximately 10 and 50%, respectively. Scanning electron microscopy (SEM) showed that hair samples retained fine fibrous structures in the cortex after extracting the KAPs and that the additional extraction of keratin caused the fibrous structures to disappear. These results indicated that KAPs may function by surrounding the fibrous structures and supporting the keratin fibers in the cortex. In this study, we propose a novel and convenient isolation procedure for KAPs and keratin from human hair.

Keywords: human hair, keratin-associated protein, keratin, purification, selective solubilization

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Introduction

The proteins in human hair comprise approximately 80% of the total mass of the hair and consist primarily of keratins, with a molecular weight of 40-65 kDa, and keratin-associated proteins (KAPs), with a molecular weight of 6-30 kDa, according to SDS-electrophoresis [1-4]. The keratin family can be further resolved into two subfamilies consisting of type I (acidic; 40-50 kDa) and type II (neutral/basic; 55-65 kDa) members. KAPs are classified based on their amino acid content into high-sulfur proteins, ultra-high-sulfur proteins, and high-glycine/tyrosine proteins. Rogers *et al*. have reported amino acid sequences obtained from the genetic analysis of KAPs as well as their detailed distribution within hair [5].

A number of investigations have focused on the biochemical properties of the keratins because they form the fibrous structures that are found in the hair cortex. On the other hand, there has been little investigation into the KAPs, which function in the amorphous space between the keratin fibers. Due to the damage that is caused to hair by bleaching or permanent wave processes, KAPs are interesting for hair care research. Kanetaka *et al.* have confirmed the elution of protein components, including sulfur and cysteic acid, when bleach-treated hair is immersed in a solution of 6% thioglycolic acid solution (TGA) as a reducing reagent [6]. Such eluted substances are presumed to be associated with KAPs, whose sulfur content is approximately 2.6 times that of keratin. Inoue *et al.* reported that when human hair was immersed in a solution containing 6% TGA, low-molecular-weight proteins (less than 15 kDa molecular weight) were observed in the solution [7].

We have developed the “Shindai method” to extract proteins from human hair easily and efficiently. This method delivers a high yield of solubilized proteins by using thiourea and urea as denaturants; the recovery was threefold higher than that of the conventional method using urea only [4]. The solubilized proteins consisted of keratin and KAPs. Previously, Gillespie reported a method of

separating KAPs and keratin from wool [1]. When the alkylated wool protein solution was mixed with zinc acetate at pH 5.8-6, the KAPs fraction was recovered as the filtrate. However, we are interested in assessing the KAPs and keratin fractions that did not undergo chemical modification occurring by this method. Kon *et al.* developed a selective isolation method for KAPs (matrix), keratin (microfibrils), high-molecular-weight proteins, and cuticles from human hair [8]. This method utilized a characteristic of KAPs that, when human hair was immersed in a 1% SDS solution containing 2 M 2-mercaptoethanol (2-ME), the KAPs were specifically solubilized from the hair samples. However, it was unknown why keratin did not elute despite the presence of the reducing agent.

We focused on the role of the hydroxyl group of 2-ME and therefore added various low molecular weight alcohols to the extraction solution. We found that elution of KAPs from hair increased with this treatment, while that of keratin was suppressed. In this study, the details of the solubilization of proteins from human hair by ethanol solutions were examined with respect to the reducing agents, pH, and temperature. By applying the findings, we propose a novel method to selectively isolate KAPs and keratin without using 2-ME or SDS.

Materials and Methods

Effect of alcohols on protein extraction from human hair

Human hair samples were obtained from numerous Japanese volunteers and did not have any chemical treatments such as bleach, hair dyes, or perms. The hair fragments were cut with scissors, mixed at 50 mg/ml with the extraction solution consisting of 25 mM Tris-HCl (pH 8.5), 2.6 M thiourea, and 5 M urea, 250 mM dithiothreitol (DTT), and diluted with distilled water or various alcohols (3 parts the extraction solution plus 1 part diluent) [4, 9]. After incubation for 24 h at 50℃, the samples were centrifuged at 12,000 g for 10 min at 25℃. The supernatants were recovered in test tubes and used to measure protein concentrations and for electrophoresis.

