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Silk Fibroin Microsphere and Its Characterization

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Using gel filtration chromatography, high molecular silk fibroin with high purity was obtained and silk fibroin microsphere particles (SFMP) could be simply made by spray dryer method. Also, some of the physicochemical properties of SFMP and morphology were investigated. The average molecular weight of pure silk fibroin protein dissolved in calcium chloride is about 61,500 g/mol as measured by gel permeation chromatography. SFMP was spherical in shape, and particles, sized average of 2 ± 10 μm, were observed by SEM and particle analyzer, respectively. Obtaining microspheres particles by spray dryer method accelerated the transition from the random coil to the β-sheet structure during spray dryer treatment. It was identified by the basic fourier transform infrared spectroscopy of SFMP. The swelling ratio of SFMP is majorly dependent on the pH of the solution, not on the occurred gelation. The characteristic structure, which might be applicable to immobilization of drugs is superior to other matrix materials for the use of biomaterials with skin affinity.

Key words: Silk fibroin, Microsphere, Gel filtration chromatography, Spray drying

Introduction

Gel filtration has been used for desalting protein solutions since the early 1960's (Hagel *et al.*, 1998). Because of the difference in molecular size between protein and the contaminating solutes, the requirements of the gel and chromatographic system are modest. Namely, an extension of the molecular sieves exclusion principle also follows the basic principles of chromatographic system. Generally, the gel is a neutral and porous material that allows molecular movement, so that small molecules passing into the interior of the gel can be eluted out. For any given gel, there is an exclusion limit: above certain molecular weight no penetration into the gel will occur. Therefore, size exclusion chromatography or gel filtration is an important technique in the area of biopolymer.

On the other hand, micro - and/or nano-particles are solid colloidal particles that range in size from about 10 to 1000 nanometers (Maeda *et al.*, 1992). They are widely used in various fields of life science such as separation techniques, clinical diagnostic assays, cosmetics and drug delivery system (Cho *et al.*, 1997; Kim *et al.*, 1998; Jeong *et al.*, 1998). However, many problems such as bio-distribution of drugs, solubility and stability of drugs, undesirable side effects, thermal instability, short blood circulation, structural fragility and lower loading efficiency, *etc.* still exist. For the utility of these biomaterials as possible, more purified and stable samples are required using, *i.e.*, gel filtration chromatography (GFC) and natural polymer of silk fibroin protein.

Recently, silk fibroin (SF) as the natural protein polymer has been applied to biomaterials such as matrix for enzyme immobilization (Yoshimizu *et al.*, 1990), mammalian cell culture (Minoura *et al.*, 1995; Inoue *et al.*, 1998), drug delivery system (Hanawa *et al.*, 1995a, b), artificial skin (Yeo *et al.*, 2000) and so on. There are several advantages of SF for biomaterials, such as biocompatible natural polymer, biodegradability, minimal inflammatory reaction and good water vapor permeability etc. because of its unique physicochemical properties and harmlessness to the human body (Tsukada *et al.*, 1994; Santin *et al.*, 1999). SF is the principal constituent of the fibrous protein of *Bombyx mori* silkworm, and is one of the sclero-proteins. Asakura *et al.* (1993) demonstrated that SF can be stabilized in the β-sheet structure form, perpendicular hydrogen bonds with respect to fiber axis,

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intermolecular between polypeptide chains using solid state NMR technique (Asakura *et al.*, 1994; Yeo *et al.*, 1994).

In this study, we have attempted to separate SF purely with high purity using GFC and to prepare silk fibroin microsphere particles (SFMPs). Additionally, some physicochemical properties and morphology of SFMP were investigated.

Materials and Method

Materials

Raw silk (*Bombyx mori*) cocoons reared on the farm affiliated with Rural Development Administration (RDA) of Korea were used as a raw material. The raw materials were degummed twice with 0.5% weight of fiber (o.w.f.), Marseilles soap and 0.3% o.w.f. sodium carbonate solution at 100°C for 1 h and then washed with distilled water. Degummed SF (35 g) was dissolved in the mixtures of $CaCl_2$: H_2O : ethanol = 1 : 8 : 2 (700 m*l*) in volume at 95°C for 5 hrs. The SF solution was obtained after dissolved fibroin solution was dialyzed against distilled water for 4 days, and then SF powder was obtained by freezying dry.

