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Article

Preparation and properties of protein films and particles from chicken feather

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A convenient procedure for protein extraction from hair, nail, and wool were applied for chicken feathers. The velocities of protein extraction by the Shindai method were 1.1–1.8 fold faster than those by the conventional method. The protein components mainly consisted of β-keratin. Protein films with white color were formed from β-keratin solution by Pre-cast, Post-cast, and soft Post-cast methods, which were effectively applicable for the conversion of α-keratin into protein films. The protein recovery from solution to solid was 60–80%. Interestingly, films were formed under the conditions at high pH and low concentrations of $MgCl_2$ and KCl where no film formation occurred in the case of hair, nail, and wool proteins. Scanning electron microscopy (SEM) showed that the surface of the translucent protein films was smooth, while that of white films was rough with porous structure and was composed of aggregated small particles. The protein particles were easily prepared from the films. The distribution of the particles was mono-disperse with the average diameter of 0.5–3 μm. These results suggested that the procedures for protein extraction and preparations of film and particle from hair α-keratin could be also effective for chicken β-keratin.

Keywords: feather keratin, protein extraction, film formation, protein particle

Introduction

Keratin is a member of intracellular protein filaments contained in hair, nail, wool, skin, and feather. The analyses of the amino acid components indicated that the cysteine content of keratins was remarkably high (6–17%), compared with the other general proteins. Keratin was classified into α- and β-keratins. Feather is mainly made from β-keratin with molecular mass of 10 kDa. On the other hand, α-keratin consists of two subfamilies, which are acidic keratins with 40–50 kDa (type I) and neutral/basic ones with 55–65 kDa (type II) [1]. The cysteine contents of chicken feather and human hair were approximately 7–8% and 17–18%, respectively [1,2]. Thus, the biomaterial properties of α- and β-keratins were quite different.

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In Japan, chickens have been consumed several hundred million per year and most of their feather was abandoned as garbage. Therefore, recycling of the feather is desirable as a valuable biomaterial resource. Martelli *et al.* [3] reported that β-keratin from chicken feather was extracted using urea, 2-mercaptoethanol, and SDS and that the protein films were prepared by casting the solutions after dialysis against distilled water. Schrooyen *et al.* [4] showed that the mechanical and physiological properties such as tensile strength, elongation, humidity, and crystallinity of feather keratin films depended on the degrees of carboxymethylated cysteine. We developed a rapid and convenient method for extracting protein from hair, nail, and wool (Shindai method) [5] and three kinds of methods (Pre-cast, Post-cast, and soft Post-cast methods) to form films from the protein solution containing hard α-keratin without chemical modification [6,7]. Recently, we reported the

preparation of hair protein particles from the films [8,9].

In this study, we applied these procedures to chicken feather to prepare protein solution, films and particles. The properties of the films and particles were also examined.

Materials and Methods

Preparation of chicken feather proteins

Feather proteins were extracted from ethanol-treated chicken feathers by Shindai method at 50°C for 24 h employing 20 mM Tris-HCl (pH 8.5), 2.6 M thiourea, 5 M urea, and 5%(v/v) 2-mercaptoethanol. The extraction was also carried out by the conventional method employing 20 mM Tris-HCl (pH 8.5), 8 M urea, and 5%(v/v) 2-mercaptoethanol [5]. After filtration and centrifugation of the extracted solution, the obtained protein solution was used to form films. Human hair protein solution was also prepared by the Shindai method for comparison.

Preparation of chicken feather protein films and particles

Pre-cast, Post-cast, and soft Post-cast methods were used for the formation of protein films [6,7]. As for Pre-cast method, the proteins in Shindai solution were premixed with guanidine-HCl (GHA) or trichloroacetic acid (TCA) or perchloric acid (PCA), and then they were poured into tissue culture dishes containing distilled water to form films. For Post-cast method, the protein solution was directly cast in tissue culture dishes containing 10 ml solution of GHA, TCA, PCA, and/or 100 mM acetate buffer (pH 4–6). For soft Post-cast method, the solution of chloride salt such as KCl, NaCl, $MgCl_2$, and $CaCl_2$ was used instead of those for Post-cast method. After standing for 0.5–1 h at room temperature, a membrane-like protein aggregate was formed and washed by rinsing with water for over 24 h and then replaced with distilled water for 6 h. The films were thoroughly dried in a silicagel box and the protein recovery as the film was calculated by weighing using an electronic balance.

