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Article

Preparation and properties of protein particles from human hair

Toshihiro Fujii,* Kaoru Nakai, Hideto Kikuta, Shunichi Kobayashi, and Toshihiro Hirai

Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-8567, Japan

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Protein microspheres were conveniently prepared from human hair without chemical modification. The particle size was dependent on the mechanical stimuli and the average diameter was approximately 0.5 μm when the wet protein films were homogenized by mechanical stimuli using a Polytron and sonicator. The protein particles mainly consisted of α-keratin types I and II. Confocal microscopic observations showed that the particles were irregular in shape and each particle consisted of smaller particle aggregates. The average size of the protein particles slightly increased when the ionic strength of the solution was high, whereas the dispersion of the particles was hardly affected by divalent cations, pH, and temperature.

Keywords: human hair, protein particle, keratin, individual product

Introduction

Collagen, a representative component of extracellular matrix proteins, and its denatured substance, gelatin, appear to be good sources for protein particles. Chowdhury and Mitra reported that low- (deoxyuridine) and high- (FITC-dextran) molecular-weight materials were entrapped in the collagen and collagen/gelatin microspheres using glutaraldehyde [1]. The binding of retinol to calf collagen microspheres caused the effective transport in mouse skin [2]. Balthasan et al. showed that gelatin nanoparticles attached to the anti-CD3 antibodies using the avidin-biotin system exhibited a slightly toxic effect at low concentrations [3]. Rising concern of bovine spongiform encephalopathy (BSE) resulted in the restricted use of a number of proteins including collagen, gelatin, casein, and albumin that have been used in the food, medical, and cosmetics fields. In order to avoid BSE, proteins and protein products from less hazardous animals and plants are beginning to be used in these fields.

We believe that some problems will be solved by using biomaterials gathered from the same species as the application object, and not from the other origins. From this viewpoint, human hair is a valuable source for the use of biomaterials containing particles, film, and fibers, because hair is collected in large quantities from specific individuals and the protein content is high (approximately 80%). In human hair, two major groups of proteins are known. One is the hard α-keratins forming microfibrous intermediate filaments and the other is matrix proteins forming a nonfilamentous matrix as intermediate filaments-associated proteins [4]. 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. Matrix proteins are classified into high-sulfur proteins (10-20 kDa) and high-tyrosine proteins (6-9 kDa). Little is known about the protein microspheres from hair proteins and α-keratins. Recently, we reported a novel method of producing protein particles from human hair protein film and used the solution as a blood analog fluid [5].

In this paper, we compared hair protein particles from three kinds of films based on the size, morphology, and protein components. The biochemical properties of the protein particles were also examined.

*Corresponding author (Fax: +81-268-21-5511, E-mail: fujiit1@shinshu-u.ac.jp)

Materials and Methods

Preparation of human hair protein particles

Human hair proteins were extracted from ethanol-treated human hair using 'Shindai solution', which is 20 mM Tris-HCl buffer solution (pH 8.5) containing 2.6 M thiourea, 5 M urea, and 5% 2-mercaptoethanol, at 50°C for 3-4 days [6]. After filtration and centrifugation of the extracted solution, the obtained ca. 5% (w/v) solution of hair protein was used to form the films. We prepared three kinds of protein films using the Pre-cast method, Post-cast method, and soft Post-cast method [7, 8]. The hair proteins in the Shindai solution were mixed with guanidine-HCl at a final concentration of 5% and then poured onto tissue cultured dishes containing distilled water (Pre-cast method). On the other hand, as for Post-cast and soft Post-cast methods, the protein solution was directly exposed in tissue cultured dishes containing 100 mM acetate buffer solution (pH 4) and 40 mM $MgCl_2$ solution, respectively, and protein aggregates were formed. After washing with tap water for 24 h or more and distilled water for 6 h, these protein aggregates, that are protein films, were crushed to pieces by mechanical processing and collected in a bottle. The protein aggregate solution was homogenized using a Polytron homogenizer to provide an apparently uniform protein particle solution. Furthermore, the protein particle solution was dispersed by ultrasonic agitation.