Pure Separation of SF

The crude salts extract were eliminated by a Sephadex G-25 media column (800 × 40 mm) equilibrated with distilled water. Five percent of calcium chloride dissolved in the SF mixed solutions was twice filtered using miracloth (Calbiochem, USA) quick filter. The column was eluted with distilled water at a flow rate of 25 m*l*/min and fraction of 35 m*l* each were collected. The main pure SF protein fractions were collected according to the salts concentration detected by the assay of their automatic conductivity monitor control.

Preparation of SFMP

The SFMP were prepared by mini spray dryer (Buch B-191 model, Switzerland) at a flow rate of 20 m*l*/min and sample inlet control temperature of 85°C.

Observation of scanning electron microscope (SEM)

The morphology of the SFMP was observed using a SEM (JEOL, JSM 5400, Japan). SFMP powders were hooked on a graphite surface, and then on a vacuum at 50°C overnight. Then, it was coated with gold/palladium using an Ion Sputter (JEOL, JFC-1100).

Measurement of particle size

Average particle size was measured by a particle analyzer using model GALAI-CIS-1 (Israel) with an automatic of particle content counter in a solution at 25°C. A particle solution was prepared by 75% ethanol of 2.5×10^{-3}% (w/v) and measured without filtering.

Measurement of swelling ratio

The swelling ratio was measured by weighing the SFMP after wiping the excess with various pH-value-adjusted-water on the surface. Swelling ratio was calculated as Ws/Wd, where Ws and Wd represent wet weight and dry weight of the SFMP, respectively. All solutions prepared for measurement of swelling ratio have the same ionic strength (I = 0.1).

Infrared (IR) measurement

A Fourier transform infrared (FT-IR) spectroscopy (Perkin elmer Spectrum 2000 spectrometer, USA) was used to confirm the structure of SF control and SFMP samples.

Molecular weight measurement

Molecular weight of the separated silk fibroin solution was measured by gel permeation chromatography (GPC) with a TSK-gel G2000 SWXL column (300 × 7.8 mm). The mobile phase was distilled water (pH 7.0). The chromatography was operated with a flow rate of 0.5 m*l*/min, column temperature at 37°C and detected with a refractive index (viscotek, LR-125, USA) detector. Pullulan P-400, P-200, P-100, P-50, P-20, P-10, and P-5 (Shodex standard P-82, Showa denko, Japan) were used as standard markers.

Results and Discussion

Separation of salts and silk fibroin

In the protein and salts to be separated purely in the silk fibroin mixing solution, there are a number of salts. In general, size exclusion chromatography is an extension of the molecular sieves exclusion principle, and it also obeys the basic principles of chromatography (Hagel *et al.*, 1998); that is, small molecules passing into the interior of the gel will be eluted out of the system. We attempted to separate the pure protein and salts in silk fibroin by Sephadex G-25 media. Sephadex G-25 seems to have become the most popular out of the hydrophilic gels in the separation of natural products. It is particularly useful in removing high molecular weight and lower material from a contaminating sample. As shown in Fig. 1, silk fibroin protein (A) and included salts of form hydrated ions compounds (B) were separated according to molecular weight, respectively. There were perfect separation of pure silk fibroin protein and low molecular of included salt of form

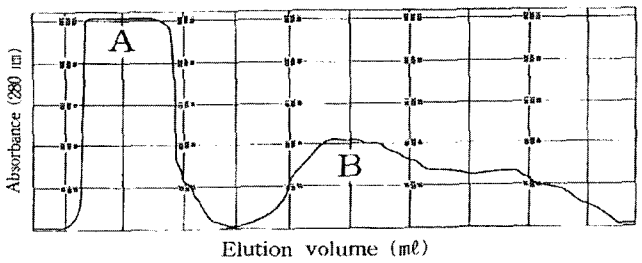

Fig. 1. Separation of high molecular silk fibroin and salts of large sample volumes by gel filtration chromatography. A, high molecular pure silk fibroin protein; B, included salts of form hydrated ions compounds.

Fig. 2. Surface morphology and characteristics by scanning electron microphotographs: SFMP with pure silk fibroin protein (×2000).

hydrated ion compounds by Sephadex G-25 media, respectively. Moreover, about 100% recovery of the silk fibroin protein is obtainable. Therefore, this method of separating of pure silk fibroin protein in calcium chloride mixed solution was found to be the optimal technique.

