Morphology of Regenerated Silk Fibroin: Effects of Freezing Temperature, Alcohol Addition, and Molecular Weight

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ABSTRACT: Regenerated silk fibroin (RSF) was prepared by dissolving in a $CaCl_2$/ethanol/H_2O solvent system, freezing, and lyophilization. The effect of freezing temperature, alcohol addition, and molecular weight on the morphological and conformational changes were investigated through scanning electron microscopy, X-ray diffraction, Fourier transform infrared spectroscopy, circular dichroism spectroscopy, and differential scanning calorimetry analysis. However, the addition of a small amount of methanol induced the morphological change of RSF to a fine-particle aggregate, which resulted from the formation of a β-sheet crystalline structure. The lower the freezing temperature was, the more the formation of aggregates was favored, and the finer powder aggregates were formed. As the amount of added hydrophilic alcohol such as methanol and ethanol increased in the silk fibroin solution, a spherical powder form was changed to fine aggregates with the enhancement of thermal stability and crystallinity. On the other hand, RSFs prepared with a hydrophobic alcohol such as 1-butanol or 1-octanol showed a lump-like or sheet-like shape of morphology without any changes in conformational transition. It is concluded that the molecular weight of the silk fibroin and the type and amount of alcohol were determining factors in the morphological features of RSF, especially the size and shape of fibroin particles. A uniform ultrafine powder of RSF with a spherical form (~ 1 μm) can be obtained when the molecular weight and the alcohol addition to the silk fibroin solution are controlled.
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Key words: regenerated silk fibroin; morphology; molecular weight; conformation; alcohol addition; freezing

INTRODUCTION

The *Bombyx mori* (*B. mori*) silk fibroin (domestic silk), which consists of mainly glycine, alanine, and sericine, is a spun fibrous protein with strong intramolecular and intermolecular hydrogen bonding. Silks have been used as textile fibers for thousands of years because of their unique gloss, handle, and mechanical properties. Recently, studies based on silk fibroin for nontextile fields, especially biomaterials applications, have been carried out extensively.[1–5]

Silk fibroin can be regenerated in various forms such as solution, powder, film, gel, filament, and so on, depending on its preparation conditions and application field. In general, the regeneration proceeds via silk fibroin solution by the treatment of acid, alkali, neutral salt, enzyme, or other compounds. Because regenerated silk fibroin (RSF) is obtained through the process of dissolution and coagulation, structural transitions and morphological changes can occur in accordance with the preparation conditions for processing. Many studies on the structural charac-

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terization of RSF have been reported.[6–11] The structural characteristics and morphological features have a great influence on the physical and mechanical properties of RSF, which should be considered when silk fibroin is used as a biomaterial.

Silk fibroin powder, the simplest form of RSF, has been used as a fundamental additive for cosmetics, and several methods have been reported for preparing the fibroin powder. Acids and alkalis are able to hydrolyze the peptide bonds of the protein chains to lower the molecular weight from thousands to the oligopeptide level. This hydrolyzed powder, which has a sharp fibrous shape several tens of micrometers in size, has disadvantages in applying; low yield, difficulties in desalting, yellowing, high crystallinity, and so on. Therefore, the application of hydrolyzed silk fibroin is limited to the field of foods, requiring good digestion with high solubility. Also, silk fibroin powder can be obtained from mechanical pulverization after mild treatment by a weak acid or alkali, with a size of less than several tens of micrometers. The application of this powder is limited to a coating material because of insolubility caused by a high molecular weight.

However, silk fibroin solution prepared by dissolution in aqueous neutral salts can be lyophilized to a powder form. Various parameters, such as molecular weight, crystallinity, solubility, size, and shape, are easily controlled during processing, and therefore, this method may have advantages for the application fields of cosmetics and drug-delivery systems. For example, RSF powder can be effectively utilized in special applications if an ultrafine spherical form of the fibroin powder is formed. Nevertheless, few studies have been reported on this powder preparation method.

In this study, RSFs were prepared by the dissolution, freezing, and lyophilizing of *B. mori* silk fiber, and the effects of various processing parameters, such as freezing temperature, alcohol addition, and dissolution time, on the morphological changes of RSF were investigated. The conformational structures and thermal properties were also studied to examine the effects of these parameters and to explain the relation between morphology and conformation.

EXPERIMENTAL

Materials

B. mori silk fiber was degummed in 0.2% on the weight of fiber (o.w.f.) aqueous solution of Marcelle soap containing 0.2% (o.w.f.) Na_2CO_3 at 90–100°C for 50 min and rinsed thoroughly with hot distilled water. The degummed silk fibroin was then dissolved in a mixture of ethanol (2*M*), calcium chloride (1*M*), and water (8*M*) at boiling point for 3 h. The resulting solution was separated from any undissolved particles by centrifugation at 7500 rpm for 30 min.

The silk fibroin solution was obtained by filtering the centrifuged solution and dialyzing it for 3 days at room temperature with a cellulose tube (molecular weight cut-off 3,500) to remove the excess salts. The concentration of the silk fibroin solution was adjusted to 2% (v/v) with polyethylene glycol as a water absorbent.

RSFs were prepared by freezing and lyophilizing the silk fibroin solution, and the series of RSFs were obtained with different processing conditions as follows;

1. Silk fibroin solution was frozen at various freezing temperatures (−10, −30, and −60°C) for 24 h and then lyophilized with an freezing dryer.
2. Different types (methanol, ethanol, 1-butanol, and 1-octanol) and various amounts of alcohol were added to the silk fibroin solution. Then, the mixture was frozen at −60°C for 24 h and lyophilized.
3. The degummed silk fiber was dissolved in a mixture of ethanol, calcium chloride, and water at boiling point for different dissolution times (1, 3, 12, 24, and 48 h). Then, methanol (5% v/v) was added to the silk fibroin solution; then, the solution was frozen at −60°C for 24 h and lyophilized.

Measurements

The surface morphology of RSF was examined with a scanning electron microscope (JSM-5410LV, JEOL, Tokyo, Japan).

The circular dichroism (CD) was measured to analyze the conformation of silk fibroin in solution with a spectropolarimeter (CD6, Jobin Yvon, Paris, France), and a Fourier transform infrared (FTIR) spectrometer (M series, Midac Co., Irvine, CA) was used for the analysis of the amide bands of RSF.

Wide-angle X-ray diffraction (XRD) curves were recorded with a D-MAX-3 diffractometer (Rigaku. Co., Tokyo, Japan) at a scanning rate of 1°/min with CuKα radiation (λ = 1.54 Å). The voltage and current of the X-ray source were 30 kV and 20 mA, respectively.

To measure the thermal properties of RSF, a differential scanning calorimeter (TA 2910, TA Instruments, New Castle, DE) was used with an aluminum hermetic sample pan at a heating rate of 10°C /min under a nitrogen atmosphere.

Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmi[12] with 12.5% acrylamide gel, which was stained with 0.25% Coomasie Brilliant Blue R-250 (Aldrich, USA).

RESULTS AND DISCUSSION

Effect of Freezing Temperature

The lyophilized RSF was prepared by freezing aqueous silk fibroin solution at temperatures of −10, −30, and −60°C, and the effect of freezing temperature on the morphology was investigated. According to the examination of optical microscopy (Fig. 1), RSF showed a smooth, sponge-like surface morphology composed of an assembly of transparent and thin layers. Different freezing temperatures, as shown in Figure 2, did not cause significant changes in the surface shape of RSF.

However, scanning electron microscopy (SEM) photographs of RSF prepared from silk fibroin solution containing a small amount of methanol (10% v/v), showed interesting morphological features because of the effect of the added alcohol. As shown in Figure 3, the sponge-like structure of thin layers (Fig. 1) was transformed into an aggregate structure of fine particles (powders) by the addition of methanol. Because methanol is a poor solvent for silk fibroin, it can promote the formation of aggregate particles, probably resulting in a structural change from a random coil to a β-sheet conformation. The structural changing effect of methanol on aqueous silk fibroin has been reported by many researchers.[13–18]

Furthermore, as the freezing temperature of methanol-added silk fibroin solution decreased from −10 to −60°C, the coarsely porous structure changed to denser and smaller particle aggregates. These morphological changes as a function of freezing temperature can be explained in terms of a temperature gradient between the fibroin solution and the cooling system. Tsukada et al.[13] suggested that at a lower cooling rate, the formation of protein aggregates would take place slowly enough to produce a less compact aggregate with large voids that had been occupied by water. As a result, larger ice crystals would be formed, and consequently, larger pores would be left after lyophilization. The higher the cooling rate would be, the more the formation of aggregates would be favored. This experiment confirmed that the freezing temperature and alcohol-induced structural changes can affect the formation of fine particle aggregates of silk fibroin.

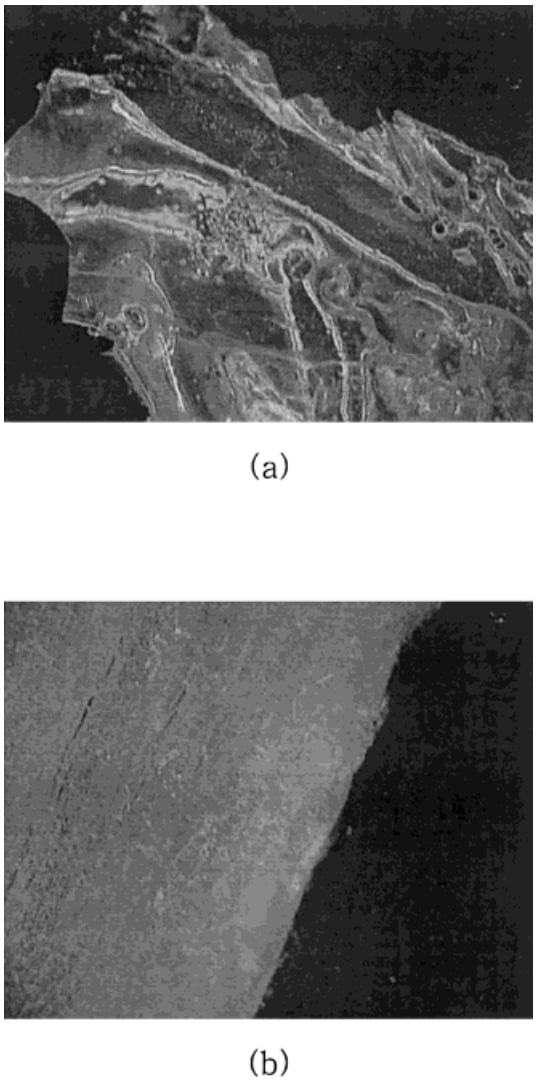

Figure 1 Optical microscope photographs of lyophilized RSF (200×): (a) surface and (b) cross-section.

To examine the change in conformation and the crystalline structure of RSF on methanol treatment and freezing, XRD and FTIR spectroscopy were used. Figure 4 shows the XRD curves of RSF obtained from aqueous silk fibroin solution with and without the addition of methanol at different freezing temperatures. A very broad peak was observed at $2\theta = 20°$ for the methanol-untreated samples, regardless of freezing temper-

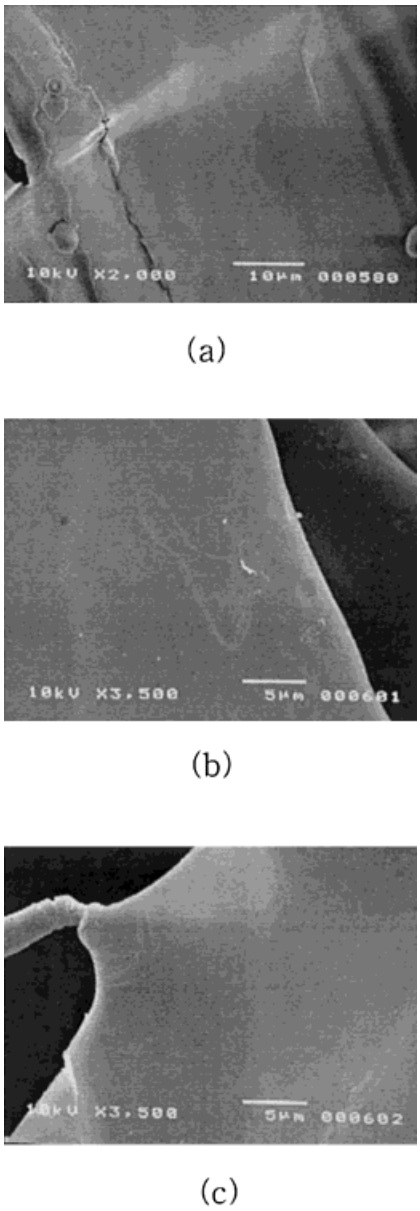

Figure 2 SEM photographs of RSF prepared at various freezing temperatures: (a) −60, (b) −30, and (c) −10°C.

ature. This is a typical characteristic diffraction pattern of amorphous silk fibroin.

