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Silk Microgels Formed by Proteolytic Enzyme Activity

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Abstract

The proteolytic enzyme α -chymotrypsin selectively cleaves the amorphous regions of silk fibroin protein (SFP) and allows the crystalline regions to self-assemble into silk microgels (SMG) at physiological temperature. These microgels consist of lamellar crystals in the micrometer scale, in contrast to the nanometer scaled crystals in native silkworm fibers. SDS-PAGE and zeta potential results demonstrated that α -chymotrypsin utilized only the nonamorphous domains or segments of the heavy chain of SFP to form negatively charged SMGs. The SMGs were characterized in terms of size, charge, structure, morphology, crystallinity, swelling kinetics, water content and thermal properties. The results suggest that the present technique of preparing SMGs by α -chymotrypsin is simple and efficient potential and that the prepared SMGS have useful features for studies related to biomaterials and pharmaceutical needs. This process is also an easy approach to obtain the amorphous peptide chains for further study.

Keywords

silk fibroin protein; α -chymotrypsin; self-assemble; peptide and microgels

1. Introduction

Microgels are microscopic hydrogel particles with tunable biological, chemical and mechanical properties [1, 2]. Natural structural biomaterials such as bone, horn, nail, skin, feather and hair are structures of self-assembled protein molecules [3]. Protein based microgels have been explored to understand their physical properties, and their potential in therapeutic applications such as tissue engineering scaffolds, controlled drug release, and biosensors [4-8]. The preparation of protein microgels can be achieved by different methods [9-12].

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The SFP from *Bombyx mori* silkworm is a promising natural polymer protein, widely studied due to its unusual amino acid composition and chemistry, and used as a biomaterial for various applications. The SFP is composed primarily of amino acids such as glycine (Gly), alanine (Ala), and serine (Ser) in a molar ratio of 3:2:1, which form typical $-(\text{Ala-Gly})_n$ - repeating motifs, accounting for ~88% of the total amino acids. Tyrosine (Tyr) accounts for 5.3 mol%, and acidic and basic amino acids total about 3.0 and 1.1 mol %, respectively [13]. This characteristic amino acidic pattern results from the contribution of three polypeptides chains, heavy chain H-fibroin with a molecular weight of ~370 kDa, light chain L-fibroin with a molecular weight of ~25 kDa and a P25 glycoprotein [14]. The L- and H-peptide chains are linked by a single disulfide bond [15]. The SFP is subdivided into four domains, N-terminus, repetitive domains, C-terminus and L-chain. The hydrophilic domains include the N- and C- termini. The N-terminus possesses negative charges with an isoelectric point (IEP) of 4.6 and the C-terminus has an IEP of 10.5. The repetitive domains consist of long hydrophobic domains of Gly and Ala with very short (12 amino acid) intermediate hydrophilic (spacer) domains with a single negative charge. The L-chain has a counterbalanced amphiphilicity and negative charge (Fig. 1) [16].

The secondary structures of SFP include random coil, alpha helix, silk I, silk II (beta-sheet) and silk III (three fold helix) [17, 18]. In the case of *B. mori*, the primary structure consists of 12 repetitive crystalline regions and 11 non repetitive charged spacers [19]. The crystalline hydrophobic domains of the amino acids comprises 94% of the sequence and each repetitive region is on average 413 residues in length [15]. Gly-Ala sequences predominate (~80%) with characteristic repeat units of GAGAGSGAGAGY and GAGAGVGY, which form the hydrophobic domains and are mainly responsible for the formation of antiparallel β -sheets [20, 21].

The combination of hydrophilicity and hydrophobicity within the SFP chains promotes self-assembly in aqueous medium which in turn results in the formation of different biomaterial forms, including microgels, micelles and vesicles. These properties of SFP also support the formation of β -sheets due to physical or chemical inputs including sonication, agitation and the addition of organic solvents, to induce the formation of β -sheet structure and insolubility in aqueous medium. SMGs can be used to improve tissue integration and facilitate drug delivery and are considered attractive biomaterials for various therapeutic applications.