Fractionation of KAPs and keratin

KAPs and keratin were separated by combining reagents including a denaturant, a reductant, and ethanol. First, the hair fragments were incubated at 50 mg/ml with a "KAPs solution" consisting of 25 mM Tris-HCl (pH 9.5), 25% ethanol, 200 mM DTT, and 8 M urea for 72 h at 50℃. The solution was filtered and centrifuged at 12,000 g for 10 min at 25℃, and the supernatant was used as the KAPs fraction. The residue obtained from filtration was washed with distilled water and dried at room temperature. Keratin was extracted from the dried hair residue by suspending it at 60 mg/ml in the Shindai solution containing 200 mM DTT and incubating the mixture for 24 h at 50℃. This suspension was also filtered and centrifuged at 12,000 g for 10 min at 25℃, and the supernatant was used as the keratin fraction.

Scanning electron microscopy (SEM)

The morphology of the hair samples after treatment with the KAPs and Shindai solutions was examined by a scanning electron microscope (Neoscope JCM-5000, JEOL Ltd., Tokyo, Japan). The samples were placed on specimen mounts using double-sided adhesive tape and were made electrically conductive by coating them with a thin layer of gold in a vacuum. The images were collected at an excitation voltage of 10 kV and 500-fold magnification [9, 10].

Protein concentration and gel electrophoresis

The protein concentration was determined by the colorimetric Bradford method using a protein assay kit (Bio-Rad) [11], using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Tricine/SDS-PAGE were performed according to the Laemmli method [12], using a 5-20% gradient polyacrylamide gel, and the Schagger and Jagow method [13], using an 18% gel, respectively. Gels were stained with 0.1% Coomassie brilliant blue R-250, 10% acetic acid, and 40% ethanol for 2 h and destained in 10% acetic acid.

Amino acid analysis

The KAPs and keratin fractions were carboxymethylated using iodoacetic acid, hydrolyzed in 6 M HCl for 24 h at 110℃ under a nitrogen atmosphere, and dried by a rotary evaporator. The samples were analyzed on an automated amino acid analyzer (JLC-500/V, JEOL Ltd., Tokyo, Japan).

Results

Effects of alcohol on protein extraction from human hair

In our research on the applications of human hair, nail, and wool proteins, we first developed a rapid and convenient procedure, called the Shindai method, for protein isolation from biomaterials containing hard keratin [4, 14]. Briefly, in this method, human hair was incubated with the Shindai solution (25 mM Tris-HCl, (pH 8.5), 2.6 M thiourea, 5 M urea, and 250 mM DTT) at 50ºC for 1–4 days. After filtration and centrifugation, the supernatant was composed predominantly of keratin and KAPs, and no significant degradation of the protein components was observed.

Kon *et al.* found that a solution containing 2 M 2-mercaptoethanol (2-ME) and 1% SDS inhibited the dissociation of keratin from hair structures [8]. Based on this characteristic, they proposed a selective preparation procedure for keratin and KAPs from human hair samples indicating that the hydroxyl group of 2-ME was presumed to affect the interactions between keratin and the KAPs molecules. Thus, we prepared solutions containing 25% alcohol (methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, or 2-methyl-1-propanol) and DTT as a reducing agent, and we mixed this solution with hair samples. After incubation for 24 h at 50ºC, the solution was centrifuged at 12,000 *g* for 10 min at 25ºC. The supernatants were recovered, and the protein concentration was measured. The protein concentrations of the samples treated with the six types of alcohol were considerably lower than that of the samples treated with distilled water (Fig. 1A). Electrophoresis showed that the alcohol-extracted solutions from the ethanol, methanol, 1-propanol, 2-propanol, and 1-butanol treatments consisted primarily of KAPs, whereas

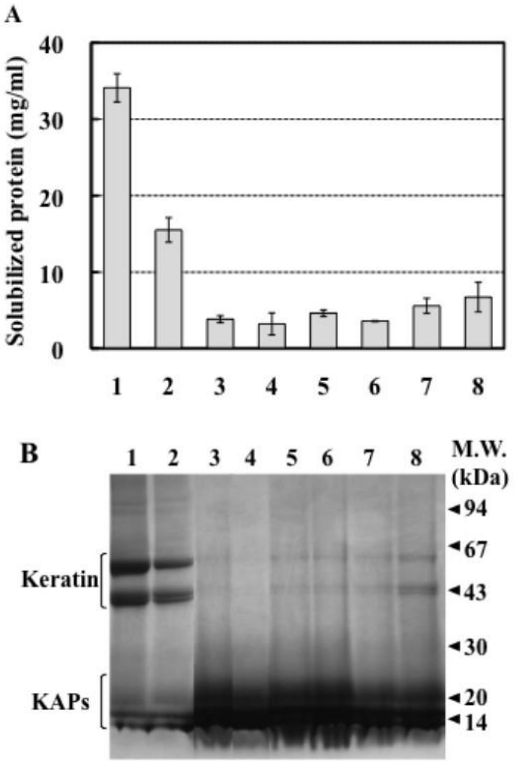


Fig. 1 Effects of various alcohols on the solubilization of proteins from human hair samples and on the solubilized protein components.