Characterization of SFMP

Fig. 2 shows SEM photographs of silk fibroin protein particles obtained by spray dryer method. The shapes of the particles were spherical, and the sizes ranged from about 2.0 μm to 10 μm. It was found that microspheres with a spherical shape could be simply prepared using spray dryer method. Also, we observed similar sized particles by particle analyzer measurement. Fig. 3 shows the particle size distribution of SFMP microspheres. These microspheres' sizes were 4 μm × 10 μm in average. Fig. 4 shows the FT-IR spectra of the pure amorphous SF structure (control) and SFMP samples. In the spectrum of the amorphous SF sample, the amide I and amide II, typical bands of amorphous SF sample were observed at around 1653 and 1531 cm^{-1}, respectively. Canetti *et al.* (1989a, b) reported that SF in aqueous solution at neutral pH had a random coil or silk I conformation.

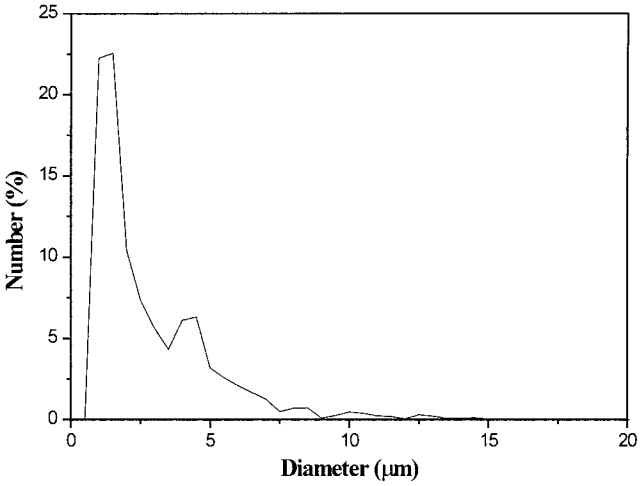

Fig. 3. Particle size distribution of SFMP.

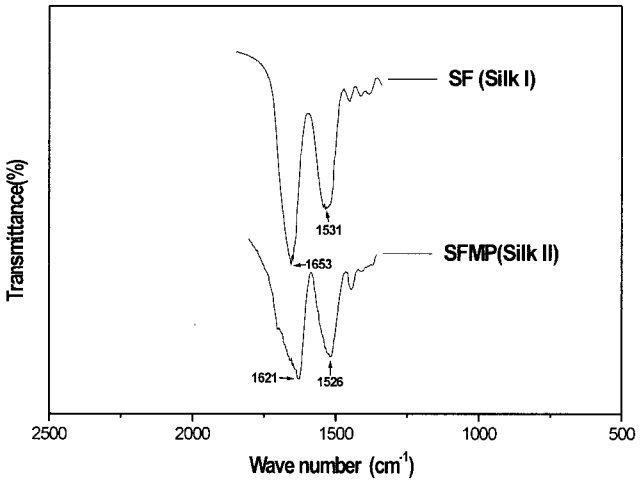

Fig. 4. Fourier transform infrared (FT-IR) spectra of SF (control) and SFMP. SF: silk I type, SFMP: silk II type.

On the other hand, in the spectrum of SFMP, these peaks were shifted to a lower frequency at 1621 and 1526 cm^{-1}, respectively. The results suggested that the structure transition from the random coil or silk I to β-sheet structure occurred during spray drying of the SFMP. From a practical point of view, moreover, this experimental system does not need another treatment of structure transition and seems to be suitable in using SFMP as a simple preparation of silk fibroin microsphere fine particles.