The objective of this study was to assess a new option for the preparation of silk-microgels, including the separation of the less hydrophobic domains of SFP using a proteolytic process. α -Chymotrypsin is a well-characterized serine protease composed of a catalytic triad serine 195 (Ser-195), histidine 57 (His-57), and aspartic acid 102 (Asp-102). The detailed catalytic mechanisms of chymotrypsin have been reported in several reviews [22-25]. α -chymotrypsin cleaves proteins selectively on the carboxyl terminal side of aromatic or large hydrophobic amino acids such as tyrosine, threonine, tryptophan, phenylalanine and methionine [26, 27] and tyrosines are in abundance in silk with about 5% of the total amino acids and located at the junctions of the hydrophobic domains

In the present study, the preparation of silk protein microgels was pursued by self-assembly of α -chymotrypsin generated silk protein peptides. The understanding of the mechanisms of silk protein self-assembly via biocatalysis would potentially be beneficial for the design and fabrication of biomaterials for various therapeutic applications. The approach described here may also provide new options to interrogate protein-self assembly by exploiting selective cleavage of peptide bonds.

2. Experimental section

2.1. Materials

Cocoons from *Bombyx mori* silkworm were obtained from Tajima Shoji Co (Yokohama, Japan). Sodium carbonate (NaCO_3) and lithium bromide (LiBr) were purchased as reagent grade from Sigma–Aldrich or Fluka (St. Louis, MO) and used without further purification. Dialysis cassettes (Slide-a-Lyzer MWCO 3.5K) were purchased from Pierce biotechnology (Rockford, IL). NuPAGE Novex Bis-Tris Mini Gels and Mw marker 460 kDa (LC5699) were purchased from Invitrogen (Carlsbad, CA). Enzyme α -Chymotrypsin (40 units/mg) was purchased as reagent grade from Sigma–Aldrich (St. Louis, MO).

2.2. Preparation of aqueous silk fibroin solution

Aqueous SFP solutions were prepared based on our published protocols [28]. Briefly, whole cocoons were cut into small pieces and were boiled in a 0.02 M aqueous solution of NaCO_3 . The remaining fibroin was rinsed thoroughly in deionized water and allowed to dry overnight. The dry fibroin was then dissolved in a 9.3 M aqueous solution of LiBr at 60°C for six hours. The LiBr was removed from the solution over the course of 48 hours by dialysis cassettes (Slide-a-Lyzer MWCO 3.5K, Pierce biotechnology, Rockford, IL) and remaining particulates were removed by centrifugation and syringe based micro-filtration (5 μm pore size, Millipore Inc., Bedford, MA). This process enables the production of 8–10 w/w % SFP in water. SFP solutions with lower concentrations were prepared by diluting the above solution with double distilled deionized water. The final SFP concentration of the solution was monitored by drying 1 ml silk solution samples in a plastic petri dish at 60°C (American Scientific Products, Constant Temperature Oven, Model DK-42) and weighing the resulting dried films. All the samples were performed in triplicates.

2.3. Preparation of silk scaffolds and microgels

The SFP solutions with lower concentrations were prepared by diluting the original SFP solution with water. Salt leaching porous silk scaffolds (SFS), to use as comparisons and controls in the present study, were prepared according to the procedure described in literature [29, 30]. The α -chymotrypsin stock solution was prepared with distilled water at the concentration of 5 mg.mL^{-1} . The stock solution of α -chymotrypsin was added to 5 mL of 2% SFP solution to obtain a final concentration 0.5 mg.mL^{-1} α -chymotrypsin in the final SFP solution. This solution was incubated at 37°C for 12 h. After the incubation period, the enzyme treated SFP solution turned milky and separated into two phases. The white colored particles at the bottom of tube were SMG.