Hair samples were incubated with the extraction solution (#1) containing 25% distilled water (#2, control), 25% ethanol (#3), 25% methanol (#4), 25% 1-propanol (#5), 25% 2-propanol (#6), 25% 1-butanol (#7), and 25% 2-methyl-1-propanol (#8) at 50℃ for 24 h. The solution was recovered and centrifuged at 12,000 g for 10 min at 25℃. The supernatant thus obtained was used to determine the protein concentration (A) and was analyzed by 5-20% SDS-PAGE (B).

the non-alcohol solutions consisted of both keratin and KAPs (Fig. 1B). These results suggested that the presence of these alcohols not only inhibited the

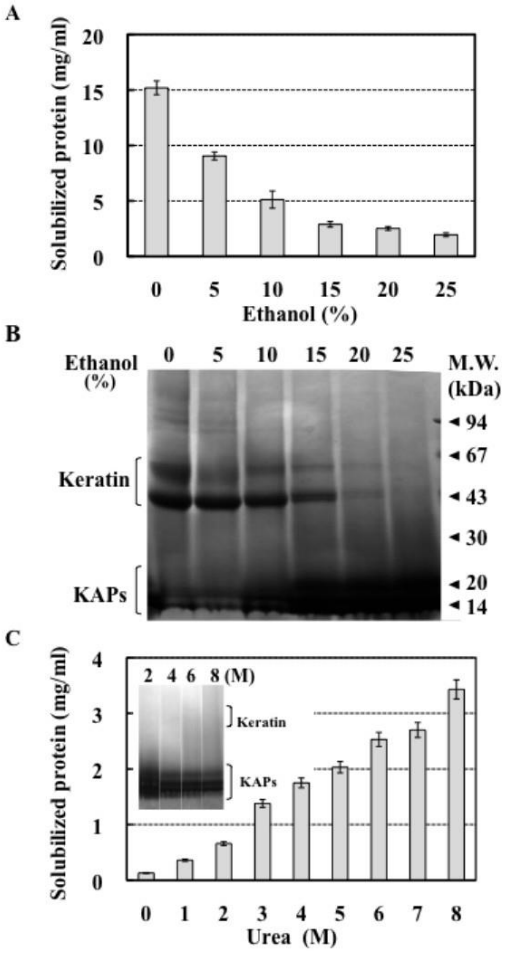


Fig. 2 Effects of the ethanol and urea concentrations on the solubilization of proteins from the hair sample.

Hair proteins were extracted with the solution containing 0-25% ethanol (A and B) and 0-8 M urea (C) at 50 ℃ for 24 h. After centrifugation at 12,000 g for 10 min, the supernatant was used to determine the protein concentration and was analyzed with 5-20% SDS-PAGE.

dissociation of keratin from the hierarchical architectures of the hair proteins but also induced the dissociation of KAPs from such macromolecules.

Characterization of KAPs extraction

Of the six types of alcohols that were tested, we selected ethanol for further experimentation because of its high safety profile. The quantities of solubilized protein upon changes in the ethanol concentration in the extraction solution were examined (Fig. 2A), and the keratin and KAPs contents were analyzed by SDS-PAGE (Fig 2B). The total solubilized protein decreased with increasing ethanol concentration. The

Table 1 Effects of the solution parameters on the amount of solubilized protein obtained from hair samples.

The standard solution contained 25 mM Tris-HCl (pH 8.5), 25% ethanol, 200 mM DTT, and 8 M urea and was incubated for 50℃ for 24 h. Hair proteins were extracted under varying conditions by changing the reducing agent (DTT and 2-ME), the pH (7.5, 8.0, 8.5, 9.0, and 9.5), and the temperature (30, 40, 50, and 60℃). After centrifugation at 12,000 g for 10 min, the supernatants were used to determine the protein concentration.

