Studies on the Effects of the Enzymatic Treatment on Silk Fine Powder

Weilin Xu,[1] Guizhen Ke,[1,2] Xuqiang Peng[1]

[1]*Wuhan Institute of Science and Technology, Wuhan 430073, People's Republic of China*
[2]*Donghua University, Shanghai 20051, People's Republic of China*

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ABSTRACT: In this study, the silk treated by steam explosion was pulverized into fine powder with a mean particle size of 1.829 μm, then the feasibility of the application of the protease treatment to produce finer and more uniform silk powder was investigated. The results indicated that after the enzymatic treatment, the mean diameter of the silk powder was reduced from 1.829 to 1.546 μm and the equivalent diameter distribution became more concentrated; thus, the uniformity of the particles was improved. The infrared spectra analysis demonstrated that the silk powder remained the β-sheet crystalline structure. X-ray diffraction analysis showed the crystallinity of the polished silk powder was very slightly strengthened. It was found that the treatment reduced some polar amino acid in the sericin; however, the composition of the kinds of the amino acids did not change. In addition, the hot water-solubility and moisture regain of the polished silk powder declined very slightly.
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Key words: silk; powder; enzymatic treatment

INTRODUCTION

Silk powder, which is one of the useful physical forms of the silk fibroin protein, has been used as an additive to cosmetic materials, functional foods, and biomaterials because of its good properties such as moderate moisture absorption and retention and considerate affinity to the human skin. Silk from the *Bombyx mori* silkworm is mostly made of fibroin and sericin, respectively taking up 72% and 28%.[1] Fibroin protein is enwrapped and conglutinated by the sericin. Generally, the sericin is removed from the fibroin before production of silk powder in some conventional methods. The silk powder used in this study was purpose-built by a dry method with an ultimate average particle diameter of approximately 2 μm. To producing the powder more easily, the raw silk fiber was pretreated by steam explosion to reduce the strength of the fiber.[2] Generally, each mechanical pulverizing technology has a limited powder size value such as a limited mean size of particles.[3] To decrease the particle size and improve the uniformity of the size distribution, at the same time to keep the configuration of crystalline in the silk powder, in this study the enzyme was used to polish the powder that developed directly from the raw silk. The effects of the enzymatic treatment on the particle size distribution, composition of the amino acids in the powder, and the shape of silk powder were presented. The main structure and crystal configuration of fibroin powder were also investigated.

Correspondence to: W. Xu (weilin_xu@hotmail.com).
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EXPERIMENTAL

Materials

Silk filament was provided by Luotian Silk Incorporation of Hubei Province, China. Silk powder was produced from the raw silk filament pretreated by steam explosion and pulverized in a purpose-built machine. The instrument has been patented in China, and the special structure has been carefully described,[4] the protease was provided by Novozymes Incorporation.

Enzymatic treatment of the powder

The silk powder was treated by protease (novozymes Alcalse®) for 30 min. The bath ratio was 1 : 30. The pH of the solution was about 8.5, and adjusted by sodium carbonate. Temperature of the solution was 55°C.

Particle size measurement

The particle size of the powder was measured by means of image analysis. The main instrument includes a VDP-1750 image analyzer (VICOM Co., America), optic microscope, and computer. During measuring, the dispersion medium was ethanol, and

TABLE I
Statistic Results of the Particle Size Distribution

Item	Original silk powder	Polished silk powder
Mean size, μm	1.829 ± 0.986	1.546 ± 0.699
Maximum, μm	4.417	3.449
Minimum, μm	0.456	0.455
0.00~0.94 μm	27.45%	28.81%
0.94~1.88 μm	27.45%	38.98%
1.88~2.97 μm	29.41%	28.81%
2.97~3.91 μm	13.73%	3.39%
3.91~5.00 μm	1.96%	0.00%

the magnification was ×400. Five samples of the original and the polished samples were measured to get the statistic data, and the results were printed out by the computer; over 20,000 single powders were measured in each sample.