The protein aggregates were recovered from wet films prepared by Pre-cast method (5% GHA), Post-cast method (5% GHA and 100 mM acetate buffer, pH 5), and soft Post-cast method (40 and 500 mM KCl). The protein particles were prepared from the aggregates by mechanical stress using a sonicator as described previously [8,9].

The protein concentrations were determined according to Bradford using bovine serum albumin as the standard [10].

Electrophoresis

Tricine/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine/ SDS-PAGE) was carried out on 10/20% discontinuous polyacrylamide gel according to the method of Schagger and Jagow [11]. Films were incubated with the Shindai solution for 1 day at 50℃ and the components were analyzed by Tricine/SDS-PAGE. Proteins in the gel were stained with 0.1% Coomassie brilliant blue R-250, 10% acetic acid and 40% ethanol for 1 h and destained in 10% acetic acid and 40% ethanol.

Scanning electron microscopy (SEM)

The morphology of the protein films was examined with Hitachi S-2380N scanning electron microscope. The samples on brass stubs using double-sided adhesive tape were made electrically conductive by coating in a vacuum with a thin layer of gold. The pictures were taken at an excitation voltage of 10 kV and a magnification of 1500× [6].

Particle size analysis

The diameters of the protein particles in the suspension and their distribution were measured using dynamic light scattering system (Zetasizer Nano-ZS, Sysmex). The mechanical stimuli were done at 8 mg/ml in distilled water, and the protein particle solution was diluted to 0.4 mg/ml in distilled water. The average particle size was expressed as both the number average diameter and the volume average one.

Results and Discussion

Preparation of protein solution and protein compositions

Figure 1A shows time courses of the protein extraction from feather vane and shaft using the Shindai and conventional methods. The protein extraction by the Shindai method almost reached to the saturation level within 1–2 h, while it required more than 8 h for the conventional method. Especially for feather shaft, the extraction efficiency

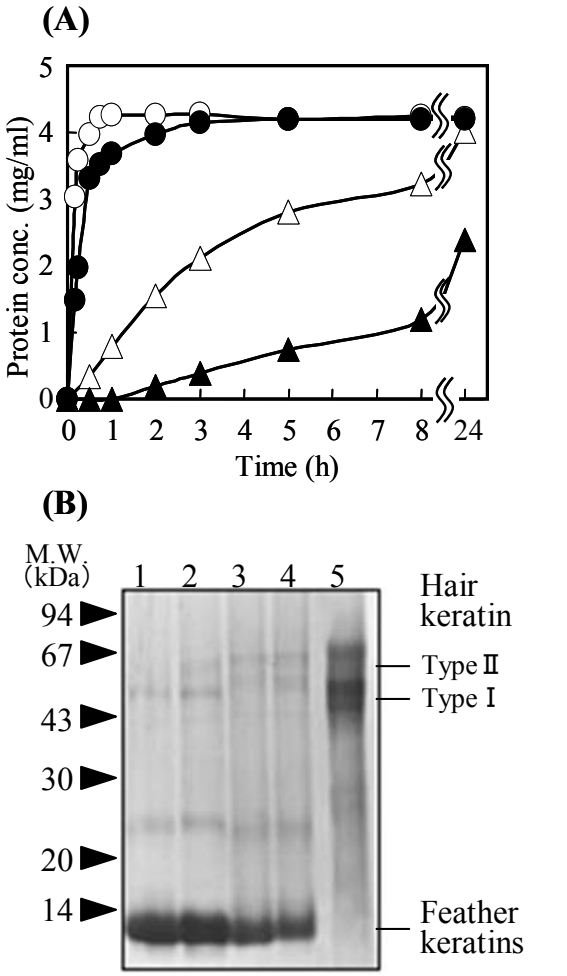

Figure 1. Time course of the extraction of protein from chicken feather vane and shaft and electrophoretical analyses. (**A**) The protein was extracted from feather vane (○,△) and shaft (●,▲) at 50°C using the Shindai method (○, ●) or the conventional method (△, ▲) and aliquots were measured for protein concentration. (**B**) The chicken feather vane and shaft proteins extracted were subjected to 10% Tricine/SDS-PAGE. Lane 1, Shindai method, feather vane; lane 2, Shindai method, feather shaft; lane 3, the conventional method, feather vane; lane 4, the conventional method, feather shaft; lane 5, Shindai method; human hair.