2.4. Sodium dodecyl sulfate polyacrylamide gels (SDS–PAGE)

Aqueous solutions of native silk and α -chymotrypsin treated SFP supernatant were subjected to SDS–PAGE by following protocols provided by NuPAGE Novex Bis-Tris Mini Gels (Invitrogen, Carlsbad, CA). After the 2% SFP solution was treated with α -chymotrypsin for different time periods, the samples were collected in Eppendorf tubes and stored at -80°C to minimize enzyme activity. A 6.5 μl sample of untreated SFP and α -chymotrypsin treated SFP were mixed with 2.5 μl NuPAGE® LDS sample buffer (4 $\times$) and the mixture was run on a NuPAGE® Novex 4–12% Bis-Tris Gel 1.0 mm, 12 well (NP0322 BOX, Invitrogen, Carlsbad, CA). Standard pure native SFP solution samples were mixed with sample buffer and run on gels as controls. The gel was run in 1X NuPAGE® MES SDS running buffer and then stained using SilverXpress® silver staining kit (Invitrogen) at voltage 200V for 35 minutes with initial current 125 mA/gel to 70 mA/gel at the end. Molecular weight marker 460 kDa (LC5699) (Invitrogen, Carlsbad, CA) was used and

digital images of the gels were captured with a Flour-S Multi Imager (Bio-Rad Laboratories, Hercules, CA) and a Samsung digital camera.

2.5. Light scattering

The dimensional analysis of SMG were carried out using a Coulter LS230 Laser Diffraction Particle Size Analyzer (Beckman Coulter Fullerton, CA, USA), equipped with small volume module plus. The particle size analysis range of Coulter LS230 is from 0.04 μm to 2000 μm . SMG suspensions were added into the cell until 30–50% obscuration of Polarization Intensity Differential Scattering (PIDS) detector was reached. Double distilled deionized water was used for background and the distribution of diameters was processed using the Fraunhofer optical model [31]. Three runs were performed on each sample.

2.6. Zeta-potential analysis

The zeta-potential of surface-charged SMG and SFS colloidal suspensions were determined using a Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK). The analysis was performed at a scattering angle of 90° and temperature 25°C.

2.7. Scanning electron microscopy (SEM)

The lyophilized SMG samples were mounted on aluminum SEM sample holder supports, fixed with double-sided adhesive tape and sputtered with gold prior to SEM observation. The morphologies of SMG samples were observed using a JEOL (JSM-5600LV, Tokyo, Japan) scanning electron microscope (SEM) at the required magnification and with accelerating voltage of 18kV. The samples were sputtered with gold prior to SEM observation.

2.8. Wide angle X-ray scattering (WAXS)

Wide-angle X-ray diffraction patterns were performed at room temperature with a Kristalloflex 810 diffractometer (SIEMENS, Karlsruhe, Germany) using a Cu K α ($\lambda=1.5406 \text{ \AA}$) as X-ray source. Scans were run in the high angle region $5^\circ < 2\theta < 65^\circ$ at scan rate of 0.040°/min and a dwell time of 2s.

2.9. Transmission fourier transform infrared spectroscopy (FTIR)

The FTIR measurements were carried out in a Jasco FTIR model 410 spectrophotometer (Jasco, Tokyo, Japan). The spectra were taken as an average of 32 scans with 4 cm^{-1} of resolution in the region of 5000 to 400 cm^{-1} .

2.10. Fourier transforms Raman (FT-Raman) spectroscopy

Fourier Transform Raman (FT-Raman) spectra were performed on an FT-Raman Module (NXR, Thermo Fisher Scientific, Madison, WI, USA). The powder samples were pressed into gold coated sample holder and spectra were collected with a laser power of 0.35 W and number of scans 1500 at a resolution of 4 cm^{-1} . The spectra were obtained in the Raman shift region between 400 and 4000 cm^{-1} using OMNIC software (Version 5.1, Thermo Fisher Scientific, Madison, WI, USA)

2.11. Deswelling kinetics

The deswelling kinetics of the samples were measured by water weight loss over time isothermally at 37°C to the dried state. The dried SFS powder was immersed in distilled water and thoroughly washed SMG with distilled water kept at 37°C for 5 hrs to obtain the samples with fully equilibrated swollen state. The swollen SMG and SFS samples were removed from the distilled water and subjected to deswelling kinetic assessments by

isothermal thermogravimetric (TGA) treatment at 37°C in a TA Q500-thermobalance (TA Instruments, Milan, Italy). The weight loss of specimens weighing 10-15 mg was tracked over 4 hr under a nitrogen atmosphere at a flow rate of 60 mL·min⁻¹.