The hair protein particles were recovered as pellets by centrifugation at 15,000 g for 20 min. The obtained pellets were mixed with the Shindai solution and incubated for 1 day at 50°C for perfect dissolution. The protein recovery as the particles from the protein solution was calculated on the basis of the protein concentrations. The protein concentrations were determined according to Bradford using bovine serum albumin as a standard [9]. The protein components were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [10].

Particle size analysis

The diameter and diameter distribution of the protein particle suspension were measured using a laser diffraction particle size analyzer (Shimadzu SALD-200V). The mechanical stimuli were done at 8 mg/ml in distilled water, and the protein particle solution was diluted to 0.2 mg/ml in distilled water or 25 mM Tris-HCl (pH 7.6) containing 75 mM NaCl. The average particle size was expressed as both the number average diameter and the volume average one.

Morphological observation

The protein particles after dying with 0.05 mg/ml rhodamine 6G were observed at the protein concentration of 0.4 mg/ml in the solution state using a confocal microscope (Bio-Rad, Radiance 2000).

Results and Discussion

Table 1. Average diameter of protein particles

Human hair protein films prepared by the Pre-cast, Post-cast, and soft Post-cast methods were disintegrated in distilled water using a Polytron homogenizer (intensity 9, for 5 min × 5 times) and a sonicator (intensity 5 and for 10 min). Samples were diluted to 0.2 mg/ml in distilled water, and the particle diameters were measured by a laser diffraction particle size analyzer. The number average diameters and the volume average ones indicated in the columns denoted with 'N' and 'V', respectively.

Conditions	Average particle diameter (μm)					
	Pre-cast		Post-cast		Soft Post-cast	
	N	V	N	V	N	V
Polytron	3.4	21	2.3	22	4.6	21
Polytron +sonication	0.6	2.5	0.6	2.8	0.5	1.7

Preparation of human hair protein particles

During the course of our continuing research on the application of human hair and nail proteins, we have developed a rapid and convenient procedure for protein extraction that was called the "Shindai Method" [6]. Using the extracted solution, we have developed novel preparation procedures for human hair protein films and called them the Pre-cast, Post-cast, and soft Post-cast methods [7]. More recently, we reported the preparation of protein particles with the average diameter of 9 μm from the wet films by soft Post-cast method and used them as a model of red blood cells [5, 8]. SEM observations revealed that the films consisted of particles with a diameter of 0.5-2 μm, filaments and their associated architecture. In addition, the fine structure was dependent on the preparation method to some extent.

From the wet hair protein films prepared by using the three methods, we prepared a protein particle suspension by mechanical stimuli (Polytron treatment). The size distribution of each protein suspension was measured using a laser diffraction particle size analyzer and the number average diameter and the volume average one were 2.3-4.6 μm and 21-22 μm, respectively (Table 1). Further stimulation of the protein particle suspension by ultrasonic treatment reduced the average diameters in protein suspension to be 0.5-0.6 μm in number average value and 1.7-2.8 μm in volume average one. The average sizes of the protein particles were slightly different among the films formed by the three methods. The soft Post-cast method was the most effective to produce fine particles after sonication among these methods. Representative size distribution data obtained from the Post-cast film by the double mechanical treatments are shown in Fig. 1. The particle sizes at the peaks were 0.6 μm in number average and 1.0 and 5.3 μm in volume average.

The original human hair protein solution mainly consisted of α-keratins and matrix proteins. In the protein particle suspension, the α-keratins were definitely certainly contained, while the content of the matrix proteins was low compared with that in the hair protein solution (Fig. 2). No significant protein degradation was found after the mechanical stimulation.