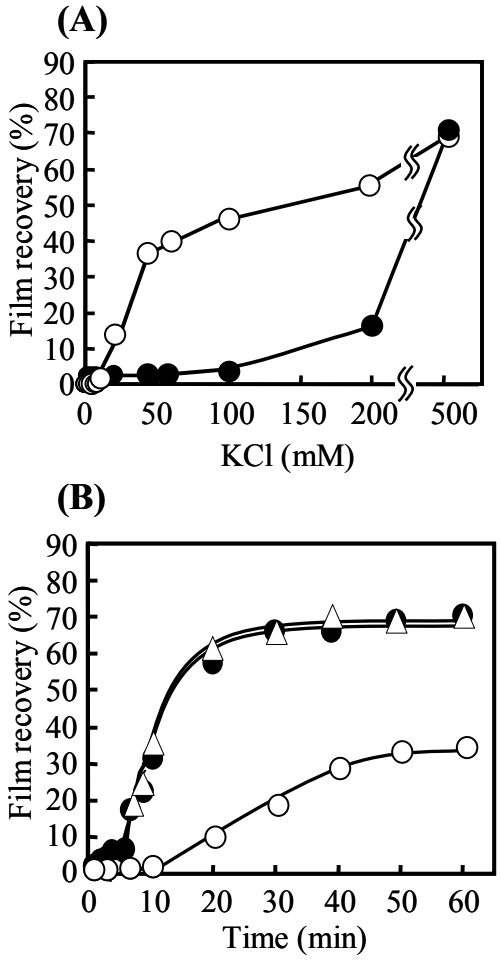

Figure 2. Effect of KCl concentration on the film recovery and time course of film formation. (**A**) The films were prepared by soft Post-cast method. Feather (○) and hair (●) protein solutions were directly exposed to various concentrations of KCl as indicated. (**B**) Feather protein solution was exposed to the developing solutions containing 40 mM KCl (○), 500 mM KCl (●), and 100 mM acetate buffer, pH 5 (△). After storing for 1 h (**A**) and various times as indicated (**B**), the resulting protein films were washed and the quantities were calculated by weighing.

was remarkably improved by the Shindai method in comparison with the conventional method. Thus, Shindai method is effectively applicable for protein extraction from hard tissues. About 80–90% of feather vane and shaft were converted into protein solutions.

The obtained proteins were analyzed by Tricine/SDS-PAGE (Fig. 1B). Proteins from feather vane and shaft by the two methods were mainly composed of β-keratin with a molecular mass of 10 kDa, as reported previously [12]. We could not find any significant difference among them. Furthermore, the composition of amino acids was almost same between in vane and shaft proteins (data not shown)

Chicken feather keratin consists of 97 amino acid residues [2]. From the amino acid sequence, pI value was calculated to be 7.8, while the values of human keratin type I and II were 4.8–6.0 and 5.9–8.5, respectively. When the sequences were compared using CLUSTALW, the identity were 13%

Method	Solvent	Chicken feather		Human Hair
		Vane	Shaft	
		Recovery (%)		
Pre-cast	5% GHA	75 ± 1	80 ± 1	76 ± 5
	5% TCA	69 ± 2	73 ± 1	86 ± 1
	5% PCA	70 ± 3	80 ± 1	79 ± 2
Post-cast	5% GHA	55 ± 5	60 ± 1	91 ± 5
	5% TCA	85 ± 3	81 ± 1	82 ± 2
	5% PCA	85 ± 2	82 ± 0	74 ± 1
	100 mM Acetate buffer, pH 4.0	60 ± 2	68 ± 5	82 ± 4
	100 mM Acetate buffer, pH 5.0	68 ± 4	70 ± 4	81 ± 6
	100 mM Acetate buffer, pH 5.5	65 ± 2	68 ± 2	0 ± 0
	100 mM Acetate buffer, pH 6.0	65 ± 1	68 ± 1	0 ± 0
soft Post-cast	40 mM KCl	38 ± 1	40 ± 2	0 ± 0
	500 mM KCl	65 ± 1	71 ± 1	73 ± 5
	40 mM NaCl	40 ± 0	51 ± 0	0 ± 0
	500 mM NaCl	60 ± 1	67 ± 1	74 ± 7
	40 mM $MgCl_2$	65 ± 2	70 ± 3	92 ± 4
	40 mM $CaCl_2$	77 ± 0	83 ± 3	98 ± 2

Table 1. Recoveries of proteins in films from chicken feather
Chicken feather (10 mg) and human hair protein (6 mg) films were prepared by Pre-cast, Post-cast, and soft Post-cast methods and the quantities of protein recovered as the films were calculated with their weights. The "recovery %" represents the ratio of the protein amount in a formed solid film to that in a cast solution.