2.12. State of water

The state of water in SMG and SFS was investigated by differential scanning calorimetry (DSC). DSC evaluations were carried out on a Mettler DSC822e module (Mettler Toledo, Milan, Italy) controlled by the STARe software under a nitrogen atmosphere at 80 mL·min⁻¹ flow rate. Equilibrated swelled state SMG and SFS samples of 3 to 5 mg were weighed in 40 µL aluminum pans and an empty pan was used as reference. The experiments were performed with 5 samples each. DSC temperature calibration was performed using indium standards. Measurements were performed according to the following protocol [32].

1. Cooling scan from 25°C to -70°C at 10° C·min⁻¹ and isotherm of 5 min at the ending temperature; and
2. Heating scan from -70°C to 50°C at 10° C·min⁻¹.

The equations used to calculate these states of water are the following:

$$w_f = Q_w / \Delta H_w \quad (1)$$

$$w_{nf} = w_t - w_f \quad (2)$$

where, w_f , w_{nf} and w_t are the weight of freezable, non-freezable and total water in swelled membranes, respectively, and Q_w is the measured heat of crystallization in Joules and ΔH_w is the enthalpy of the ice-water transition obtained from pristine water, which is 393 J·g⁻¹ measured on cooling.

2.13. Thermogravimetric analysis (TGA)

TGA experiments were performed in the TA Instruments Series TA Q500 (TA Instruments, Milan, Italy) with a Thermogravimetric Analyzer Software. Sample weights were between 9-13 mg and were scanned at 10°C·min⁻¹. The temperature range was 30°C to 900°C under a 60 mL·min⁻¹ flow rate of nitrogen.

2.14. Differential scanning calorimetry (DSC)

Thermodynamic parameters DSC of the samples were assessed on a Mettler DSC822e module (Mettler Toledo, Milan, Italy) controlled by the STARe software under a nitrogen atmosphere at 80 mL·min⁻¹ flow rate with three scans. Samples of 3-9 mg were weighed in standard 40 µL aluminum pans and an empty pan was used as reference. Measurements were performed in accordance with ASTM D 3418 methods under a nitrogen flow rate of 80 mL·min⁻¹ according to the following protocols:

1. First heating scan from -20°C to 180°C at 10°C·min⁻¹ and 3 min of isothermal conditions at the end;
2. First cooling scan from 180°C to -20°C at -10°C·min⁻¹ and 3 min of isothermal conditions at the end
3. Second heating scan from -20°C to 300°C at 10 °C·min⁻¹.

2.15. Statistical analysis

Statistical significance between multiple groups was calculated by a one-way analysis of variance (ANOVA). The Student's t test was used for the statistical significance of

differences between average values of two independent groups at 95% confidence ($p < 0.05$).

3. Results and discussion

3.1. Effect of proteolysis

α -chymotrypsin was added to a SFP solution at room temperature, which turned milky after incubation at 37°C for 5 minutes. This milky color was due to the α -chymotrypsin selectively interacting with the amorphous linker regions in the SFP and allowed the crystalline regions to self-assemble into microgels. The untreated solution of SFP was used as a control. After incubation for 1 hr, the enzyme treated SFP solution formed microgels and settled at the bottom of the container. The transparent supernatant was withdrawn at different time intervals and characterized by SDS-PAGE (Fig. 2). Untreated SFP solution (control) was run for comparison. A darker smear appeared at the upper part of gel for the control, which corresponds to heavy chain peptides H-fibroin comprised of the crystalline hydrophobic domains with a molecular weight of ~370 kDa. The smaller L-fibroin with a molecular weight of 25 kDa and a P25 glycoprotein of SFP were visible on the gels, whereas the heavy peptide chains disappeared from the SDS-PAGE when the enzyme treated SFP supernatant was run. This observation indicated that α -chymotrypsin selectively cleaved the amorphous regions of SFP and allows the crystalline regions to self-assemble into SMG at physiological temperature.