The recoveries of net proteins in the protein solution from the human hair and that in protein

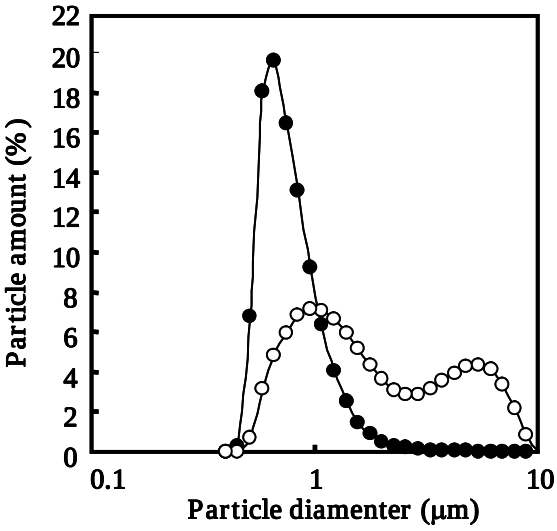

Fig. 1. Particle diameter distribution. Hair protein particles were prepared from the films formed by the Post-cast method. After treatment of the protein suspension at 8 mg/ml by the Polytron plus sonication, the particle diameter at 0.2 mg/ml was measured using a laser diffraction particle size analyzer. The plots for number average diameters and volume average ones are exhibited with closed and open circles, respectively.

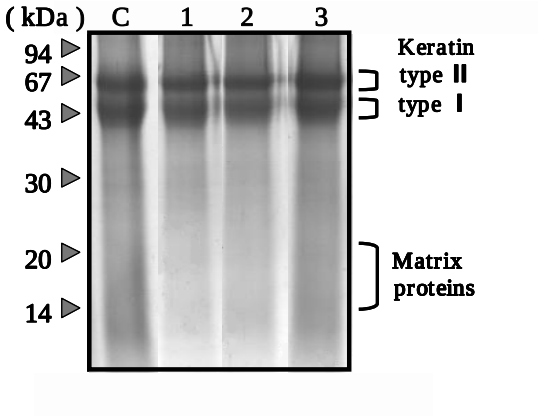

Fig. 2. SDS-PAGE analysis of the protein particles. Hair protein particles were prepared from the films formed by the Pre-cast (lane 1), Post-cast (lane 2), and soft Post-cast (lane 3) methods. After centrifugation at 15,000 g for 20 min, the pellets were incubated in the Shindai solution for 1 day at 50°C, and the extracts were analyzed by SDS-PAGE. C, original hair proteins.