SEM analysis

Scanning electron microscopy (SEM) analysis was carried out with an X-650 microscope, at 10 kV acceleration voltage, after gold coating.

FTIR analysis

The FTIR spectra were obtained by means of American Avtar360 infrared spectrophotometer with attenuated total reflectance, scanning ranged from 4000 to 650 cm^{-1}.

X-ray diffraction analysis

The silk powder was dispersed onto a stub and placed within the chamber of Analytical X-Ray powder diffractometer (Japanese Dmax-rA, wavelength = 1.54 Å, CuKα radiation) with 40-kV generator intensity and 50-mA generator current. The sample was then scanned from 2θ = 5–45°, in step of 0.02°.

Amino acid analysis

Silk powder was hydrolyzed in 6 mol/L HCl aqueous solution for 24 h at 110°C in a vacuum hydrolytic tank, then the solution was diluted to a certain concentration with 0.02 mol/L HCl. The experiment was carried out in Amino Acid Analyzer (Hitachi 835–50).

Hot-water solubility measurement

The degree of hot-water solubility of the silk powder was tested: 3 g(absolute dry weight) of the silk powder treated by protease was put into 150 mL of distilled water and boiled at 100°C for 15 min; the undissolved fraction of the silk powder was fully dried and then weighed. The percentage of hot-water insoluble silk powder is calculated from the equation:

$$\text{percentage of hot-water insoluble silk powder} = W/3 \times 100\ (\%)$$

where W stands for the fully dried weight (g) of the undissolved silk powder.

Moisture regain of the sample was tested under the same conditions, and the moisture regain is defined as the ratio of the mass of water to the mass of fully dry silk powder:

$$\text{Moisture regain}(\%) = (W_2 - W_1)/W_1 \times 100$$

where W_1 and W_2 represent the fully dried weight and conditioned weight of the tested samples.

RESULTS AND DISCUSSION

Particle size distribution of the silk powder

As shown in Table I, both of the two samples had very small particle size with the mean equivalent diameter of 1.829 and 1.546 μm, respectively. According to the statistic data, in the polished silk fibroin powder there were about 96% of the particles less than 3 μm in diameter, while only about 84% particles less than 3 μm in the original silk powder. The standard deviation of the polished powder (0.669) was also smaller than that of the original powder (0.986), which also indicated that after enzymatic treatment, the distribution of silk fibroin powder became more homogeneous and concentrative.

The histograms for the diameter distribution of the silk powder were shown in Figures 1 and 2, respectively. Obviously the histogram 1 had a wider distributing span than histogram 2, and the kurtosis skewed to the left side. It showed that the protease treatment

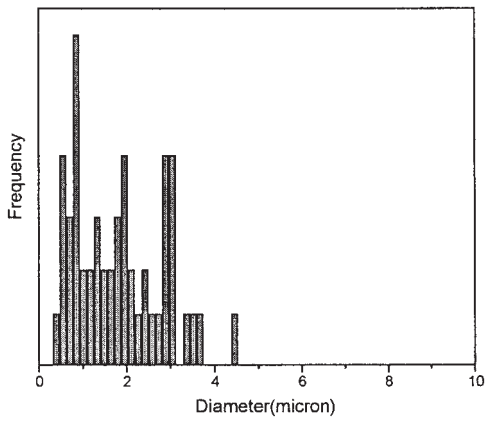

Figure 1 Equivalent diameter distribution of the original silk powder.

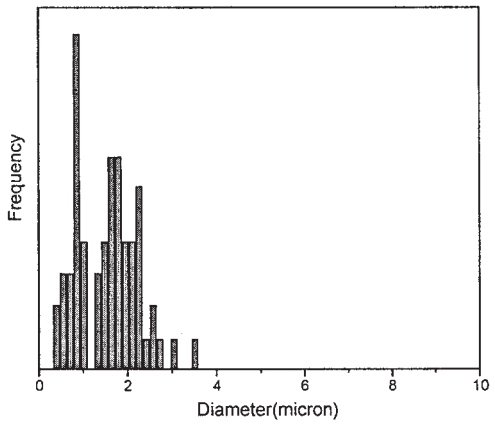

Figure 2 Equivalent diameter distribution of polished silk powder.

effectively polished the silk powder and then made the silk powder finer and more even.