3.2 Light scattering

Light scattering has a direct dependence on particle size. Dynamic Light Scattering (also known as PCS -Photon Correlation Spectroscopy) measures Brownian motion and relates this to the size of the particles. The light scattering experiment results revealed that the microgel suspensions consisted mainly particles of ~10-50 μm average diameter (Fig. 3). The suspensions were stable and could be stored in a closed glass bottle at 4°C for 30 days without further aggregation.

3.3 Zetapotential

Zeta potential is a measure of the magnitude of electrostatic interactions between charged surfaces. The measurement of zeta potential brings detailed insight into the dispersion mechanism and colloidal stability of prepared silk microgels. The zeta potential represents the surface charge which occurs in the presence of an aqueous solution when functional groups dissociate on surface or ions adsorb onto surfaces from the solution.

The obtained zeta potential results of SMG indicates that SMG were negatively charged -24 ± 2.9 mV, where as the SFS powder suspension showed a charge of $+1.5 \pm 0.8$ mV (Fig. 4). Since the SMGs are a result of the self-assembly of the crystalline hydrophobic domains of SFP heavy peptide chains.

3.4. Scanning electron microscopy (SEM)

The morphological characteristics of the samples were examined by SEM as shown in Fig. 5. The microstructure of microgels holds a prominent influence on cell proliferation, function, and migration, all of which are key issues in biomedical applications of polymeric hydrogels. The microscopic view shows a porous surface. The gel particle size was about ~50 μm with pores ranging around 4 μm . The microgels showed a spongy interconnected structure with roughness on the surface. The porous surface could also arise from the freeze drying process to which the SMG was subjected to freezing to a temperature of about -20°C followed by vacuum drying has been shown to produce porous structures.

3.5. Wide angle X-ray scattering (WAXS)

The WAXS diffraction patterns of SMG and SFS images are shown in Fig. 6. The SMG XRD spectra shows narrow and intense peak while SFS shows broad and low intense peak. The differences between diffraction patterns suggest that SMG is more crystalline than SFS. This is again due to the selectivity of α -chymotrypsin to foster the self-assembly of the crystalline regions of SFP to form SMG. Crystallinity of the SMG alters its morphology and physiochemical properties such as degradation, stiffness, yield stress, modulus and tensile strength, solubility, optical and electrical properties, which can have a direct impact on applications. High crystallinity may lead to the formation of microchannels, and at the same time the large surface area enhances biomolecule release. In some cases biomolecules do not tend to diffuse through crystalline domains and as a result the route that the biomolecules travel is longer and can provide a controlled release system.

3.6. Structural analysis by FTIR

The FTIR spectra of silk SMG in comparison with SFS are shown in Fig. 7. The spectra of SMG are partly overlapped by that of SFS with some band shifting. The general characteristic absorption peaks of silk random coil appears at 1650 cm^{-1} for amide-I, 1540 cm^{-1} for amide-II, 1240 cm^{-1} for amide-III and 740 cm^{-1} for amide-IV. SFS presents peaks at 1628 cm^{-1} and 1523 cm^{-1} that can be attributed to β -sheet formation in contrast to SMG peaks at 1647 and 1539 cm^{-1} . These results suggest that α -chymotrypsin modifies SFP structure due to self assembly of the crystalline hydrophobic domains of SFP chains and other interactions between the heavy chains.

