Preparation and Characterization of Low Molecular Weight Silk Fibroin by High-Temperature and High-Pressure Method

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ABSTRACT: A low molecular weight silk fibroin powder (LMSF) was prepared through high temperature (200°C) and high pressure (20 kgf/cm^2), without any addition of chemicals. The carbonized adducts produced during this process were then removed by treatment with activated charcoal. The yield of LMSF by this preparation method was over 60% after the removal of carbonized adducts by using activated charcoal. Amino acid analysis showed an observable decrease in contents of serine and tyrosine in LMSF prepared by this method, as compared to those prepared by neutral salt. The molecular weight of this LMSF was also observably decreased with an increase in the reaction time. From the measurements of differential scanning calorimeter (DSC) and thermal gravimetric analyzer (TGA), thermal properties of LMSF through high temperature and high pressure were also decreased as compared to those produced by neutral salts. In addition, wide-angle X-ray diffraction (WAXD) patterns showed that the crystallinity of LMSF differed from that of the original silk fibroin. It can be said that the preparation method of LMSF in this study is a simple, economical, and environmentally compatible process with many advantages. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 85: 2890–2895, 2002

Key words: thermal properties; differential scanning calorimetry (DSC); thermogravimetric analysis (TGA); X-ray

INTRODUCTION

Recent studies of low molecular weight silk fibroin (LMSF) have shown various uses in biomaterials[1–6] or health supplementary diets.[7,8] Natural silk fibroin, however, cannot be dissolved in water or other solvent because of its high molecular weight, crystallinity, and orientation,[9] restricting its broader application. Until now, available methods of preparing water-soluble silk fibroin powder have resulted in a decrease of molecular weight through the hydrolysis of silk fibroin by neutral salts,[10,11] strong acids,[12–14] or enzymes.[15,16] LMSF prepared by the use of neutral salts is mainly used in biomaterial applications[4–6] and its molecular weight is about 50,000 to 200,000.[12,17] On the other hand, LMSF produced by the other two methods is used in health supplementary diets because of its low molecular weight and high absorption rate to the body.[7,8] The methods, however, have demonstrated many drawbacks. First, chemicals used to prepare

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LMSF are known to be toxic to humans and the environment and must be removed by dialysis or ion exchange chromatography.[10–14] These processes are also impractical for both experimental and industrial applications because of their sophistication, time consumption, and high expense. In addition, the release of the toxicoids into the environment has complicated its study by scientists.

Enzymatic hydrolysis, which consists of two steps, is more complex than chemical hydrolysis. In the first step, where there is a use of chemicals, a mild hydrolysis must be carried out because of its high molecular weight, crystallinity, and orientation. This replicates the chemical problems listed above. In the second step, an enzyme is added to the pretreated silk fibroin solution in a suitable condition, when enzymatic hydrolysis of silk fibroin occurs.[15,16] A lengthy reaction time, as well as a prohibitive price for enzymes, prevents common industrial application.

In this study, LMSF was prepared by the technique of high temperature and high pressure without any use of chemicals. The process is relatively simple and is expected to overcome the problem listed above. The thermal properties and crystallinity of LMSF produced by this preparation method were investigated by using a differential scanning calorimeter (DSC), a thermal gravimetric analyzer (TGA), and wide-angle X-ray diffraction (WAXD). The molecular weight and the amino acid composition were determined by gel-filtration chromatography (GFC) and an amino acid analyzer, respectively.

EXPERIMENTAL

Preparation of LMSF

Silk fibroin was acquired through a degumming process of raw cocoon shell with Na_2CO_3 to remove sericin and other impurities and then treated with pure water under 200°C and 20 kgf/cm^2 for 6, 12, and 18 h in the reactor (Next Instrument, Korea). This reactor was made out of Hastelloy-C, known as the most stable metal agent against chemicals and high temperature. After vacuum filtration passed through Whatman filter paper (qualitative 2), the filtered LMSF was boiled in the presence of activated charcoal (Kanto, Japan) to remove the carbonized products produced during the previous process. Pure LMSF powder was obtained by vacuum filtration and lyophilization.