Generally speaking, most of the silk fibroin powder produced by mechanical pulverization consists of the particles of the short fibroin rather than nearly globular particles, and when the powder is used as an additive for cosmetic and pharmaceutical preparations, it will lead to various difficulties that prevent us from making good use of the excellent properties of silk fibroin.[5] Thus, it is important to produce a homogeneous fine powder of fibroin in globular particulate form rather than in fibrous form. In our experiment, most of fine silk powder particles in the untreated sample and the treated sample were observed in round shape rather than fibrous form.

Although the particles tested were very small, and most were in circular shape, the actual shape of the particles may differ in thousands way and there still existed some irregular shape such as fibrous, claviform, and spindly form in both samples. There are some particular images shown in Figures 3 and 4, and there were some evident powder agglomerates in each sample, which are shown in Figures 5 and 6.

It is noticed that the particles with irregular form were very difficult to be dispersed into individuals

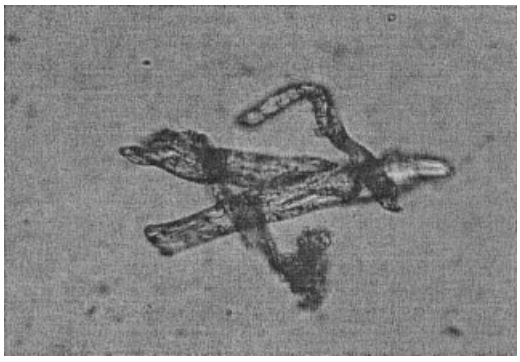

Figure 3 Irregular particles in the original sample (× 400).

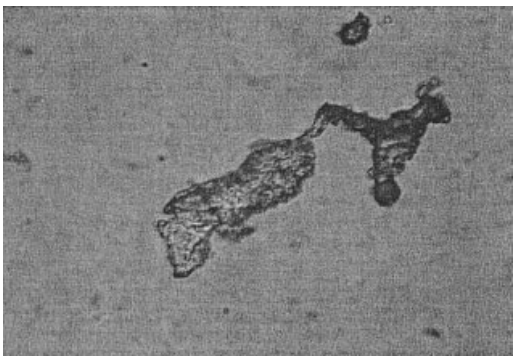

Figure 4 Irregular particles in the powder polished by protease (×400).

during the preparation of the samples and the powder was prone to form relatively large agglomerates. So further study is still necessary to get super fine silk powder with better dispersing and uniformity and less conglomeration.

FTIR analysis

The infrared spectra of the two kinds of silk fibroin fine powder are shown in Figure 7. They showed similar characteristic absorption bands. For example, amide I(vC=O) band appeared at ~1644 cm^{-1} in the original silk powder, and in the polished powder it appeared around ~1626 cm^{-1}. In both spectra, Amid II(δNH + vCN) and amid III(vCN + δNH) bands, respectively appeared around ~15 cm^{-1} and ~1230 cm^{-1}. These showed there were no new functional groups and no new chemical bonds produced during the treating by the protease. The IR spectra of both samples showed the characteristic bands around 1630, 1230, 1070 cm^{-1} of a β-sheet conformation.[6] However, there are still some minor differences that can be observed. For example, the whole reflectance intensity of spectra 2 increased, namely the absorption intensity of

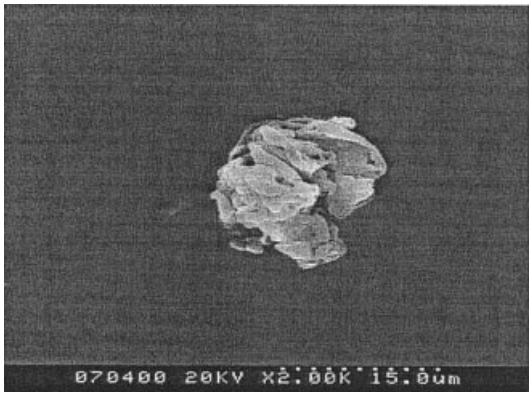

Figure 5 Agglomerates form of silk powder in the original silk powder.