(hHa4 of type I) and 16% (hHb6 of type II) [13,14], respectively, indicating that both keratins were quite different in the primary structure. Our results suggest that the Shindai method is effective for the extraction of proteins irrespective of primary structure of keratins. In addition, it was found useful even for the proteins with high contents of disulfide bond.

Preparation of protein film

Based on the conventional method, we developed the method to prepare films from the protein solution of hair, nail, and wool mainly consisting of α-keratin. Then, we established the methods named Pre-cast, Post-cast, and soft Post-cast methods [5,6]. Table 1 shows the amount of protein recovered as film consisting of feather and human hair proteins using these methods. The film-like aggregates were formed from the feather protein solutions like hair protein solution. The recoveries of feather proteins as films were 40–85% and somewhat lower than the values for hair proteins. No significant difference was found between the vane and the shaft [5,6], so that the subsequent experiments were undertaken by using the mixture of feather vane and shaft. Interestingly, feather proteins formed the film at slightly acidic pH and lower concentrations of KCl or NaCl. Under these conditions hair protein solution could not be converted to the film.

The film formation of feather and hair proteins was examined as a function of KCl concentrations (Fig. 2A). Feather protein films were generated at the developing solution more than 20 mM KCl, whereas higher concentrations of KCl more than 200 mM were required for film formation from hair proteins. These two proteins were quite different in the sensitivity for KCl in applying soft Post-cast method. Recently, we found that hair α-keratin films prepared by soft Post-cast method (50 mM $CaCl_2$) significantly interacted with Ca^{2+} after washing enough them with distilled water (unpublished data). It can therefore be presumed that α- and β-keratins contain direct binding sites for K^+ and the bindings promote the conversion from the solution to the film.

Next, we examined the time course of film formation of the feather proteins when KCl (40 and 500 mM) and acetate buffer (pH 5) were used as the developing solution (Fig. 2B). At 500 mM KCl or

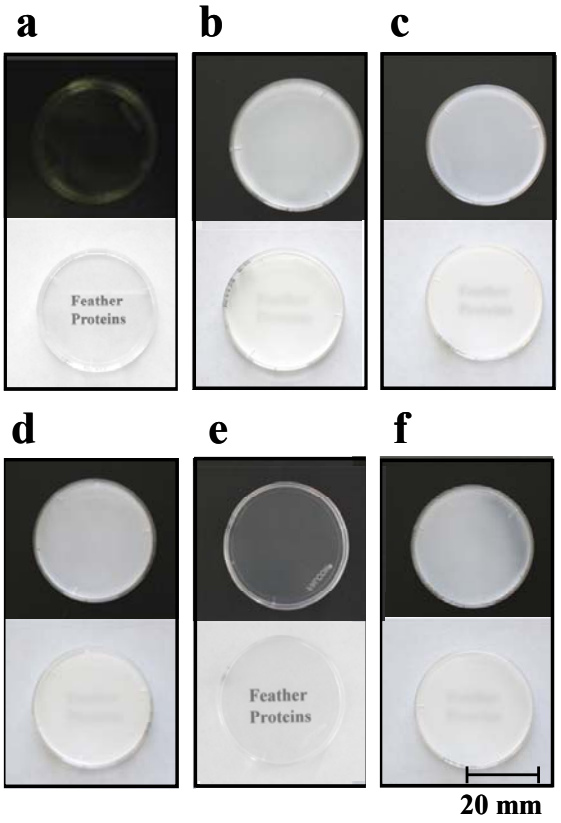

Figure 3. Photographs of feather vane protein films by obtained by Pre-cast, Post-cast, and soft Post-cast methods. **a**, Control (tissue culture dish); **b**, Pre-cast method (5% GHA); **c**, Post-cast method (5% GHA); **d**, Post-cast method (100 mM acetate buffer, pH 5); **e**, soft Post-cast method (40 mM KCl); **f**, soft Post-cast method (500 mM KCl).