Factors			Solubilized protein (mg/ml)
Reducing reagent (mM)	DTT	100	2.1 ±0.2
		200	2.7 ±0.1
		300	2.9 ±0.3
	2-ME	100	0.1 ±0.01
		200	0.4 ±0.07
		300	0.6 ±0.04
pH		7.5	2.5 ±1.1
		8.0	3.2 ±1.2
		8.5	3.7 ±1.4
		9.0	4.0 ±1.7
		9.5	4.9 ±2.3
Temperature (℃)		30	1.1 ±0.6
		40	2.1 ±0.6
		50	3.7 ±0.5
		60	4.6 ±0.2

KAPs content increased at greater than 10% ethanol, while the keratin content decreased steadily and almost disappeared at 25% ethanol. Because 25% ethanol is calculated to be 4.3 M, approximately two times the concentration of ethanol was required as for 2-ME [8].

Figure 2C shows the effect of urea concentration on the protein solubilization in the presence of 25 mM Tris-HCl (pH 8.5), 25% ethanol, and 200 mM DTT. The quantity of solubilized protein increased linearly with the urea concentration. The protein concentration (2 mg/ml) at 5 M urea was similar to that obtained by the Shindai solution, indicating that thiourea (2.6 M) will not contribute to the extraction of KAPs from hair samples. The solubilized protein concentration obtained from the solution containing 25 mM Tris-HCl (pH 8.5), 25% ethanol, 200 mM DTT, and 8 M urea was 1.7 times higher than that from the alcohol-diluted solution containing 25% ethanol and 200 mM DTT (3.4 mg/ml versus 2 mg/ml, respectively). All protein fractions recovered at 1 to 8 M urea consisted of only the KAPs solution.

Various conditions for the extraction of KAPs, including different reducing agents, pH, and temperatures, were examined and are presented in Table 1. In the solution consisting of 25 mM Tris-HCl (pH 8.5), 25% ethanol, and 8 M urea, the addition of DTT was more effective than that of 2-ME (5-20 fold), indicating that DTT is a stronger reductant than 2-ME. The quantity of recovered protein was increased by increasing the pH value of the solution (pH 7.5-9.5). When the incubation temperature was changed over the range from 30 to 60℃, the quantity of recovered protein increased with the increase of temperature. Therefore, the solution consisting of 25 mM Tris-HCl (pH 9.5), 25% ethanol, 200 mM DTT, and 8 M urea was identified as the KAPs extraction buffer and named the KAPs solution. When hair samples were incubated with the KAPs solution at 50 mg/ml for 24, 48, or 72 h at 50℃, the quantity of KAPs was 3.5, 5.6, and 6.9 mg/ml, respectively. The protein concentration was almost saturated at 72 h (3 days). Taken together, we considered this to be a selective method

for extracting KAPs from human hair.

Selective fractionations of KAPs and keratin from human hair

Using the effects of ethanol on protein solubilization from human hair, we attempted to establish a convenient method for the fractionation of KAPs and keratin from human hair (Fig. 3A). First, the hair samples (1 g) were cut

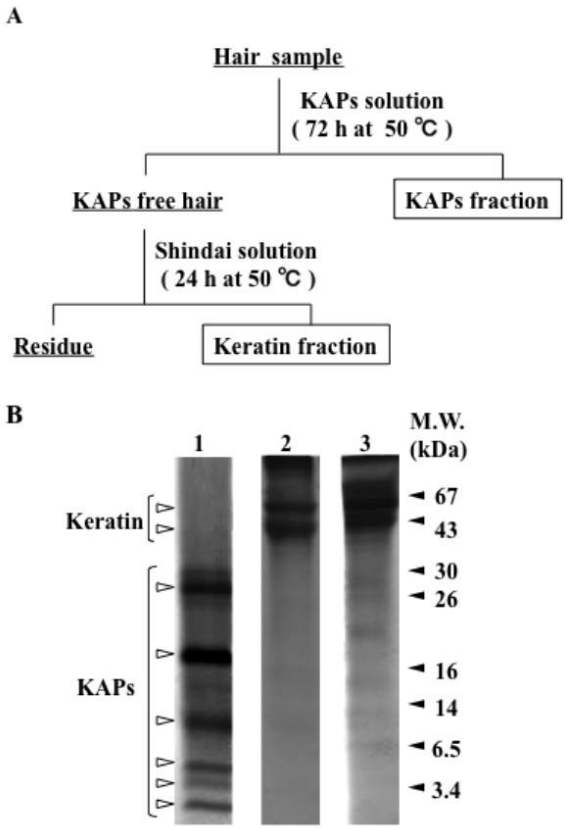


Fig. 3 Scheme for the fractionation of KAPs and keratin from human hair.