Thermal Property and Crystallinity of LMSF

The thermal properties of LMSF powder were determined by the use of a differential scanning calorimeter (DSC 2910 TA Instruments, USA) in the range of 70 to 300°C and a thermal gravimetric analyzer (TGA 1000 Rheometric Scientific, U.K.) in the range of 70 to 500°C under nitrogen. Crystallinity was measured from wide-angle X-ray diffraction by using a D5005 Bruker (Germany) with Ni-filtered Cu-Kα radiation under 40 kV, 40mA.

Molecular Weight and Amino Acid Composition of LMSF

Molecular weight of LMSF was determined by gel filtration chromatography. The column (2.5 $\times$ 50 cm Pyrex, Germany) was filled with Sephadex G-15 (Sigma) and swelled in phosphate buffer solution (0.1*M*, pH 7.4). The molecular weight marker kits were purchased from Sigma, containing aprotinin (MW 6500), cytochrome C (MW 12,400), and carbonic anhydrase (29,000). The marker kits and LMSF were passed through the column and a UV detector (M720 Youngin, Korea) continuously at a flow rate of 14 mL/min. The absorbance at 280 nm was measured. The amino acid composition was measured by using the Pico-Tag system (Waters, USA)

RESULTS AND DISCUSSION

Preparation Method of LMSF

Table I shows the yield of LMSF prepared by high-temperature and high-pressure method. A very low amount of LMSF below 35% could be obtained under 170°C and 4 kgf/cm^2. However, the yield of LMSF was increased with the increase of reaction temperatures and a marked increase in yield was observed at over 80% in the conditions of 200°C and 20 kgf/cm^2. It was discussed that, at higher temperature, the cleavage of the main chain occurs more and more. The mechanism of the hydrolysis of silk fibroin by this method is fundamentally different from that by neutral salts, acids, or enzymes. In a general system of $CaCl_2$/ethanol/water solvent, $CaCl_2$ plays a major role in breaking the peptide chains of silk fibroin, while ethanol and water play a role in

Table I Yield of the LMSF by High Temperature and High Pressure with Reaction Time

Temperature and Pressure	Time (h)	Yield (%)[a]
170°C, 4 kgf/cm^2	24	32
180°C, 7 kgf/cm^2	24	47
	30	47
200°C, 20 kgf/cm^2	6	87
	12	82
	18	85

[a] Yield (%) = [(final weight of LMSF at the above conditions before treatment with activated charcoal)/(initial weight of silk fibroin)] × 100.

breaking the hydrogen bond and the hydrophobic interaction.[11] Acidic hydrolysis is carried out throughout the cleavage of a Gly-Ala linkage in the main chains of silk fibroin by the attack of acids.[18] Enzymatic hydrolysis is a more specific method. For example, a protease from *Streptomyces griseous* (EC 3.4.24.31) attacks both sides of tyrosine in the peptide chains of silk fibroin.[16] In the case of high-temperature and high-pressure method, however, the cleavage of peptide chains and the breakage of hydrogen bonds or hydrophobic interaction may occur at the same time. Some of the silk fibroin was carbonized under more critical conditions (200°C and 20 kgf/cm^2), as shown in Figure 1.

Yields of LMSF after the Removal of Carbonized Adducts

The solution containing both pure LMSF and carbonized adducts appeared as a deep brown color because of the adducts produced. The browning or yellowing sources[19] can be removed by ion exchange chromatography or through treatment of activated charcoal.[20] To remove the carbonized adducts in our experiments, a suitable amount of activated charcoal was added to the solution and then boiled for 1 h. From a UV spectra, it was found that the degree of the removal of carbonized adducts was closely concerned with the amount of activated charcoal (Fig. 1). The absorbance at 325 nm, which was observed because of the carbonized adducts, disappeared with an addition of activated charcoal. It is explained that the carbonized adducts were absorbed into activated charcoal through the hydrophobic interaction between the adducts and the activated charcoal. As the amount of activated charcoal increased, the yield of pure LMSF decreased, as shown in Table II. Although the amount of activated charcoal was directly proportional to the degree of removal of the carbonized adducts (Fig. 1), the yield of pure LMSF decreased remarkably (Table II) with an