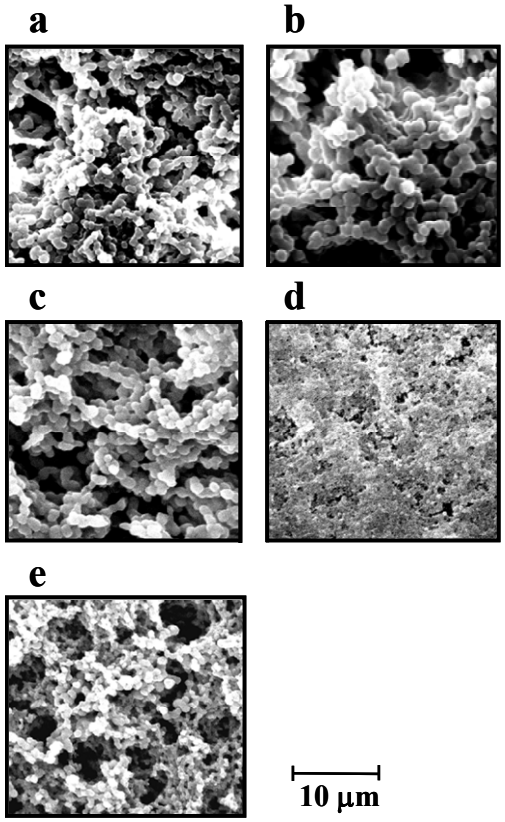

Figure 4. Morphological observation of feather protein films. **a**, Pre-cast film (5% GHA); **b**, Post-cast film (5% GHA); **c**, Post-cast film (100 mM acetate buffer, pH 5); **d**, soft Post-cast method (40 mM KCl); **e**, soft Post-cast method (500 mM KCl).

acetate buffer (pH 5), the half-times required for the maximal film formation were almost 10 min, whereas the time was fairly longer at 40 mM KCl. Similar phenomena were observed when NaCl was used in place of KCl (data not shown). The curves also showed the presence of a lag time for film formation. It may be due to the formation of nucleation center in a similar way as the assembly of microtubules.

The addition of plasticizers such as sorbitol, glycerol, and polyethylene glycol to feather keratin films prepared by casting method caused the increase of moisture content and water vapor permeability [3]. Thus, we examined the effect of sorbitol and glycerol (10–50 mg/g of feather protein) to the films prepared by Post-cast (acetate buffer, pH 5) and soft Post-cast (40 and 500 mM KCl) methods. These reagents had little effect on the flexibility of the films (data not shown).

Morphological observations of the protein films

When the films containing approximately same amount of protein were prepared on tissue culture dishes, the films by soft Post-cast method using 40 mM KCl was clear enough to show the letter on the paper after drying, whereas the films made by the other conditions were white and opaque (Fig. 3). We attempted to image the films using SEM (Fig. 4). The fine structure of the white films prepared by Pre-cast method (guanidine-HCl), Post-cast method (GHA and acetate buffer, pH 5), and soft Post-cast method (500 mM KCl) exhibited aggregates of particles with a diameter of 0.5–3 μm and formed heterogeneous porous surface. On the other hand, the surface of the translucent films prepared by soft Post-cast method (40 mM KCl) was smooth and the particles over ca. 0.5 μm in diameter were not found. Similar images were observed in the human hair films [6,7].

Protein compositions of the films

The protein films by Pre-cast (5% GHA), Post-cast (100 mM acetate buffer pH 5), and soft Post-cast (40 and 500 mM KCl) methods were stored in the dry state for 4–6 weeks at room temperature. The protein of the films was re-extracted using the Shindai solution for 1 day at 50 ℃, and the components were analyzed by Tricine/SDS-PAGE (Fig. 5). The extracted solution of all the films mainly consisted of β-keratin and no significant difference in composition was found among the films examined here. Furthermore, protein hydrolysis was not found during the film formation nor during the storage period, indicating that feather keratin was remarkably stable after forming film-like aggregates like hair and wool keratins.

Preparation and properties of protein particles

Recently, we reported a novel preparation method of protein particles from human hair protein films and used the suspension as a blood analog fluid [8,9]. A particle suspension was easily prepared from the wet feather protein films by mechanical fracture with a sonicator and the size distribution of each protein suspension was measured with Zeta-Sizer. The average diameters were 1.9–2.6 μm without the particles (0.6 μm) prepared by soft Post-cast method using 20 mM KCl (Table 2). When soft Post-cast method was used in film formation, the average diameters of the particles increased with increasing the concentration of KCl (Fig. 6A). This seems to correlate directly with the fine structure of the films as shown in Fig. 4, since the film prepared at 40 mM KCl consists of smaller particles than the film obtained at 500 mM KCl. In the hair protein particles [9], the number-average size was apparently distributed in a monodisperse manner, while two peaks were observed in the distribution of volume-average size. On the other

