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Silk microgels formed by proteolytic enzyme activity

Sangram K. Samal [a,b,c], Mamoni Dash [a,c], Federica Chiellini [a], David L. Kaplan [b,*], Emo Chiellini [a]

[a] BioLab-UdR-INSTM, Via Vecchia Livornese, University of Pisa, Pisa 1291, Italy
[b] Department of Biomedical Engineering, 4 Colby Street, Tufts University, Medford, MA 02155, USA
[c] Polymer Chemistry & Biomaterials Research Group, Ghent University, Krijgslaan 281, S4-Bis, B-9000 Ghent, Belgium

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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 (SMGs) at physiological temperature. These microgels consist of lamellar crystals in the micrometer scale, in contrast to the nanometer-scaled crystals in native silkworm fibers. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and zeta potential results demonstrated that α-chymotrypsin utilized only the non-amorphous 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, and that the prepared SMGs have useful features for studies related to biomaterial and pharmaceutical needs. This process is also an easy way to obtain the amorphous peptide chains for further study.

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].

The silk fibroin protein (SFP) from *Bombyx mori* (*B. mori*) silkworm is a promising natural polymer protein that has been 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 $-(-Ala-Gly)_n-$ 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 polypeptide 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, whereas 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 comprise 94% of the sequence and each repetitive region is an average of 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 the β-sheet structure and insolubility in aqueous medium. Silk microgels (SMGs) can be used to

* Corresponding author. Tel.: +32 16176270851; fax: +32 16176273231.
E-mail address: David.Kaplan@tufts.edu (D.L. Kaplan).

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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 SMGs, 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 of 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]. Tyrosines are in abundance in silk, comprising about 5% of the total amino acids, and are 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 ponds.

2. Experimental section

2.1. Materials

Cocoons from the *B. 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 the 460 kDa molecular weight marker LC5699 were purchased from Invitrogen (Carlsbad, CA). The enzyme α-chymotrypsin ($\geqslant$40 units mg^{-1}) 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 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 6 h. The LiBr was removed from the solution over the course of 48 h by dialysis cassettes (Slide-a-Lyzer MWCO 3.5K) and the remaining particulates were removed by centrifugation and syringe-based microfiltration (5 μm pore size, Millipore Inc., Bedford, MA). This process enables the production of 8–10 wt.% 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 (Constant Temperature Oven Model DK-42, American Scientific Products) 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), for use as comparisons and controls in the present study, were prepared according to the procedure described in the literature [29,30]. The α-chymotrypsin stock solution was prepared with distilled water at a 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 gel electrophoresis (SDS–PAGE)

Aqueous solutions of native silk and α-chymotrypsin-treated SFP supernatant were subjected to SDS–PAGE by following the protocols provided by NuPAGE Novex Bis-Tris Mini Gels (Invitrogen). 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×) and the mixture was run on a NuPAGE® Novex 4–12% Bis-Tris Gel 1.0 mm, 12 well (NP0322 BOX, Invitrogen). Standard pure native SFP solution samples were mixed with sample buffer and run on gels as controls. The gel was run in 1× NuPAGE® MES SDS running buffer and then stained using SilverXpress® silver staining kit (Invitrogen) at a voltage of 200 V for 35 min, with an initial current of 125 mA per gel falling to 70 mA per gel at the end. The 460 kDa molecular weight marker LC5699 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 the Coulter LS230 is from 0.04 to 2000 μm. SMG suspensions were added into the cell until 30–50% obscuration of the polarization intensity differential scattering (PIDS) detector was reached. Double-distilled deionized water was used as the 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 potentials 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 a temperature of 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 JSM-5600LV scanning electron microscope (JEOL, Tokyo, Japan) at the required magnification and with an accelerating voltage of 18 kV. 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 (λ = 1.5406 Å) X-ray source. Scans