Swelling measurement

Hanagawa *et al.* (1995a, b) reported that the silk fibroin gel characterizations were studied as a function of adjusted pH and the concentration of silk fibroin were used on new oral dosage materials. Gelation was not observed when pH range of the mixture was adjusted to 2.0, whereas in the case of pH 3.0 and 4.0, gelation was

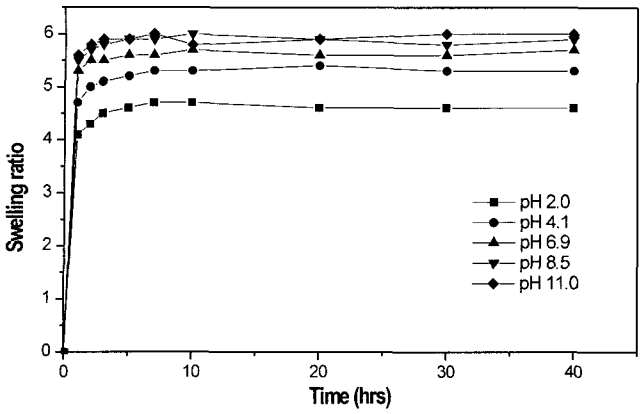

Fig. 5. Swelling ratio of SFMP with various pH values. Each point represents the mean±SD of at least 3 experiments.

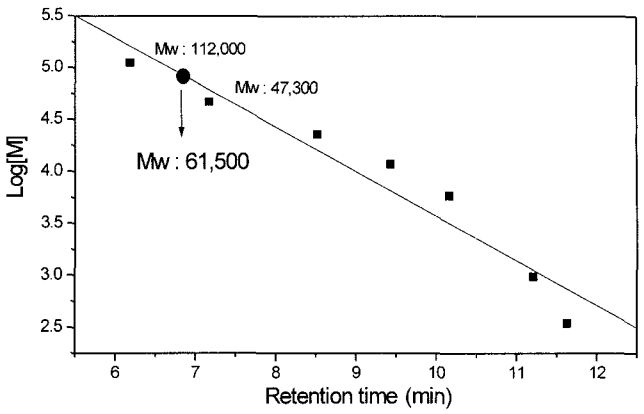

Fig. 6. Calibration plot of weight average molecular weight (Mw) with pullulan standard (■) *vs.* retention time on silk fibroin aqueous solution (●).

observed. This can be explained by presuming the existence of an optimal pH for gelation when it does not transform structure. Also, Shimura *et al.* (1973) demonstrated that the gelation of silk fibroin took place in its acidic solution, and was due to the conformational change from the random coil structure to the β-sheet structure by intermolecular hydrogen bonds between polypeptide chains. On the other hand, SFMP prepared in our study was already in a β-sheet structure form when it was treated in the spray dryer, and in a very stable state. Therefore, in the case of this SFMP, swelling stability throughout storage should be considered. Through these results, we consider that SFMP is structurally irreversible, and this SFMP seems to be suitable to use for biomaterials.

Fig. 5 shows the swelling ratio of SFMP microspheres particles according to incubation time with various pH values. A whole range from acid to alkali was shown to be dependent on the solution pH, not on occurred gelation. These results show that swelling ratios are dependent upon the solution pH and that it increases as the pH increases. It is thought that if immobilized dose of polymer-polymer or polymer-oligomer complex such as SFMP-enzyme or SFMP-drug system is taken in different pH solution, pH will be expected to increase in the electrostatic repulsion effect.

Molecular weight measurements

Molecular weight and its distribution is one of the basic descriptive characteristics of polymers. Molecular weight and its distribution have remarkable influences on thermal deformation temperature, tensile strength, shock resistance, fusing fluidity, reactivity, hardening, weatherability, and other properties of polymers. The molecular weight calibration curve of pullulan standard *vs.* pure separated SF aqueous solution average molecular weight (Mw) are presented in Fig. 6. The molecular weight of SF aqueous solution sample was 61,500 ± 3,000 when calculated by elution time. Chen *et al.* (1991) demonstrated that the average Mw of calcium chloride dissolved in silk fibroin is approximately over 60,000 according to the SDS-PAGE study (Chen *et al.*, 1991), which is consistent with our GPC results.

Pure separation of high molecular silk fibroin is obtainable using Sephadex G-25 gel filtration chromatography. And silk fibroin nano-like particle could be simply obtained by spray dryer method, and with the structural transition from the random coil to β-sheet structure during spray dryer, treatment was accelerated or completed. The various pH range of swelling ratio is dependent on the solution pH, but not on ocurred gelation. For these reasons, it is expected that the SFMP will exist in a powder form, which is applicable to structural irreversible system, and will be used for the matrix of drug, enzyme, additive to cosmetics and wound healing paste as the biomaterials. Also, it is expected that the SFMP is superior to other matrix materials considering its superior chemical and/or microbiological stability.

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