Figure 6 Agglomerates form of silk powder in the polished silk powder.

spectra 2 was weakened. The amid band of the polished silk powder became narrower and slightly shifted to a lower wave number (1626 cm^{-1}). The shifts may result either from a change in the spectral components caused by the presence of different conformations in the crystalline and amorphous regions of silk fibroin or from a slightly different crystalline environment.

X-ray diffraction analysis

Just like other natural fibroin proteins, silk fibroin primarily consists of two kinds of the structure, including the crystalline structure and the amorphous structure. The crystalline polypeptide contains a basic sequence of the —$(Ala\text{-}Gly)_n$—, and the amorphouontains also contain amino acids with bulky and polar side chains, particularly in tyrosine, vas polypeptide cline, and acidic amino acids. The crystalline structure of silk fibroin in the solid state is characterized by a typical dimorphism, with two different chain conformations known as silk I and silk II forms that corresponds with well-established β-sheet structure.[7]

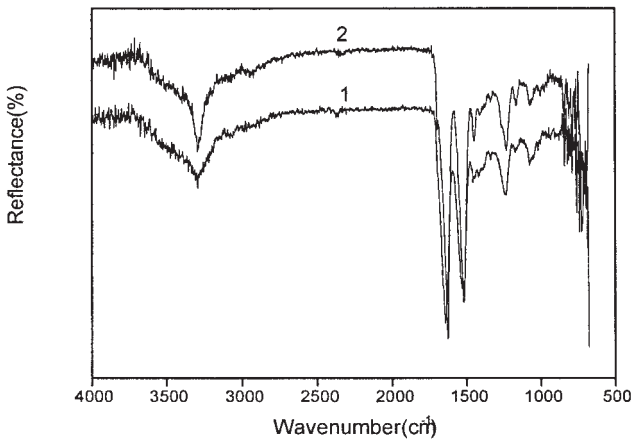

Figure 7 IR spectra of the two powder samples. Curve 1: spectra of the original powder; curve 2: spectra of the polished powder.

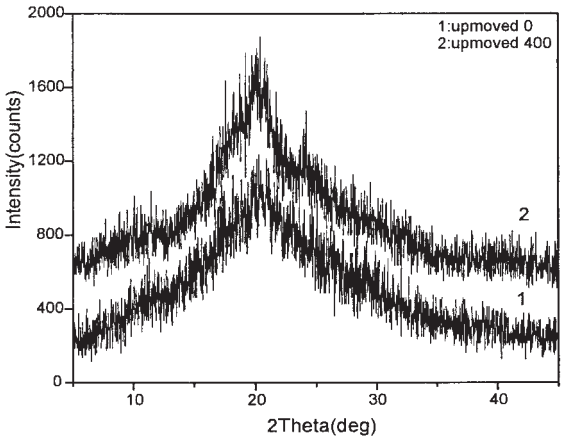

Figure 8 X-ray diffraction pattern of powder samples. Curve 1: spectra of the original powder; curve 2: spectra of the polished powder

X-ray diffraction curves of the two kinds of silk powder are presented in Figure 8. Both of them showed a major peak around 21°, which is the typical peak of silk fibroin known as β-sheet crystalline structure with high orientation.[8] The results indicated that enzymatic treatment did not change the macromolecular conformation and the crystal configuration in fibroin. But sample 1 contained more diffraction intensity of amorphous region than that of sample 2, and the sharpness of the major diffraction peak of sample 2 became stronger and sharper. The results agreed well with the removal of the sericin, which is of amorphism. The removing of the sericin meant the reduction of the amorphous region so it accordingly increased the proportion of crystallinity of the powder. We found that if silk powder with very low crystallinity was fully dried after absorption of moisture or water, the powder usually tended to tightly coagulate together; thus, to hold the property intrinsically provided in silk, it is necessary to keep the original crystalline structure unchanged.