However, the methanol-added RSF showed a major peak at $2\theta = 20°$ and two minor peaks at about 9° and 24°, known as characteristic peaks of a β-sheet crystalline structure. The peak at $2\theta = 20°$ corresponds to the reflection of the (201) plane and indicates the structural repeating distance of 4.3 Å, and the minor peaks at $2\theta = 9°$ and 24°, are reported as β-crystalline spacing of 9.8 and 3.7 Å, respectively.[19,20]

However, there was little effect of freezing temperature on the crystalline structure of the X-ray diffractograms. It was expected that at higher freezing temperatures, the time for chain interactions would be somewhat prolonged, and this could result in a slight difference in conformational change and crystalline structure. Therefore, FTIR spectroscopy was applied to study the conformational changes of silk fibroin molecules. Figure 5 shows the FTIR spectra of RSF prepared at different freezing temperatures.

The absorption bands sensitive to conformations could be detected in the spectral regions of amide I, II, III, and V modes, and the characteristic bands of β-sheet conformations appeared at 1630 (amide I), 1530 (amide II), 1265 (amide III), and 700 cm^{-1} (amide V).[17,21,22] As shown in Figure 5, there was no positive indication of the formation of a β-sheet structure. However, as the freezing temperature of the silk fibroin solution increased from −60 to −10°C, the absorption band at 1265 cm^{-1}, a β-sheet characteristic peak of amide III mode, appeared as a shoulder.

It seems that the β-sheet crystalline structure may be slightly formed at a somewhat higher freezing temperature. A slow cooling or freezing rate usually gives a slight increase in crystallinity and the size of the particles. According to the results of this study, the structural changes were hardly affected by the freezing temperature in the range of −60 to −10°C.

The effect of freezing temperature on the thermal behavior of RSF was evaluated on the basis of differential scanning calorimetry (DSC) measurement. Figure 6 shows a characteristic DSC thermogram of amorphous silk fibroin for RSF samples, independent of freezing temperature. The first broad endotherm at about 120°C appeared because of the evaporation of absorbed water, and the main endotherm at 285°C was attributed to the thermal decomposition of silk fibroin. The exothermic peak, which was caused by the crystallization of silk fibroin, also appeared in the vicinity of 200°C. The relevant thermal changes induced by heating caused conformational transitions of fibroin molecules, which resulted in the exo–endo transitions in DSC thermograms. When RSF was prepared at lower freezing temperatures, the crystallization exotherm appeared at a much lower temperature.

Effect of Alcohol Addition

When methanol was added to the silk fibroin solution, the molecular conformation and the mor-

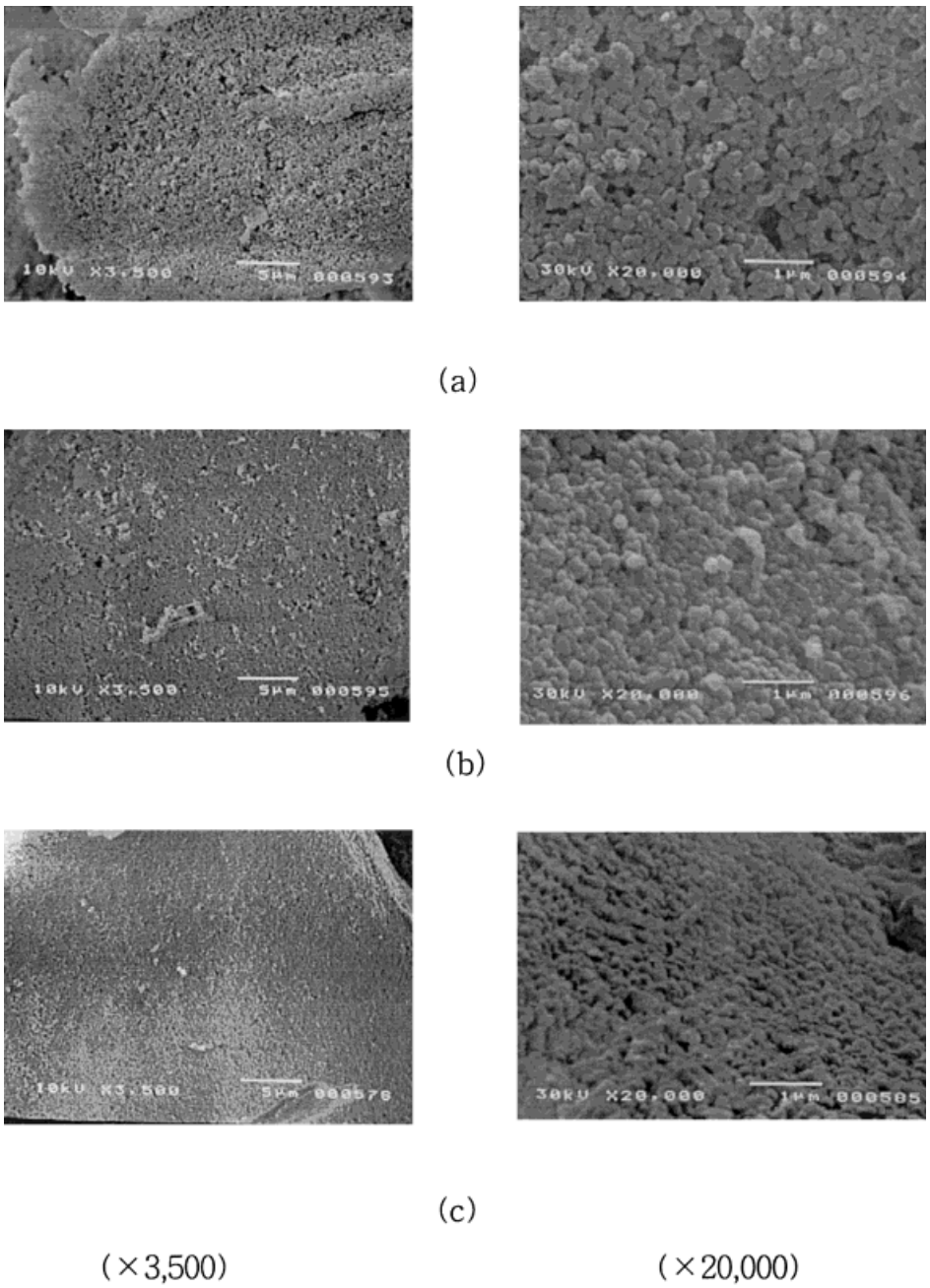

Figure 3 SEM photographs of RSF prepared with methanol (10% v/v) at various freezing temperatures: (a) −10, (b) −30, and (c) −60°C.

phology of particle aggregates was changed as previously shown in Figures 3 and 4. Therefore, it was interesting to investigate the effects of alcohol types and concentrations on the morphological changes of RSF. Figure 7 shows SEM photographs of RSF prepared with different alcohol additives in fibroin solution. Easily crushed aggregates of fine powder were formed for RSF when methanol or ethanol was added. The particle size of the powder aggregate was somewhat larger when ethanol was used.

