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Impact of silk biomaterial structure on proteolysis

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ABSTRACT

The goal of this study was to determine the impact of silk biomaterial structure (e.g. solution, hydrogel, film) on proteolytic susceptibility. In vitro enzymatic degradation of silk fibroin hydrogels and films was studied using a variety of proteases, including proteinase K, protease XIV, α-chymotrypsin, collagenase, matrix metalloproteinase-1 (MMP-1) and MMP-2. Hydrogels were used to assess bulk degradation while films were used to assess surface degradation. Weight loss, secondary structure determined by Fourier transform infrared spectroscopy and degradation products analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis were used to evaluate degradation over 5 days. Silk films were significantly degraded by proteinase K, while silk hydrogels were degraded more extensively by protease XIV and proteinase K. Collagenase preferentially degraded the β-sheet content in hydrogels while protease XIV and α-chymotrypsin degraded the amorphous structures. MMP-1 and MMP-2 degraded silk fibroin in solution, resulting in a decrease in peptide fragment sizes over time. The link between primary sequence mapping with protease susceptibility provides insight into the role of secondary structure in impacting proteolytic access by comparing solution vs. solid state proteolytic susceptibility.

1. Introduction

Silk secreted from *Bombyx mori* silkworms has emerged as a useful protein polymer due to its biodegradability and utility in biomaterials for regenerative medicine and drug delivery [1–4]. *B. mori* silk is composed of a number of proteins: fibroin (heavy chain, light chain and P25), the key structural components, and sericin, the glue-like outer layer that coats fibroin during fiber spinning and formation of cocoons [5]. The fibroin heavy chain can be divided into four different regions based on amino acid chemistry and sequence: the N-terminus, C-terminus, 11 spacer regions and 12 large repeat bulk domains. The N-terminus, C-terminus and 11 spacer regions are hydrophilic and form the non-repetitive, amorphous regions of the assembled proteins. The 12 large bulk domains are hydrophobic and predominantly consist of the repeating hexapeptides GAGAGS and GAGAGY [6], which form the dominating crystalline β-sheet regions responsible for the strength and stability of silk biomaterials [7].

Silk fibroin can be formed into a variety of different biomaterials, such as hydrogels, sponges and films [8]. Hydrogels are water-insoluble networks of polymer chains, and can be composed of a number of synthetic (e.g. polyethylene glycol or polyvinyl alcohol) or naturally derived (e.g. collagen or hyaluronic acid) polymers [9]. To fully utilize a hydrogel in a biomedical context, an understanding of the degradation process is essential to determine the utility of the material for specific medical needs. For example, natural polymers, such as collagen and hyaluronic acid, have been used as filler materials for soft tissue augmentation. These materials work well as short-term cosmetic fillers, but exhibit low volume persistence due to the presence of endogenous enzymes in the body [10]. An understanding of the enzyme kinetics associated with these materials is important in order to design crosslinking agents that inhibit their degradation and prolong their volume retention in the body [11,12].

Although silk fibers are defined by the US Pharmacopeia as non-degradable materials because they retain tensile integrity (>50%) after 60 days in vivo [13], recent studies have demonstrated biodegradation of silk fibroin when prepared without waxes and coatings, and with tunability of β-sheet (crystalline) content via augmentation of processing conditions [8,14–19]. Several silk fibroin degradation studies investigating porous sheets, yarns, powders and films have demonstrated a role for proteases (including protease XIV, α-chymotrypsin and collagenase) in the degradation process [20,21]. In addition, we have recently reported studies of silk fibroin degradation [16,22] which provide the foundation for the additional study of the process with other proteases, including matrix metalloproteinases. The results of these studies on fibers and films suggest that silk degrades via surface erosion, with little bulk degradation observed.

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Matrix metalloproteinases (MMPs) are a family of naturally occurring enzymes that degrade extracellular matrix proteins [23]. In their natural environment, they contribute to a range of physiological mechanisms useful in cell and blood vessel growth, cell death, reproduction and the embryonic development, as well as tissue remodeling and wound healing [24]. MMPs are classified into different classes (i.e. collagenases, gelatinases, stromelysins) based on their in vitro substrate specificity [23]. Within each class, MMPs recognize specific peptide sequences common to that substrate. Some of these known recognition sequences are found within the amino acid profile of the silk fibroin heavy chain. Therefore, we would predict that MMPs would degrade silk fibroin materials. Understanding how MMPs interact with silk fibroin hydrogels and films will provide insight into silk biomaterials related to degradation in vitro and in vivo [25]. In the current work, the degradation of silk fibroin using both proteases and MMPs was studied, with a focus on how the material format impacts access to proteolysis. The proteolytic degradation of silk fibroin materials was characterized using two types of enzymes: serine proteases (proteinase K, protease XIV, α-chymotrypsin and collagenase) and MMPs (MMP-1, interstitial collagenase, and MMP-2, gelatinase A). Additionally, we sought to assess bulk degradation of silk through the use of hydrogels due to the open porous structure, which promotes transport and access of the enzymes throughout the material, in comparison to films, where such access is limited. It was hypothesized that hydrogels would provide a useful biomaterial format to define the differences in surface vs. bulk enzymatic degradation processes, while films would emphasize differences in silk proteolytic susceptibility with respect to surface erosion. Together, the findings provide a foundation towards predictive outcomes when these biomaterials are used in vivo.