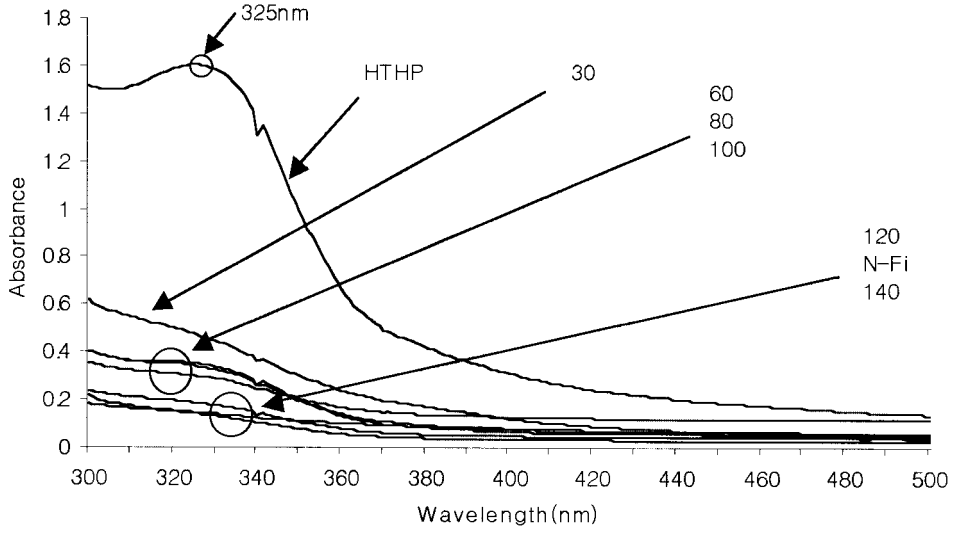

Figure 1 UV spectra of the solution of LMSF after treatment with activated charcoal. HTHP and N-Fi represent the LMSF solution before the treatment of activated charcoal and that prepared by neutral salt ($CaCl_2$), respectively. The numbers 30–140 mean the amounts of activated charcoal used in the removal of carbonized silk fibroin per total dissolved silk fibroin and percentage dimension.

Table II Yield of LMSF as the Amounts of Activated Charcoal Used in the Removal of the Carbonized Materials

Amount of Activated Charcoal per Fibroin Solutes (wt %)	Yield (%)[a]
30	76
60	69
80	67
100	62
120	60
140	59

Note. LMSF was prepared under 200°C and 20 kgf/cm^2 without any use of chemicals.

[a] Yield (%) = [(final weight of LMSF treatment with activated charcoal)/(initial weight of silk fibroin)] × 100.

excessive addition of activated charcoal, indicating that some pure LMSF was also absorbed into activated charcoal. The results suggest that the optimum amount of activated charcoal should be 60–80%, which should correspond to a yield of over 65%.

Molecular Weight and Amino Acid Composition of LMSF

The average molecular weight of LMSF, which means the molecular weight of the peptide species giving the most intense peak in the chromatogram, according to the reaction time, was listed in Table III. LMSFs, prepared by high temperature and high pressure with difference reaction times, showed a wide distribution of molecular weight. A gradual decrease of the average molecular weight was observed with an increase in reaction time. When silk fibroin reacted in conditions of 200°C and 20 kgf/cm^2 for 18 h, the average molecular weight was about 6000. In comparison, LMSF by neutral salts was reported to be 50,000–200,000.[12,17] The wide distribution of molecular weight may be attributed to the nonspecific and rough method by using high temperature and high pressure, as mentioned above.