Human hair fragments (1 g) were cut with scissors and immersed in 20 ml of the KAPs solution. After incubation for 72 h at 50℃, the solution was filtered and centrifuged at 12,000 g for 10 min, and the supernatant was used as the KAPs fraction (Lane 1). The residue was washed with distilled water and used as KAPs-free hair. The washed hair was further incubated with the Shindai solution at 50℃ for 24 h, to extract the keratin (Lane 2) (A). As a control experiment, human hair was also incubated with the Shindai solution at 50℃ for 24 h, and a mixture of keratin and KAPs was extracted (Lane 3). The protein components were analyzed by Tricine/SDS-PAGE (B).

with scissors and incubated with the KAPs solution (20 ml) at 50℃ to solubilize the KAPs. After incubation for 72 h with shaking, the hair fibers became an aggregate like muddy paste and were filtered. The filtrate was centrifuged at 12,000 g for 10 min at room temperature, and the supernatant was recovered and used as the KAPs fraction. The residue from the filtration was thoroughly washed with distilled water and dried at room temperature. The aggregates thus obtained were used as the KAPs-free hair sample. We then used the Shindai solution (25 mM Tris-HCl (pH 8.5), 2.6 M thiourea, and 5 M urea containing 200 mM DTT) for the solubilization of keratin from the KAPs-free hair samples. The incubation was performed at 50℃ for 24 h, and then, the suspension was filtered [9, 10]. The filtrate was centrifuged at 12,000 g for 10 min, and the supernatant was recovered and used as the keratin fraction.

The fractions thus obtained were analyzed by Tricine/SDS gel electrophoresis (Fig. 3B). Our KAPs

Table 2 Amino acid compositions of the hair proteins.
Hair protein fractions were prepared as described in Fig. 3. The amino acid composition is expressed as the number of residues per 100 residues.

	This study		Literature[7]	
	KAPs frac.	Keratin frac.	KAPs	Keratin
Aspartic acid	2.4	7.9	2.9	8.3
Threonine	10.4	5.9	10.7	6.9
Serine	12.5	9.3	12.0	8.0
Glutamic acid	8.5	15.8	8.4	17.2
Proline	12.7	5.4	12.3	5.1
Half-Cystine	23.9	9.3	23.5	9.0
Glycine	6.1	5.4	6.2	5.2
Alanine	2.1	6.2	2.0	6.3
Valine	5.2	6.1	5.5	6.0
Methionine	0.3	0.6	0.0	1.1
Isoleucine	1.7	3.6	2.0	3.6
Leucine	3.0	9.2	3.3	9.2
Tyrosine	1.9	2.8	1.6	2.5
Phenylalanine	1.3	2.0	1.4	2.0
Histidine	1.0	0.9	1.0	0.7
Lysine	0.6	2.4	0.6	3.5
Arginine	6.2	7.5	6.6	5.4

fraction consisted of seven polypeptides with molecular masses of 3.5, 4.4, 5.2, 7.8, 15, and 28 kDa according to the electrophoresis. This fraction did not contain significant amounts of keratin and other high-molecular-weight proteins. However, the keratin fraction, which did not contain significant amounts of KAPs, consisted primarily of keratin type I and II polypeptides. From 1 g of hair samples, approximately 120 and 510 mg of protein were recovered in the KAPs and keratin fractions, respectively.

As a control, we also prepared a hair protein fraction (KAPs + keratin) by using the Shindai solution containing 250 mM DTT as previously described [9, 10]. The hair protein solution contained both keratin type I and II polypeptides and low-molecular weight KAPs polypeptides (Fig. 3B). The recovery from the hair protein fraction was 610 mg, which was mostly in agreement with the summation of the KAPs and keratin fractions.

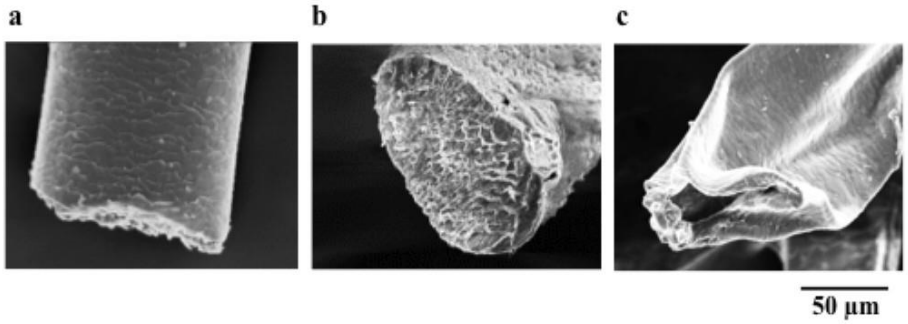
Amino acid composition of the KAPs and keratin fractions