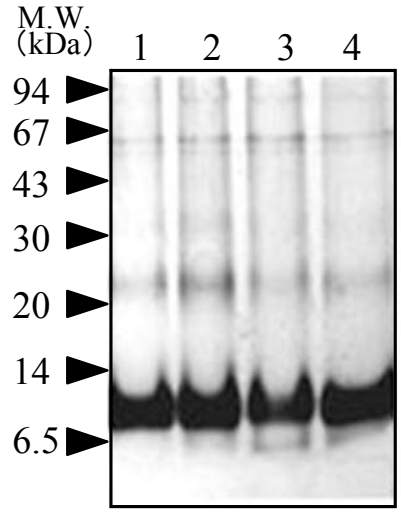

Figure 5. Tricine/SDS-PAGE of the extracted proteins from feather protein films. Extracted proteins were analyzed by 10% Tricine/SDS-PAGE. Lane 1, Pre-cast film (5% guanidine-HCl); lane 2, Post-cast film (100 mM acetate buffer, pH 5); lane 3, soft Post-cast film soft Post-cast method (40 mM KCl); lane 4, soft Post-cast method (500 mM KCl).

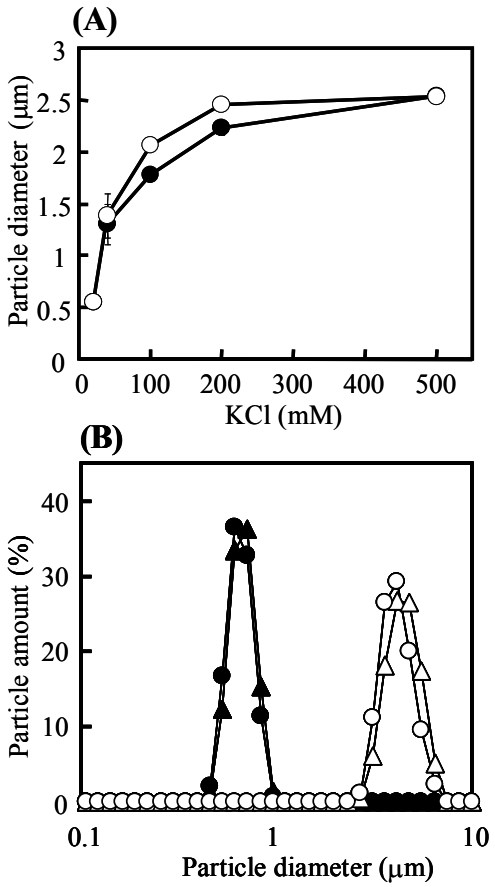

Figure 6. Effect of KCl on average diameter of protein particles and particle diameter distribution. (**A**) Feather protein particles were prepared from the films formed at various concentrations of KCl as shown in Fig. 2. After treatment of the protein suspension at 8 mg/ml by sonication, the particle diameters were measured at the concentration of 0.4 mg/ml. The plots exhibited the number average diameters (○) and volume ones (●). (**B**) The size distributions of the particles from the films formed at 20 mM (●,▲) and 500 mM KCl (○,△) were exhibited. The plots for number-average and volume-average diameters are shown with circles (○, ●) and triangles (△,▲), respectively.

Method	Solvent	Average particle diameter (μm)	
		N	V
Pre-cast	5% GHA	1.85 ± 0.02	2.08 ± 0.04
Post-cast	5% GHA	2.39 ± 0.05	2.61 ± 0.09
	100mM Acetate buffer, pH 5.0	2.00 ± 0.02	2.11 ± 0.02
soft Post-cast	20mM KCl	0.55 ± 0.05	0.55 ± 0.05
	500mM KCl	2.50 ± 0.01	2.53 ± 0.04

Table 2. Average diameter of feather protein particles
Chicken feather protein films prepared by three methods were disintegrated in distilled water using a sonicator at the 85% intensity of full power for 1.5 min ×8 times. Samples were diluted to 0.4 mg/ml in distilled water and the particle diameters were measured. The number average diameters and the volume average ones indicated in the columns denoted with 'N' and 'V', respectively.

hand, in the feather protein particles, the values of number-average and volume-average diameters were highly close, indicating that they are monodisperse protein particles (Table 2 and Fig. 6B). The reason for this difference will be caused the distinct biochemical characteristics of both keratins, that is, the molecular mass and cysteine content of feather β-keratins were lower than those of α-keratins in hair, nail, and wool [1,2].

In this report, we applied some processing methods developed in human hair, nail, and wool to chicken feather. It was found that our methods are convenient to prepare not only the protein solution but also the protein materials with the form of films and/or particles without chemical modification.

Acknowledgments

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