3.7. Structural analysis by FT-Raman

Raman spectroscopy is a useful tool for studying the conformation of SFP and SFP based biomaterials. In the present study the conformational characterization of SFS and SMG powder were performed by using Fourier transforms Raman (FT-Raman) spectra and are shown in Fig. 8. The SFS displays strong characteristic conformationally sensitive bands at 1664 cm^{-1} , 1230 cm^{-1} , 1083 cm^{-1} which are attributed to β -sheet. There are also relatively weak bands at 1261 cm^{-1} associated with α -helices. In SMG, bands appear around 1658 cm^{-1} , $1274\text{--}1245\text{ cm}^{-1}$ and 1106 cm^{-1} . The sharp narrow amide-I peak reflects the uniformity of chain conformation in both SFS and SMG samples. The band at 1641 cm^{-1} appears in SFS and disappears in SMG, which suggests that the enzymatic treatment cleaves phenylalanine, tryptophan and tyrosine residues. Moreover, the SFS shows an intense band at 852 cm^{-1} and at 829 cm^{-1} , attributed to tyrosine residues, where as in SMG the peak appears at 863 cm^{-1} . The Raman spectra of the SFS and SMG differed significantly in peak positions and intensities. This shows that salt leaching and enzymatic process alter the natural conformation of the silk fibroin protein differently.

3.8. Deswelling kinetics

The deswelling kinetics of SMG and SFS provides information about the movement of water in the microgels. The deswelling profiles of the SMG and SFS were recorded isothermally at 37°C during 90 minutes and weight loss data are shown in Table 1.

Fig. 9 shows the relative weight loss of SMG and SFS during 90 minutes of isothermal treatment at 37°C . The SMG released more water than SFS. The deswelling behavior was significantly affected by the structural difference of SMG in comparison with SFS. The SMG and SFS show a similar pattern of deswelling until 15 minutes. The difference in behavior of SMG and SFS occurs at 30 minutes, due to the larger surface area of SMG compared to SFS. This characteristic leads to faster deswelling with a weight loss of about

35.4% for SMG and 22.3% for SFS. After this time frame, SMG shows a 2.1% weight loss and SFS a 0.8% loss during the 30 – 90 minute time.

3.9. State of water

Biomedical or pharmaceutical activity of polymeric devices depends significantly on how water molecules associate with the polymer matrix. For example, DSC analysis was employed to assess the type of water present in polymers [33]. This type of investigation of water structure in hydrogels was based on the hypothesis that on cooling, free water freezes at the same temperature as pure water, whereas bound water does not freeze even at very low temperature, due to its strong interaction with the polymer matrix [34]. According to this hypothesis the endotherm measured when warming the frozen gel represents the melting of free water and this value will yield the amount of free water present in the hydrogel.

The state of water absorbed in SMG and SFS was studied by DSC to investigate structural changes. Total water (W_t), free water (W_f) and non free water (W_{nf}) contents are reported in Fig. 10. The total and freezable water content in SMG were higher when compared to SFS. The non-free water content was higher in SFS in comparison with SMG, whereas the total water and free water content showed higher values in SMG. These results are in agreement with previous deswelling results.

3.10. Thermogravimetric analysis (TGA)

The thermal properties of the microgels were determined by using thermo gravimetric analysis. The TGA and DTG curves of SMG and SFS are shown in Fig. 11. The thermal degradation curve of SMG and SFS are similar. The onset of degradation temperature of SMG was 234°C and was lower when compared to the SFS onset temperature of 257°C. The thermal degradation step was observed with maximum rate at 297°C and 295°C, corresponding to 38% and 35.8% for SMG and SFS, respectively. There was a shoulder at 397.8°C corresponding to 22.1% in the SMG, which was more prominent than that of SFS with values of 410°C and 18.3%, respectively.

3.11. Differential scanning calorimetry (DSC)

The DSC curves of SMG and SFS are shown in Fig. 12. The reorganization of SMG was observed at 79.3°C, which was higher than SFS at 74.6°C. A sharp melting peak of SMG was observed at 290°C, whereas SFS showed a small melting peak at 289°C. These results also correlate with the result observed in the DTG peaks of TGA. The degradation was followed by melting in both SFS and SMG.