However, the morphology of RSF prepared with 1-butanol or 1-octanol addition was totally different from that of methanol- or ethanol-prepared RSF. They showed a lump-like or sheet-like shape of dense aggregates with a soft surface and toughness. The octanol-added RSF exhibited a macroscopic morphology similar to

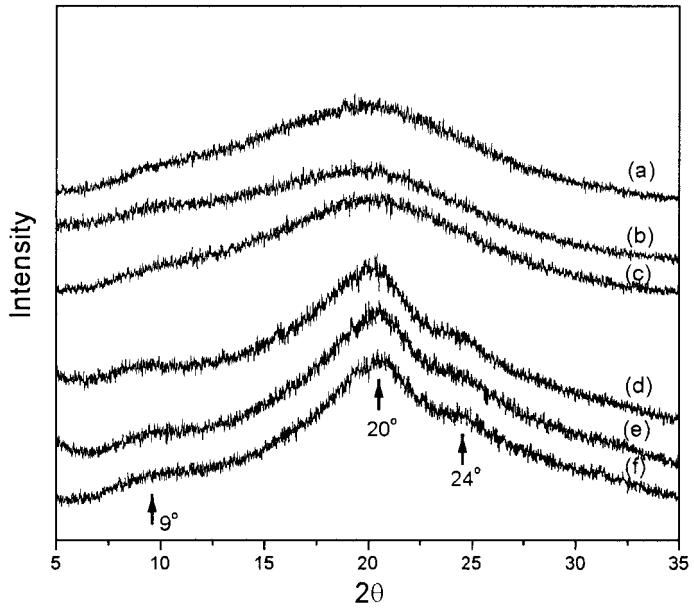

Figure 4 X-ray diffractograms of RSF prepared with and without methanol (10% v/v) at various freezing temperatures: (a) without methanol, −60°C; (b) without methanol, −30°C; (c) without methanol, −10°C; (d) with methanol, −60°C; (e) with methanol, −30°C; and (f) with methanol, −10°C.

RSF prepared without alcohol addition. The branched and tangled network structure of aggregates was obtained when 1-octanol added, despite the fact that a smooth surface appeared at a higher magnification of the SEM photograph.

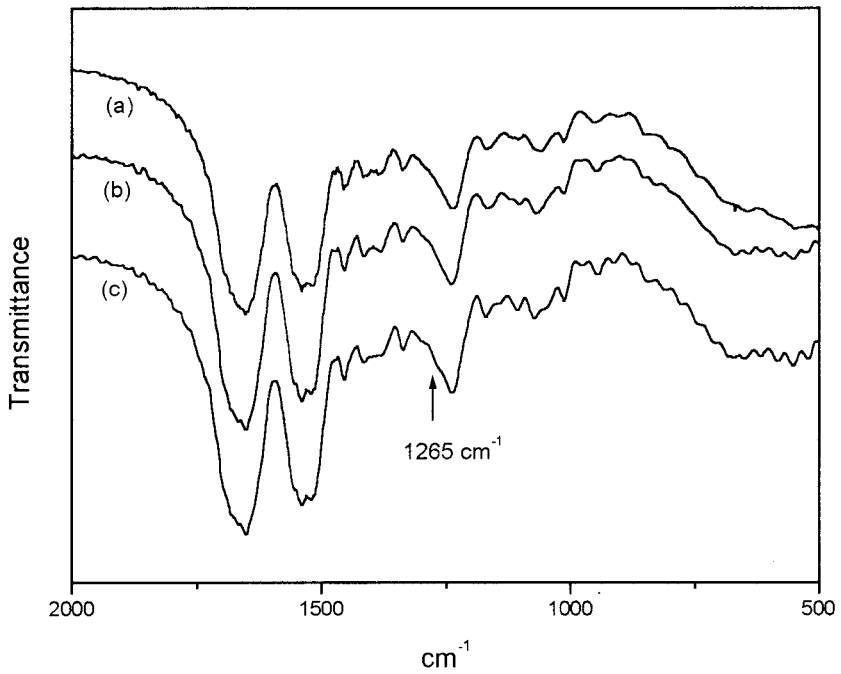

Figure 5 FTIR spectra of RSF prepared with methanol (10% v/v) at various freezing temperatures: (a) −60, (b) −30, and (c) −10°C.

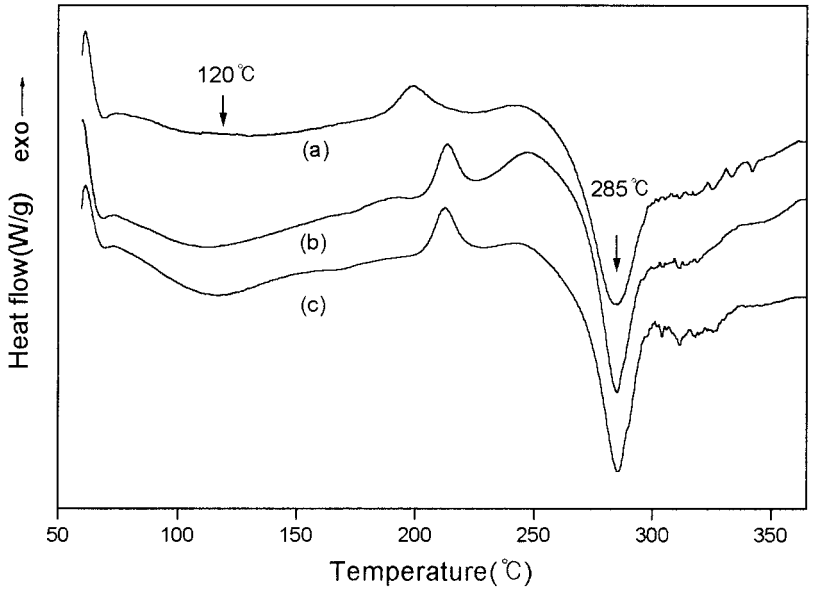

Figure 6 DSC thermograms of RSF prepared at various freezing temperatures: (a) −60, (b) −30, and (c) −10°C.

The differences in the morphological structures of RSF can be explained by the conformational differences of alcohol-added silk fibroin. Figure 8 shows the CD spectra of silk fibroin solution prepared with various types of alcohol additives, whereas Figure 9 shows the X-ray diffractograms of RSF. When methanol or ethanol was added to silk fibroin, both spectra showed that the β-sheet conformation was clearly formed. However, 1-octanol did not change the conformation of silk fibroin into the β-sheet structure; the silk fibroin remained in a random-coil conformation. When 1-butanol was added, a mixed type of conformation was observed with β-sheet, random-coil, and α-helix structures.