2. Experimental section

2.1. Silk fibroin solution

Silk fibroin solution was prepared as we have previously described [26]. Briefly, 30 grams of cocoons from *B. mori* silkworms were cut into fragments and boiled for 20 min in an aqueous solution of 0.04 M Na_2CO_3 to remove the sericin coating from the fibroin fibers. Cocoons were then washed in deionized water to further remove sericin and Na_2CO_3. Remaining silk fibroin was left to dry overnight in a flow hood. Next, the extracted silk fibroin was dissolved in a 9.3 M LiBr solution for 4 h in a 60 °C oven in order to breakdown and solubilize the protein in water, yielding a 20% (w/v) solution. This solution was dialyzed in 3500 Da cut-off dialysis tubing for 3 days to remove LiBr. The final concentration of the silk fibroin solution generated was diluted to approximately 3% (w/v) and contained silk fibroin heavy chain, light chain and P25 proteins. The silk solution was not tested for active endogenous proteases or protease inhibitors remaining from the native silk cocoon. However, if present, they would be in all the materials and accounted for in the control samples.

2.2. Preparation of silk hydrogels

Silk fibroin solution was sonicated with a Branson 450 Sonifier (Branson Ultrasonics, Danbury, CT, USA), consisting of the Model 450 power supply, converter, externally threaded disruptor horn and 1/8″ (3.175 mm) diameter-tapered microtip, at 20% of the maximum amplitude for 5 s. Sonication physically induces β-sheet crosslinks via alteration in hydrophobic hydration of the protein chains and accelerated sol–gel transitions, as we have reported previously [15]. After sonication, 75 μl aliquots of solution were cast onto 4 mm diameter polydimethylsiloxane (PDMS) discs and allowed to sit on the bench top overnight.

2.3. Preparation of silk films

A 75 μl aliquot of silk fibroin solution was cast onto a 4 mm diameter PDMS disc and allowed to sit at room temperature overnight. These films were water annealed by placing them inside a vacuum chamber containing a small water reservoir and pulling an 80 kPa vacuum for 1 h. We have previously shown that water annealing increases the β-sheet content of the silk films and therefore makes them water insoluble [27]. Films were removed from the vacuum chamber and allowed to air dry for 12 h on the bench top.

2.4. Preparation of enzymes

Proteinase K from *Engyodontium album* (specific activity: ⩾30 units per mg protein), protease XIV from *Streptomyces griseus* (specific activity: ⩾3.5 units per mg protein), α-chymotrypsin from bovine pancreas (specific activity: ⩾40 units per mg protein) and collagenase from *Clostridium histolyticum* (specific activity: >125 collagen digestion units per mg solid) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Solutions of proteinase K, protease XIV, α-chymotrypsin and collagenase were freshly prepared by dissolving the enzyme powder in deionized water. An Amplite™ Universal Fluorimetric Protease Activity Assay kit (AAT Bioquest®, Inc., Sunnyvale, CA) was used to normalize digestion rate. From the derived data, enzyme concentration was determined based on equal enzymatic activity (160 RFU min^{-1}), proteinase K: 1.8 units ml^{-1}; protease XIV: 0.1 units ml^{-1}; α-chymotrypsin: 12 units ml^{-1}; collagenase: 75 units ml^{-1}.

MMP-1 (interstitial collagenase, *E. coli* expressed, ⩾700 units per μg protein) and MMP-2 (gelatinase A, yeast expressed, ⩾25 units per μg protein) were purchased from Enzo Life Sciences (Farmingdale, NY). MMP-1 and MMP-2 were reconstituted in assay buffer (50 mM HEPES, 10 mM $CaCl_2$, 0.05% Brij-35, pH 7.5) and preliminary studies determined that concentrations of 8750 units of MMP-1 per ml and 313 units of MMP-2 per ml were necessary to detect degradation of silk materials.

2.5. Degradation of silk materials by serine proteases and MMPs

Silk fibroin hydrogels and films were removed from the PDMS discs and incubated in deionized water for 24 h to leach out any soluble peptides. Materials were then immersed in 40 μl of enzyme solution (or deionized water for control) and incubated at 37 °C. Enzyme solution was removed and replaced with fresh enzyme once every 24 h for up to 5 days. After removal, enzyme solution was heated to 100 °C for 15 min to denature the enzymes per the manufacturer's instructions and stored at −80 °C until analysis. At each time point, one set of silk materials was removed from incubation and washed with deionized water for 48 h, with water changes every 12 h, to remove adsorbed protein. Materials were then dried in an airflow hood for 3 days and stored dry at room temperature until analysis.

Silk films were also treated with ethylenediaminetetraacetic acid (EDTA)-inhibited MMP for 48 h. To inhibit the MMP, 10 mM EDTA was added to 40 μl of a solution of MMP in assay buffer and incubated at 37 °C for 1 h. Films were placed in solutions of inhibited MMP enzyme and incubated at 37 °C for 48 h with refreshment at 24 h. Films and supernatant were removed at the end of the study and analyzed for degradation.

To ascertain the effects of MMP degradation on aqueous silk fibroin proteins, a silk fibroin solution was diluted to 3% (w/v) and split into 50 μl aliquots. Next, 350 units of MMP-1 or 12.5 units of MMP-2 were added to the appropriate treatment group and allowed to react for 6, 24 and 72 h at 37 °C. Enzyme was refreshed only once at 36 h for appropriate groups. After treatment, samples

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