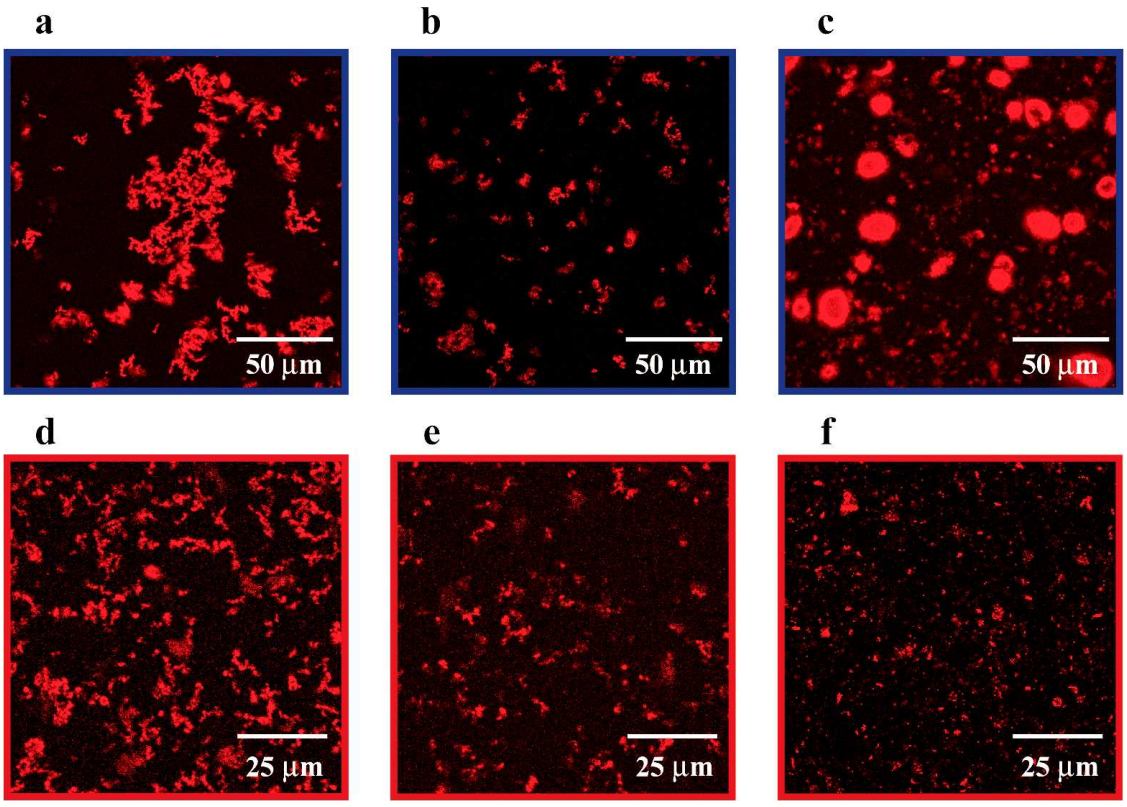

Fig. 3. Confocal microscopic observations of human hair protein particles. The protein particles fabricated from the films prepared by the Pre-cast (a and d), Post-cast (b and e), and soft Post-cast (c and f) methods were incubated with rhodamine for 30 min at 25°C. After washing, the samples were dispersed by the Polytron (a-c) and ultrasonic (d-f) treatments and then observed by confocal microscopy.

films from the hair protein solution were 50-60% and 60-85%, respectively [6-8]. Thus, it is possible to obtain 300-500 mg of a protein particle suspension from 1 g of human hair that can usually be bio-synthesized within 4-7 days on the head.

Morphology of the hair protein particles

When the protein particles were prepared from the films formed by the Pre-cast, Post-cast, and soft Post-cast methods, a fine structure in the solution was observed using a confocalmicroscope (Fig. 3). The protein particles were irregular in shape and had a rough surface with cracks. Some of small particles with the diameter of 0.5-2 μm seemed to be aggregated together. These images agreed with the scanning electron microscopic observations (data not shown). Although the fine structure was different in the hair protein films prepared by the three methods, the size and morphology of the protein particles were only slightly affected by the preparation methods. Therefore, in order to reveal the particle-particle interactions, the following experiments were undertaken using the protein particles from the Post-cast film (100 mM acetate buffer, pH 4).

Effects of ionic strength, divalent cations, pH, and temperature on hair protein particles

The effects of the ionic strength, divalent cations, pH, and temperature on the size and protein composition of the hair protein particles were examined (Table 2). After letting the particle suspension (4 mg/ml) stand under various conditions for 1-3 days, the samples were vigorously shaken by the hand, and the size and the protein components were examined. The average diameters, especially the volume average diameters, increased with the increasing ionic strengths during the period, suggesting the hydrophobic interaction promotes the aggregation

Table 2. Effects of various factors on average diameter of hair protein particles

Samples (8 mg/ml) under various conditions were stored at 25°C and diluted to 0.2 mg/ml in the solution as mentioned above. The particle diameters were measured as shown in Table 1.

Conditions	Average particle diameter (μm)					
	0 day		1 day		3 days	
	N	V	N	V	N	V
Control	0.7	1.8	0.8	2.5	0.8	2.1
100mM NaCl	0.7	1.7	0.8	2.6	0.8	2.3
500mM NaCl	0.7	1.8	1.1	5.7	1.2	4.2
100mM KCl	0.7	1.8	0.8	2.5	0.8	2.3
500mM KCl	0.7	1.9	1.1	4.8	1.1	4.0
2mM EDTA	0.7	1.8	0.8	2.1	0.8	2.2
5mM $MgCl_2$	0.7	1.8	0.8	2.4	0.8	2.1
2mM $CaCl_2$	0.7	1.7	0.8	2.2	0.8	2.1
pH 6.0	0.7	1.9	0.7	1.9	0.8	2.1
pH 9.0	0.7	2.0	0.8	2.2	0.8	2.7
4°C	0.7	2.0	0.7	1.9	0.7	2.2
37°C	0.7	2.0	0.7	2.2	0.7	2.6
50°C	0.7	2.0	0.8	2.2	0.8	2.7