The conformational and morphological differences may be attributed to the hydrophilicity or hydrophobicity of alcohol used as an additive in the silk fibroin solution. When a hydrophilic alcohol such as methanol and ethanol was added to the silk fibroin solution, the molecular chains of the fibroin interacted quickly and strongly with one another throughout a narrow range. Then, the chains were rearranged in a regular array to some extent, which changed the conformation from a random coil to a β-sheet structure. The crystallization could occur, and this resulted in the formation of fine powder aggregates, which appeared in microscopic observation.

On the contrary, a hydrophobic alcohol such as 1-octanol was immiscible with aqueous silk fibroin, and the phase separation could take place between two mixtures of hydrophilic silk fibroin and hydrophobic 1-octanol, which formed excluded regions in the aqueous environment. As a result, neither the conformational change nor crystallization occurred. The protein chains themselves might have assembled with relative ease and interacted slowly with a wide range. Consequently, a lump-like or branched network structure was formed after freezing and lyophilization.

In addition to the alcohol type, the alcohol concentration of RSF may have also affected the morphology and the conformation of RSF. Because methanol has been widely used for the crystallization of silk fibroin, the effect of methanol contencentration in silk fibroin solution was investigated in this study.

Figure 10 shows the morphological structure of RSF prepared with various amounts of methanol additive in solution. According to this SEM photograph, a coarse and sheet-like structure of particles was transformed into a dense and solid-like structure as methanol concentration increased. The size of the particles became smaller when the methanol content was increased. Moreover, very fine fibrous aggregates could be formed for the sample containing large amounts of alcohol in silk fibroin solution.

These morphological structures of RSF could be attributed to the difference in crystallization behavior, which depended on the amount of meth-

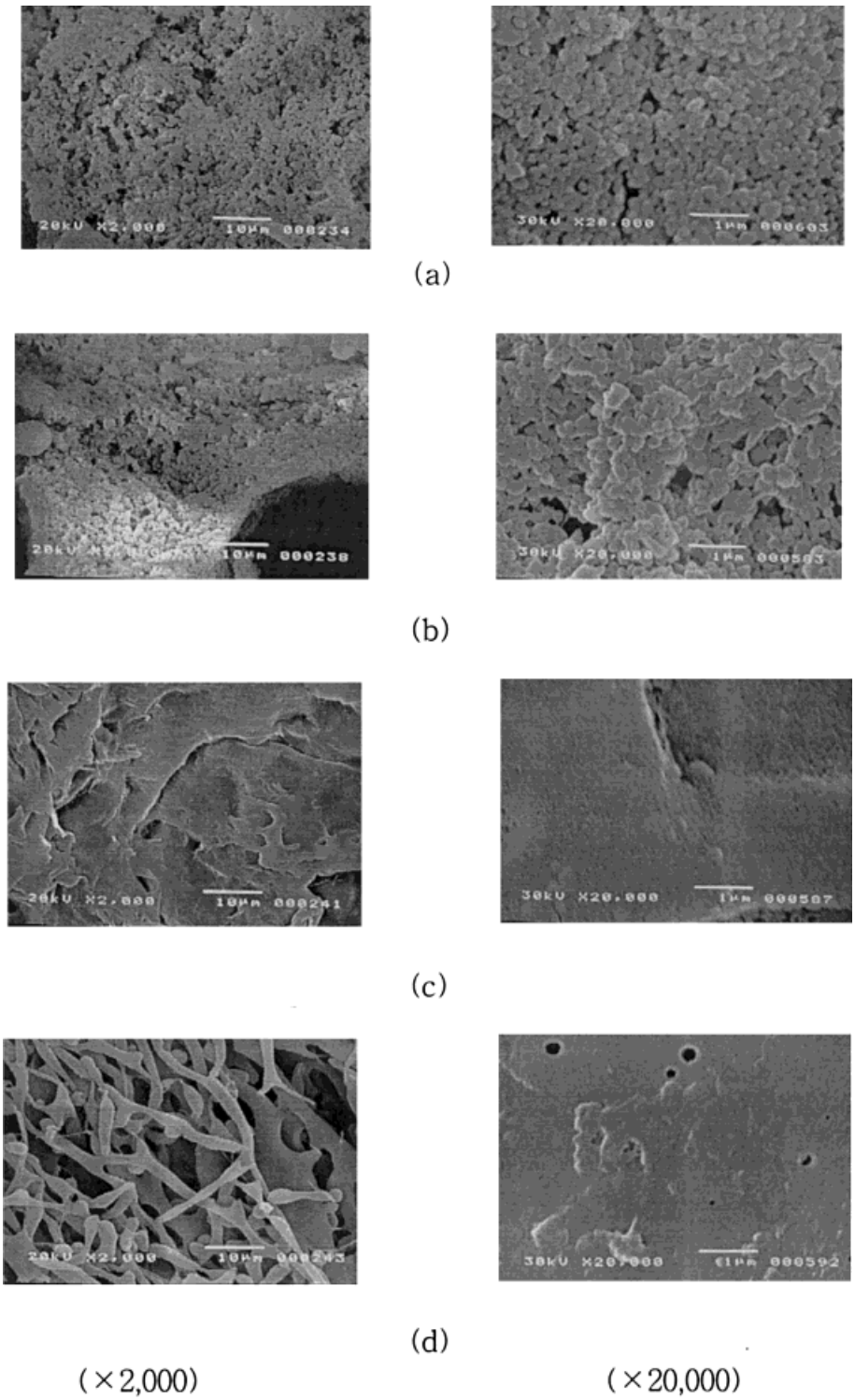

Figure 7 SEM photographs of RSF prepared with various alcohol additives in 10% alcohol-content solution: (a) methanol, (b) ethanol, (c) 1-butanol, and (d) 1-octanol.

anol added. As shown in Figure 11, the β-sheet structure was clearly observed in the FTIR spectra of RSF prepared with a more than 15% concentration of methanol, whereas a random-coil structure was observed with 5% methanol concentration. It seems that the intensity of the absorp-

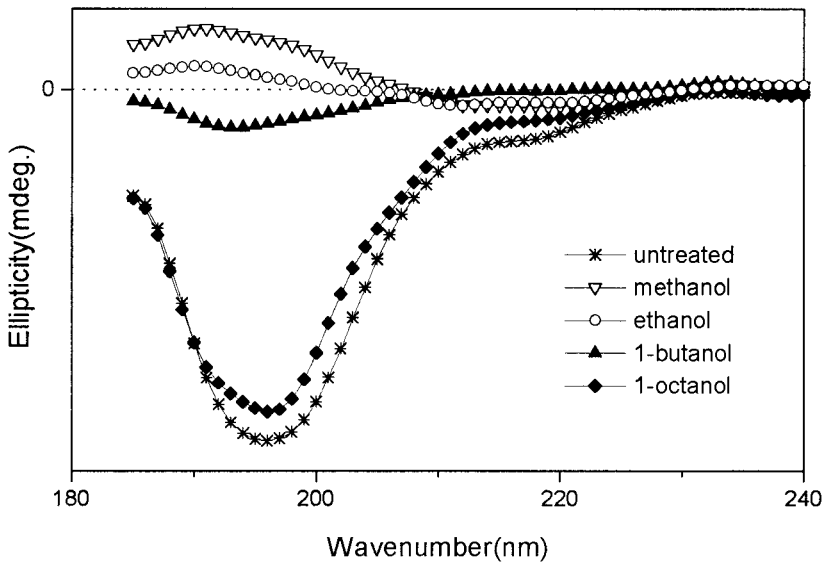

Figure 8 CD spectra of silk fibroin solution prepared with various alcohol additives (10% concentration).

tion bands at 1265, 1520, and 1622 cm^{-1} because of the β-sheet conformation increased with the methanol content but was not evident. Therefore, XRD was used to further examine the structural characterization.