Because both keratin and KAPs are considered to be multi-protein polypeptides, we examined their amino acid compositions to identify them. The half-cystine content of the KAPs fraction was 2.5 times higher than that of the keratin fraction (Table 2). The contents of aspartic acid, threonine, glutamic acid, proline, alanine, and leucine differed between the KAPs and keratin fractions. The amino acid compositions of the KAPs and keratin fractions in this study were mostly in agreement with those in the literature [8]. Based on these results, KAPs and keratin could be fractionated by using a combination of different solutions, and the obtained samples were of adequate purity.

SEM observation of hair samples after selective extraction

Morphological change of hair samples was observed by scanning electron microscopy. The surface of hair samples after treatment of KAPs solution, that is, KAPs free hairs in the lateral direction was retained cuticle structures and apparently unchanged as untreated hairs. However, the sectional

Fig. 4 Morphological observation of the hair samples after protein extraction.
(a) Untreated hair, (b) KAPs-free hair, and (c) KAPs-free hair residue after extraction by the Shindai method.



view indicated a number of fibers with several micrometers in diameter were projected from the cortex region (Fig. 4a and 4b). It seemed that the glue substance surrounding the cortex microfibers had removed and disappeared. After further treatment with the Shindai solution, the form of the hair samples was irregular and became flat and the sectional view showed the fibrous structures had disappeared (Fig. 4c). On the other hand, the cuticle was resistant to these treatments and the structure was maintained [4, 8].

Discussion

In this study, we established a novel purification method for KAPs and keratin from human hair. Previously, an alternative isolation technique for KAPs and keratin was reported that utilized their differential solubilities in different concentrations of 2-ME [8]. In this conventional method, human hair samples were incubated with the buffer (25 mM Tris-HCl (pH 8.3), 2 M 2-ME, and 1% SDS) for 72 h at 50℃, and the KAPs were selectively released and recovered in the supernatant after centrifugation. After the extraction of the KAPs, the hair sample was further incubated with the buffer (25 mM Tris-HCl (pH 8.3), 0.4 M 2-ME, and 1% SDS) for 144 h at 50℃, and the keratin was recovered. Compared with the conventional method [8], there are three advantages to our method. ① SDS, a commonly used detergent, has been known to interfere with chemical and biological

analyses, and its complete removal is difficult. No detergent was used in our solution. ② The KAPs solution contained ethanol and DTT, while 2-ME and SDS were used in the conventional method. In Japan, 2-ME has been designated as a toxic substance since 2008. ③ Processing by our method was complete within five days, while six to twelve days were required for all of the operations in the conventional method.

Some types of hair damage are thought to arise from structural changes to KAPs. Kon *et al.* applied their method for the analysis of protein composition in hair samples and found that the keratin content had decreased in the end and middle regions of hair samples after perm treatment [8]. Inoue *et al.* reported that low-molecular-weight proteins such as S100A3 and ubiquitin were eluted from perm-treated hair [7, 15]. This phenomenon will be a useful index of hair damage. We have developed a convenient procedure for preparing a keratin film consisting primarily of keratin and KAPs from human hairs [16]. The keratin film can be used as a hair alternative to accurately evaluate the effects of reductive damage from UV irradiation, perm, and heat treatments [9, 10, 17, 18]. As with hair samples, reductive treatment by TGA caused the selective release of KAPs from the keratin film, and the amount of protein that was eluted was two thousand times greater than the amount eluted from hair samples [17, 18]. Procedures have been developed for the preparation of films and sponges from wools and human tissues containing keratins and their related proteins [19]. Cellular adhesion and proliferation of mouse fibroblasts on keratin substrates was comparable to those on collagen materials [20]. Keratin sponges were also used as scaffolds for long-term cell cultivation [21]. In mammalian hair, KAPs are believed to control the steric configuration of the keratin filaments. Recently, Fujikawa *et al.* reported that KAP2, one of the high-sulfur KAPs, was prepared by a gene expression system and induced self-aggregation and interacted with the head domain of the keratin molecule [22]. Because the molecular interactions of KAPs with keratin and other KAPs have not yet

been fully studied, the method presented in this paper will be useful for analyzing protein architectures in human hair tissue in the future.

Acknowledgements

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