Table III The Average Molecular Weight of LMSF Prepared Under a Condition of 200°C and 20 kgf/cm^2 with Reaction Time

Reaction Time (h)	Molecular Weight		
	Minimum	Average[a]	Maximum
6	8200	13,600	23,500
12	4400	7200	14,400
18	3600	6000	11,400

[a] Average means the molecular weight of the peptide species giving the most intense peak in the chromatogram.

Table IV Composition of Amino Acids According to the Methods Preparing LMSF

Amino Acid[a]	Content of amino acid (mol %)			
	Original Silk Fibroin	LMSF		
		$CaCl_2$[b]	HCl[c]	HTHP[d]
Gly	46.5	49.8	48.4	53.8
Ala	28.2	25.4	30.6	36.3
Ser	11.3	10.9	12.3	1.5
Tyr	4.6	4.0	0.1	1.9
Val	2.1	1.9	2.1	1.6
Asp	1.4	1.3	2.1	0.1
Lys	1.2	1.2	0.3	0.1
Glu	1.1	1.1	1.4	1.9
Thu	0.8	0.8	1.1	0.1
Leu	0.8	0.6	0.3	0.2
Phe	0.6	0.6	0.1	0.2

[a] Some amino acids (Ile, Arg, Pro, Try, Met, Cys, and His) were not shown in the table.

[b] Treatment of silk fibroin with $CaCl_2$/ethanol aqueous solution.

[c] Treatment of silk fibroin with 2N HCl at 100°C for 48 h.

[d] Treatment of silk fibroin with water under 200°C and 20 kgf/cm^2.

The results of the amino acid composition from amino acid analysis were tabulated in Table IV. The content of the amino acids containing hydroxyl group, such as serine and tyrosine, decreased for LMSF prepared by high-temperature and high-pressure method, whereas there was little change in the contents of other amino acids. It is expected that the carbonized adducts are caused by amino acids containing the hydroxyl group.[19,20] From Table IV, although serine was mostly removed, the content of tyrosine was moderately reduced at LMSF by this method after the treatment with activated charcoal. It can be proposed that serine acts as a major factor for inducing the carbonized adducts, whereas tyrosine plays a negative role.

Thermal Properties and Crystallinity of LMSF

Thermal properties of LMSF were measured from DSC and TGA. As shown in Figures 2 and 3, LMSF prepared by neutral salt, $CaCl_2$ (N-Fi), decomposed at about 290°C, whereas the thermal

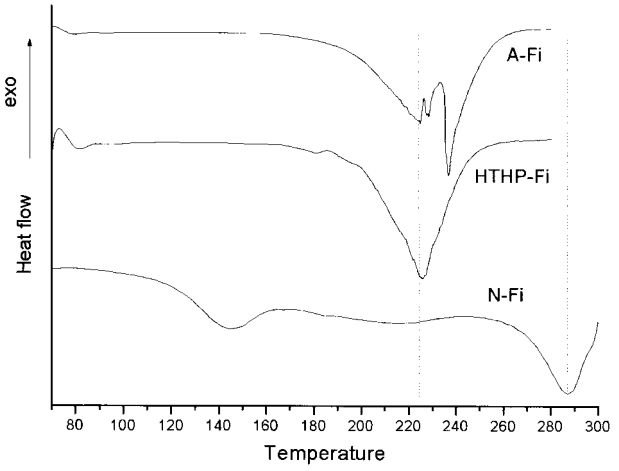

Figure 2 DSC curves of the LMSF powder to be hydrolyzed by hydrochloric acid (A-Fi), high-temperature and high-pressure (HTHP-Fi), and neutral salt, $CaCl_2$ (N-Fi).