Figure 12 shows the X-ray diffractograms of RSF. When more than 15% methanol content was added to the silk fibroin solution, the conformation of silk fibroin was transformed from a random coil to a β-sheet crystalline structure. This was consistent with the FTIR results, indicating that the crystallization and the formation of the β-sheet conformation can be induced by sufficient amounts of methanol in the silk fibroin solution. It was also observed in the X-ray diffractograms when the area under the diffraction curves was

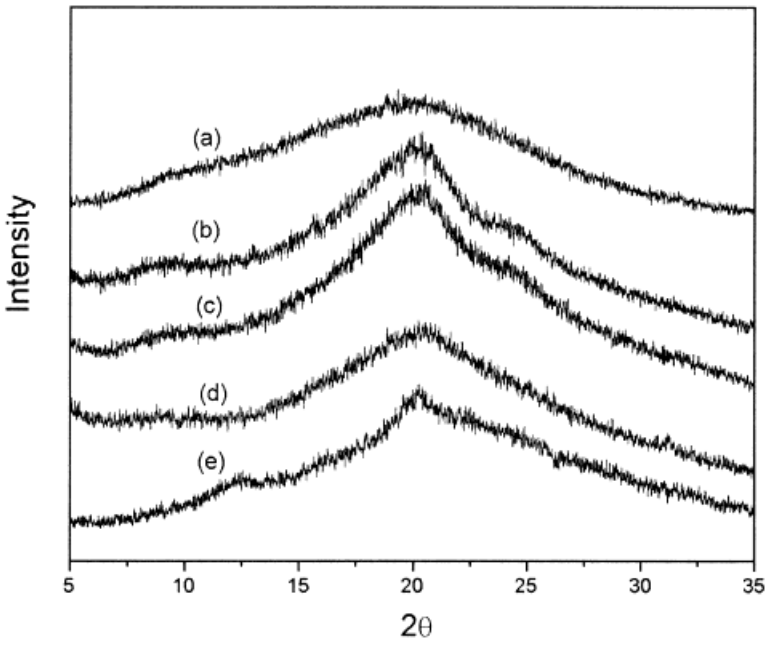

Figure 9 X-ray diffractograms of RSF prepared with various alcohol additives in solution: (a) untreated, (b) methanol, (c) ethanol, (d) 1-butanol, and (e) 1-octanol.

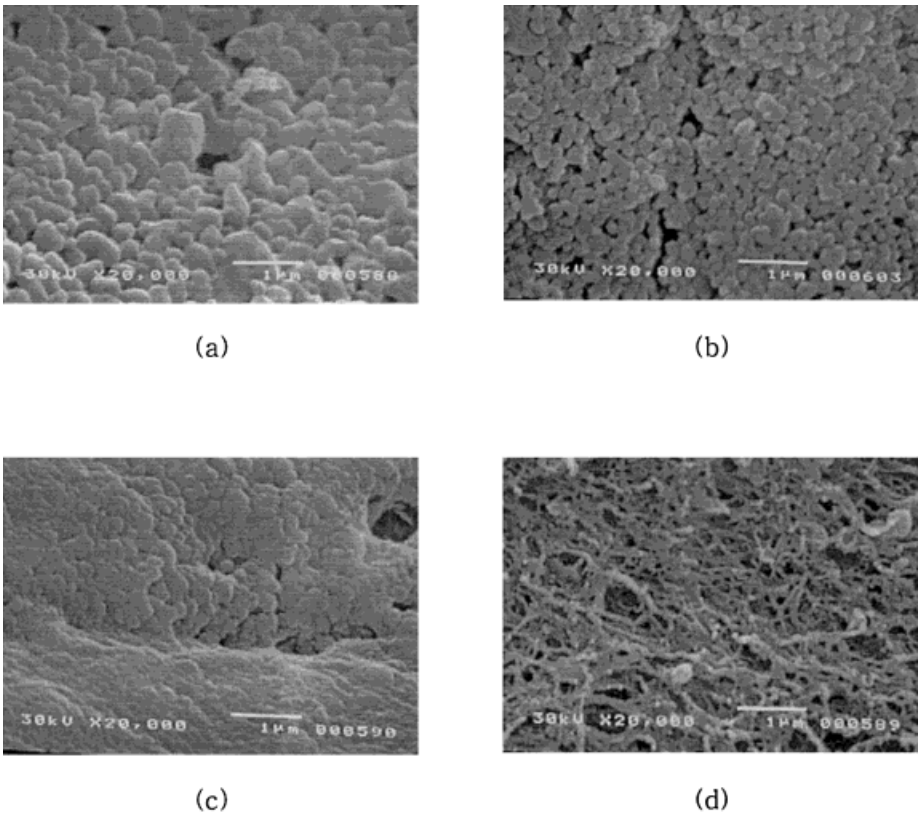

Figure 10 SEM photographs of RSF prepared with various methanol concentrations: (a) 5, (b) 15, (c), 25, and (d) 35%.