The effect of enzymatic treatment on amino acid component

Silk of some species contain high amounts of the essential amino acids; thus, it is very welcome to be used as cosmetic and additives of food. Amino acids are the "building blocks" of the body; besides building cells and repairing the tissue, they can provide the energy to kill the invading bacteria and viruses; they are part of the enzyme and the hormonal system; they build nucleoproteins (RNA and DNA); they carry oxygen throughout the body, and participate in muscle activity.[9]

TABLE II
Amino Acid Composition and Content (mg %)

Silk amino acids	Original silk powder	Polished silk powder
Histidine	0.59	0.34
Isoleucine	0.99	0.97
Leucine	1.00	0.83
Lysine	1.04	0.58
Methionine	0.32	0.61
Phenylalanine	1.24	1.2
Threonine	2.68	1.16
Tryptophane	/	/
Valine	3.06	2.9
Alanine	25.96	29.95
Glycine	28.33	33.53
Proline	0.57	0.61
Glutamic acid	3.89	2.67
Aspartic acid	5.34	2.37
Cystine	0.36	0.43
Tyrosine	8.47	8.99
Arginine	1.74	1.68
Serine	14.47	11.14
NH_3	0.57	0.61

The results of amino acid analysis of the pulverized silk powder are presented in Table II. The default of data of tryptophane acid was due to the breakage of tryptophane during hydrolysis. As was shown in Table II, after enzymatic treatment the kinds of the amino acids in the powder remain unchanged, and three simple amino acids (glycine, alanine, and serine) together accounted for a major proportion in the total amino acids. But the content of each amino acid changed a little, especially the relative proportion (%) of some amino acids with high polarity and hydrophilicity. The difference was mainly attributed to the removal of the sericin after the protease treatment. Sericin contains a great deal of aspartic acid, serine, glutamic acid, etc.; thus, after the treatment, the relative content of these amino acids decreased. However, the essential amino acids (which cannot be manufactured by the body) such as phenylalanine, methionine, and valine almost remained unchanged; this is very important when the powder is used in the biomaterials.

Solubility and moisture regain of silk powder

Water-soluble silk fibroin powder has been usually produced by means of hydrolysis with hydrochloric acid or neutral salt or the like.[10] However, sometimes the silk powder with the crystalline structure is needed for special materials developing; thus, in this case it is important to keep the crystalline structure of fibroin in the powder. Hot-water insolubility of the two kinds powders were tested; they were 79.66 and 92.57%, respectively, for the original powder and polished powder. This was mainly due to the removing of the sericin in the powder.

On the other hand, the moisture regain of the silk powder was 9.89 and 8.14%, respectively, for the original powder and the polished powder.

As for further application of this silk superfine powder below 3 μm in an average particle diameter provides excellent formability, improved adhesive property to the skin or the like, improved extensibility, and an improved sense of touch and the like.[11] Then the crystalline superfine silk powder manufactured in the present process is hopeful in applications to raw materials for use in cosmetics such as lip sticks, eyebrow paints, hair dyes, eyeliners, powder blushes, and foundations, and for applications to ink additives, resin composite raw materials, and raw materials for paints.

CONCLUSIONS

The feasibility of the application of protease treatment to produce finer and more uniform crystalline silk powder had been investigated. After enzymatic treatment, the mean diameter of silk powder was reduced from 1.829 to 1.546 mm, and the equivalent diameter distribution became more concentrated and the uniformity of particles was improved. After the polishing, the silk powder remained β-sheet crystalline structure and crystallinity increased slightly. The amino acid analysis results showed the kinds of the amino acids did not change, but the proportion of some amino acids decreased a little due to the removing of the sericin. Owing to the removal of some amino acids with polar side chains, hot-water insolubility increased and the moisture regain of polished silk powder declined in some degree.

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