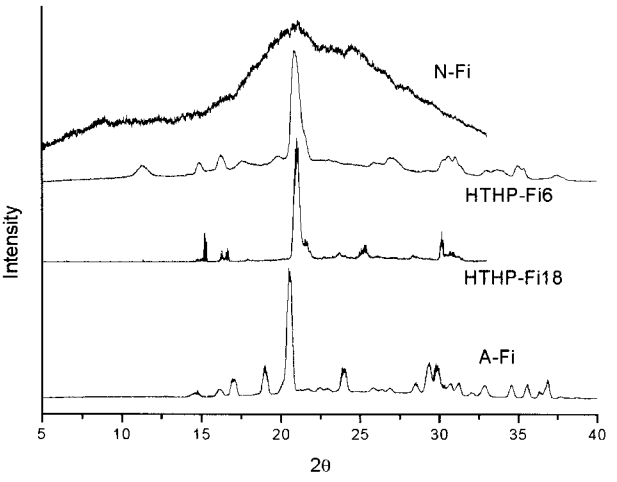

Figure 4 WAXD patterns of the LMSF powder to be hydrolyzed by hydrochloric acid (A-Fi), high-temperature and high-pressure for 6 h (HTHP-Fi6) and 18 h (HTHP-Fi18), and neutral salt, $CaCl_2$ (N-Fi).

decomposition temperature of LMSF by high-temperature and high-pressure method (HTHP-Fi) occurred at about 220°C. The thermal behavior of LMSF by hydrochloric acid (A-Fi) was similar to that of HTHP-Fi. It is expected that the apparent decrease of the temperature was caused by a vigorous cleavage of the main chain. The main chain cleavage may affect the crystallinity as well as the molecular weight, as mentioned above. From the measurement of WAXD patterns (Fig. 4), the N-Fi showed that both amorphous regions and crystalline regions existed together, shown as a broad peak. On the other hand, HTHP-Fi and A-Fi showed a sharp diffraction pattern at $2\theta = 20.9°$ and 20.6°, assigned to β-sheet structure,[21] respectively. The difference of the peak between HTHP-Fi and A-Fi may be explained as a distortion in crystalline portions, where the Gly-Ala linkage is easily accessible by acid when LMSF is prepared by acid.[18] From these results, it can be confirmed that the thermal decomposition temperature is closely related to the molecular weight and crystallinity of LMSF.

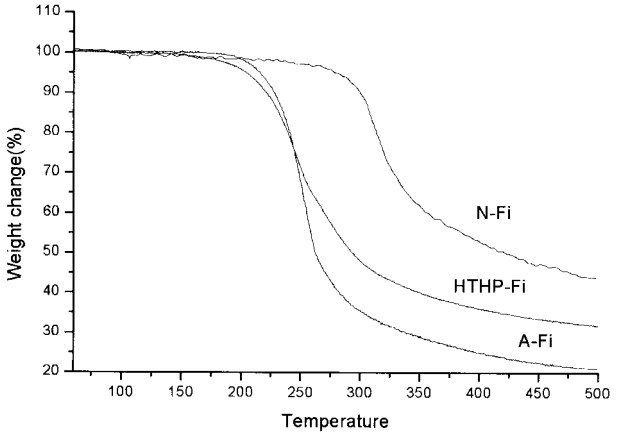

Figure 3 TGA curves of the LMSF powder to be hydrolyzed by hydrochloric acid (A-Fi), high-temperature and high-pressure (HTHP-Fi), and neutral salt, $CaCl_2$ (N-Fi).

CONCLUSION

Because the preparation method of LMSF by high temperature and high pressure is not specific but rather rough, a large distribution of molecular weight is observed. However, it is expected that the molecular weight of LMSF can be controlled by the reaction time in this method. LMSF by this method shows a deteriorated thermal property and a change of crystallinity when compared with LMSF by neutral salts. As a result of amino acid analysis, this method caused some loss of certain amino acids due to the introduction of carbonized adducts produced.

Even though there is a slight disadvantage that the hydroxyl side group containing amino acids, such as serine and tyrosine, is carbonized at a critical condition, the preparation method proposed in this study may have many advantages, such as a simple handling in both experimental and industrial point of view, the elimina-

tion of chemical use, an environmental-compatible process, and affordability. Additionally, the impurities and adducts to be produced during the reaction can be easily removed through the treatment of activated charcoal.

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