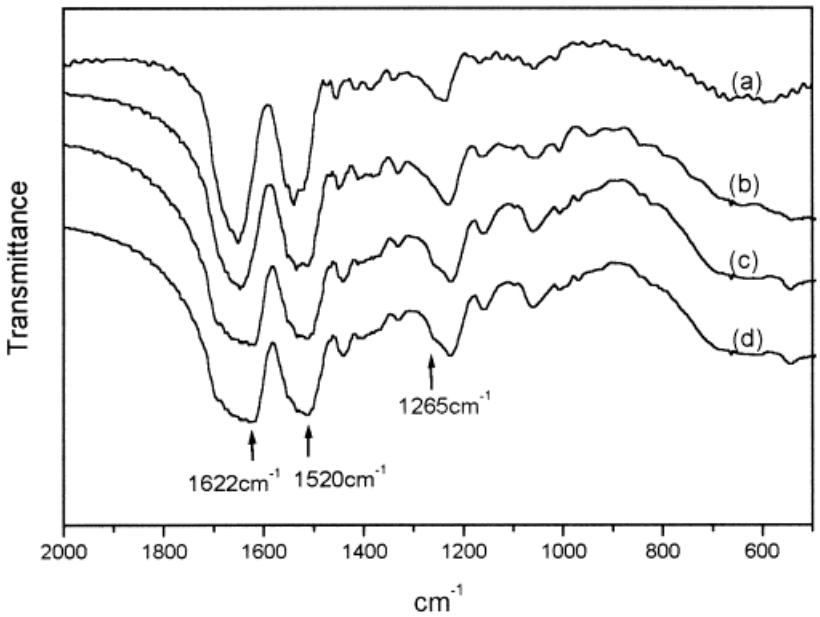

Figure 11 FTIR spectra of RSF prepared with various methanol concentrations in solution: (a) untreated and (b) 5, (c) 15, and (d) 35%.

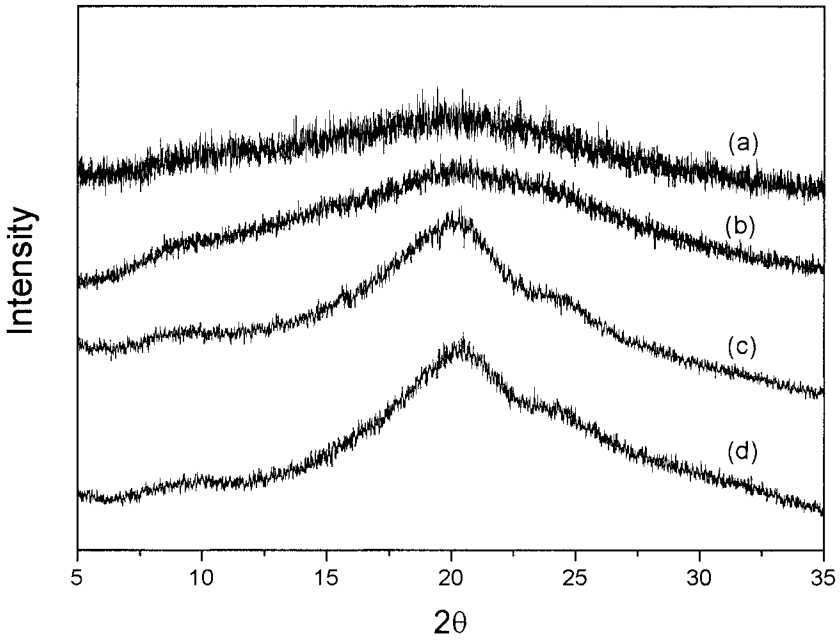

Figure 12 X-ray diffractograms of RSF prepared with various methanol concentrations: (a) untreated and (b) 5, (c) 15, and (d) 35%.

considered that the crystallinity might have increased with the increase in methanol content.

The structural changes caused by methanol treatment usually affect the thermal behavior of polymers. Figure 13 shows the DSC thermograms of RSF prepared with different methanol contents in silk fibroin solution. The endothermic peak of maximum decomposition and the initial decomposition temperature slightly increased to higher temperature.

In addition, the exothermic peak at about 195°C, attributed to the recrystallization of silk

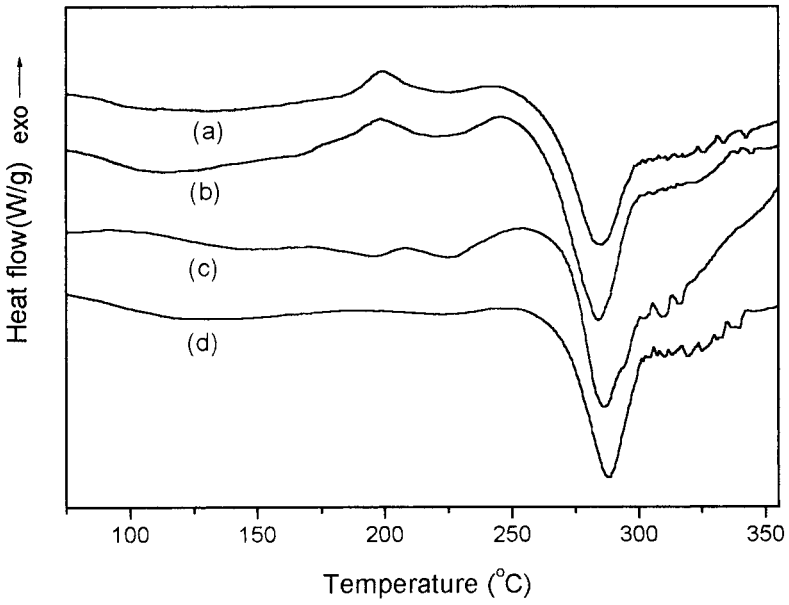

Figure 13 DSC thermograms of RSF prepared with various methanol concentrations in solution: (a) untreated and (b) 5, (c), 15, and (d) 35%.

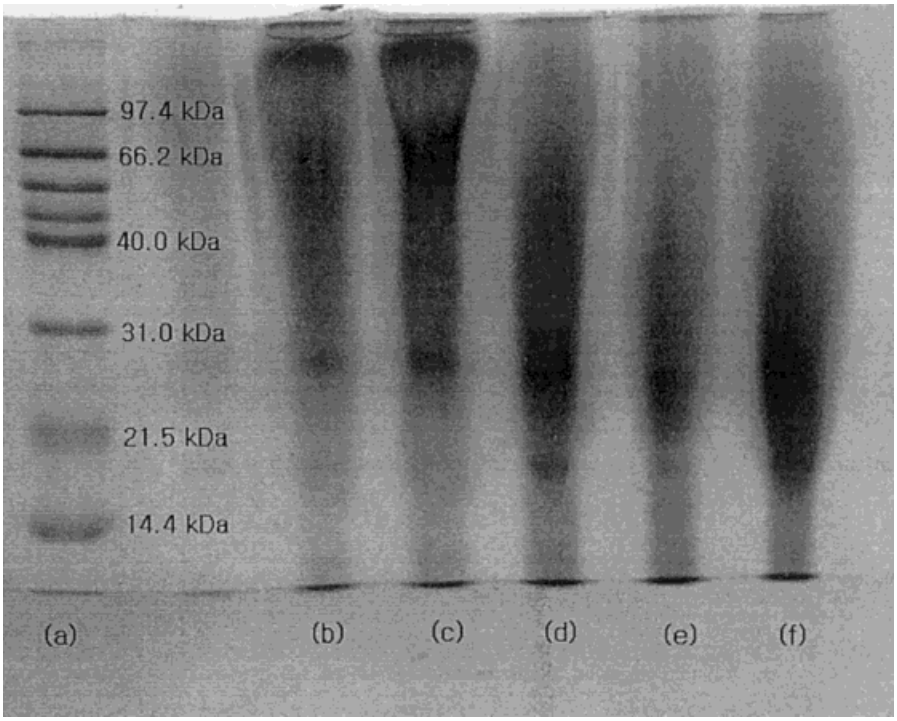

Figure 14 SDS-PAGE (12.5%) of silk fibroin solution prepared with various dissolution times: (a) molecular-weight marker and (b) 1, (c) 3, (d) 12, (e) 24, and (f) 48 h.