4. Conclusions

In the present study a simple fabrication process for the preparation of silk protein microgels was described using a biocatalytic approach based on treatment of the protein with α -chymotrypsin. The enzyme selectively cleaved the SFP resulting in peptide fragments that then self-assembled via their hydrophobic features to form protein microgels. The microgels were physico-chemically characterized and found to be negatively charged and highly crystalline. The negatively charged silk microgels can form ionic complexes with cationic drugs and could be useful for delivery of cationic drugs *in vivo*. SEM analysis indicated the SMGs had a porous microstructure while the WAXS data indicated higher crystallinity compared to SFS. The higher crystallinity of SMG alters the morphology and physiochemical properties which can affect the behavior of these materials for biomedical applications such as drug delivery. The total and freezable water content in SMG were higher when compared to SFS, in agreement with the deswelling features. The thermal degradation curves of SMG

and SFS were similar whereas a sharp melting peak for SMG was found, in contrast to SFS which showed a small melting peak.

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Fig. 1.
The SFP amino acids polypeptide chain and its charge distribution.

Fig. 2. SDS-page of SFP and proteolytic enzyme α -chymotrypsin treated SFP at different times at 200V for 35 minutes with initial current 125 mA/gel and a 70 mA/gel at the end (a) A= protein marker (LC5699, Invitrogen, Carlsbad, CA), G= 2% SFP before enzyme treatment, B= Cp 1hr, C= Cp 2hrs, D= Cp 4hrs, E= Cp 8hrs, F= Cp 12hrs (Cp = α -chymotrypsin).

Fig. 3. SMG particle size by light scattering analysis using a Coulter LS230 Laser Diffraction Particle Size Analyzer. SMG suspensions were added into the cell until 30–50% obscuration of PIDS detector was reached, analysis range 0.04 μm to 2000 μm.

Fig. 4. The charge of (a) SMG and (b) SFS colloidal suspensions were determined using a Zetasizer 3000HS. The analysis was performed at scattering angle of 90° and temperature 25°C.

Fig. 5. Morphologies of SMG and SFS were observed using a JEOL JSM-5600LV SEM at (a) lower magnification and (b) higher magnification with accelerating voltage of 18kV.

Fig. 6. WAXS diffraction patterns of (a) SMG and (b) SFS obtained by Kristalloflex 810 diffractometer using a Cu K α ($\lambda=1.5406$ Å) as X-ray source. Scans were run in the high angle region $5^\circ < 2\theta < 65^\circ$ at scan rate of $0.040^\circ/\text{min}$ and a dwell time of 2s.

Fig. 7. FTIR spectra of SMG and SFS (a) scanned in the range 4000-500 cm^{-1} and (b) enlarged view, scanned over the range 2000-500 cm^{-1} .

Fig. 8. FT-Raman spectra of SMG and SFS (a) scanned in the range 3500-400 cm^{-1} and (b) enlarged view, scanned over the range 1800-400 cm^{-1} .

Fig. 9.

Deswelling kinetics spectra of (a) weight % and (b) derivative weight by water weight loss over time isothermally at 37°C to the dried state over time 4 hr under a nitrogen atmosphere at a flow rate of 60 mL·min⁻¹ by TGA, TA Q500-thermobalance.

Fig. 10. State of water in SMG and SFS, where W_t = total water, W_f = free water and W_{nf} = non free water.

Fig. 11.

TGA spectra of SMG and SFS (a) residual weight % and (b) DTG curves scanned at $10^{\circ}\text{C}\cdot\text{min}^{-1}$ with temperature range was 30°C to 900°C under a $60\text{ mL}\cdot\text{min}^{-1}$ flow rate of nitrogen by TA Instruments Series TA Q500.

Fig. 12. DSC second heating curves of SMG and SFS at scan $10^{\circ}\text{C}\cdot\text{min}^{-1}$ using Mettler DSC822e module with STARe software under a nitrogen atmosphere at $80\text{ mL}\cdot\text{min}^{-1}$ flow rate.

Table 1

Deswelling weight loss of silk SMG and SFS by TGA isothermal analysis.

Sample	0 – 15 min (%-wt)	15 – 30 min (%-wt)	30 – 90 min (%-wt)
SMG	54.3	35.4	2.1
SFS	54.3	22.3	0.8