of particles to some extent under high-salt conditions. Whereas, the diameters were not significantly changed by the addition of EDTA, $MgCl_2$, and $CaCl_2$. Furthermore, the average diameters were hardly affected over wide pH (pH 6-9) and temperature ranges (4-50°C). The data listed in Table 2 suggests that the conditions of less than 100 mM salt and nearly neutral pH at 4°C are suitable for the purpose of longtime storage of protein particles. After the suspension was stored under such conditions for 3 days, no detectable degradation was observed by SDS-PAGE (data not shown). We have reported that the keratin of the protein particle was only slightly degraded even after storage for 8 months at 4°C [5]. These data suggested that human hair protein particles were extremely stable not only at the molecular level, but also at the microspherical level.

In this report, we presented a procedure for the preparation of stable protein particles with a diameter of 0.6-4.6 μm from human hair and some properties of the protein particles. The particle samples with fine size and high recovery as a protein were obtained from the films prepared by soft Post-cast method and Post-cast method, respectively. We have succeeded in trapping alkaline phosphatase with a molecular mass of 95 kDa in the hair protein film using the Post-cast method [7]. The protein particles were prepared from the film, indicating that high-molecular-weight components such as alkaline phosphatase will be incorporated into the particles without chemical modification. The application of protein particles is being sought in the cosmetic and medical fields [1-3,11,12]. Human hair protein particles will be promising as safe and biocompatible materials, because the hair can be individually collected to be self-originating products.

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References

1. Chowdhury D.K. and Mitra A.K. (1999) Kinetics of in vitro release of a model nucleoside deoxyuridine from crosslinked insoluble collagen and collagen-gelatin microspheres. Int. J. Pharm. **193**, 113-122.
2. Swatschek D., Schatton W., Muller W.E.G., and Kreuter J. (2002) Microparticles derived from marine sponge collagen (SCMPs): preparation, characterization and suitability for dermal delivery of all-trans retinol. Eur. J. Pharm. Biopharm. **54**, 125-133.
3. Balthasar S., Michaelis K., Dinaurer N., Briesen H., Kreuter J., and Langer K. (2005) Preparation and characterization of antibody modified gelatin nanoparticles as drug carrier system for uptake in lymphocytes. Biomaterials **26**, 2723-2732.
4. Gillespie, J.M. (1990) The proteins of hair and other hard α-keratins, in "Cellular and Molecular Biology of Intermediate Filaments" (Goldman, R.A. and Steinert, P.M. eds.), Plenum Press, New York, pp.95-128.
5. Kobayashi, S., Morikawa, H., Ishii, S., and Fujii, T. (2005) Development of blood analog fluids using human hair protein particles. JSME Int. J. Ser. C, **48**, 494-498.
6. Nakamura, A., Arimoto, M., Takeuchi, K., and Fujii, T. (2002) A rapid extraction procedure of human hair proteins and identification of phosphorylated species. Biol. Pharm. Bull. **25**, 569-572.
7. Fujii, T., Ogiwara, D., and Arimoto, M. (2004) Conventient procedures for human hair protein films and properties of alkaline phosphatase incorporated in the film. Biol. Pharm. Bull. **27**, 89-93.
8. Fujii, T. and Ide Y. (2004) Preparation of translucent and flexible human hair protein films and their properties. Biol. Pharm. Bull. **27**, 1433-1436.
9. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.
10. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.
11. Lee, C.H., Singla, A., and Lee, Y. (2001) Biomedical application of collagen. Int. J. Pharm. **221**, 1-12.
12. Muller, R.H., Radtke, M., and Wissing, S.A. (2002) Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Ad. Drug Deliv. Rev. **54**, S131-S155.

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