fibroin, shifted to a somewhat higher temperature but disappeared with the increase of methanol content. At 35% methanol concentration, the exo–endo transition region was completely obscured in DSC thermograms, indicating that no recrystallization occurred. This was caused by the stable and perfect crystalline formation of the β-sheet conformation induced by the interactions between fibroin molecular chains on methanol addition. Therefore, the morphological features of RSF, such as the form, shape, and size of particle aggregates, could be markedly affected by the structural changes, depending on alcohol type and concentration.

Effect of Molecular Weight

Different molecular weights and molecular-weight distributions of RSF could be obtained by controlling the dissolution time in the $CaCl_2$/ethanol/H_2O solvent system. Figure 14 shows the gel electrophoresis result of a silk fibroin solution prepared at various dissolution times ranging from 1 to 48 h. The bands of molecular-weight distributions were spread out so widely that it was hard to differentiate the molecular weight in accordance with dissolution time. However, as the dissolution time increased, the major band of molecular weight shifted from a higher range (40–100 kDa) to a lower range (20–40 kDa).

The effect of molecular weight was examined on the morphology of RSF, and Figure 15 shows the SEM photographs of RSF prepared at different dissolution times. A large and irregular shape of fibroin particles was obtained for the sample of high molecular weight, whereas small and spherical shapes were formed for the low-molecular-weight samples. The fibroin chains with low molecular weight were free to interact with methanol in the silk fibroin solution, and consequently, spherical fine shapes of fibroin particles could be uniformly formed. The size and shape of particles also did not change much with dissolution times higher than 12 h when the molecular weight distribution were assumed to be same.

CONCLUSIONS

It was concluded that the morphology and structural conformation of regenerated *B. mori* silk fibroin are affected by freezing temperature, type and amount of alcohol added, and molecular

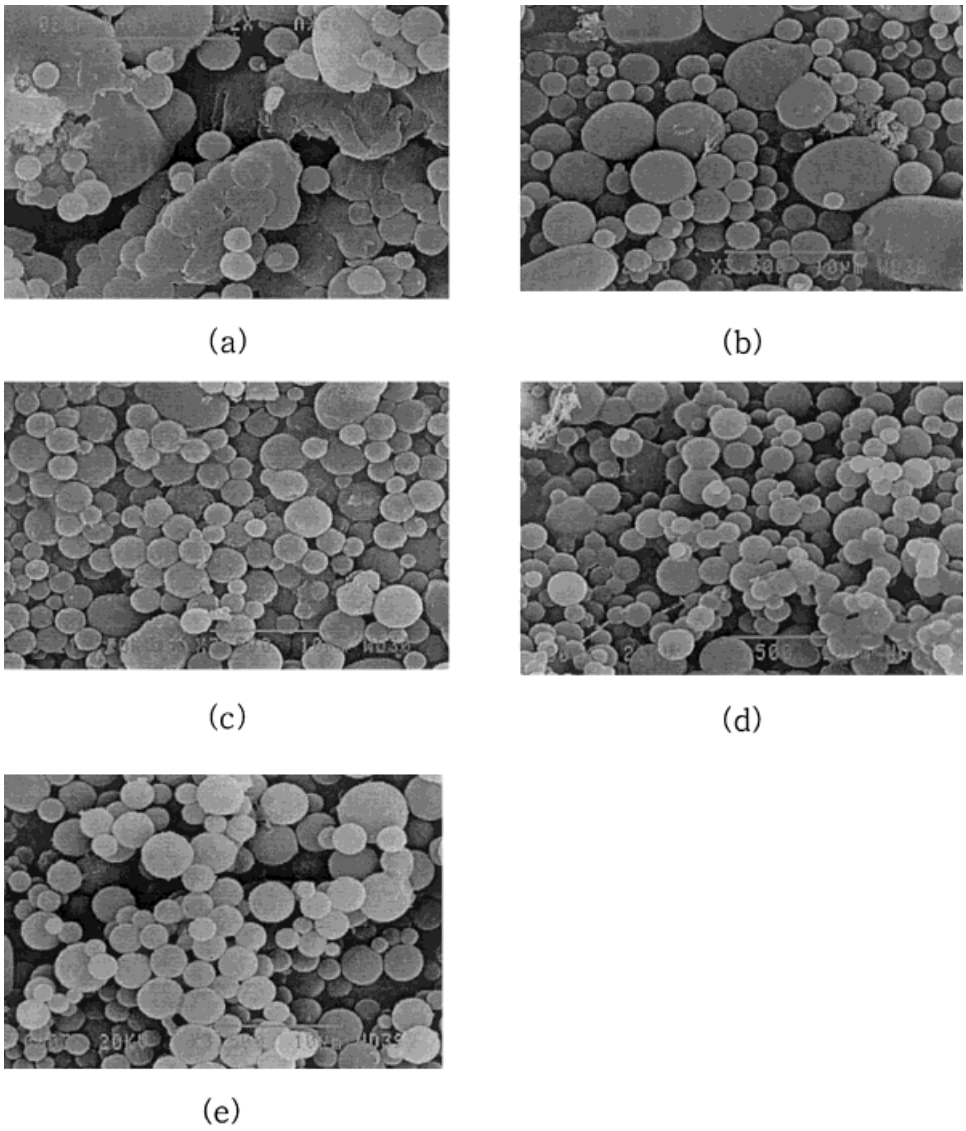

Figure 15 SEM photographs of RSF prepared with various dissolution times: (a) 1, (b) 3, (c) 12, (d) 24, and (e) 48 h.

weight. The fine powder form of aggregates can be obtained at lower freezing temperatures with the addition of a somewhat higher content of hydrophilic alcohol such as methanol or ethanol for a lower molecular weight silk fibroin. The morphological features are related to the conformational changes from a random coil to a β-sheet structure, which is attributed to the crystallization of silk fibroin.

If the preparation conditions and parameters of RSF are selected properly, the shape, size, and crystallinity can be controlled, which has a significant influence on solubility and swelling. Consequently, RSF can be used as effective tool for controlled release and is expected to be a promising biomaterial.

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