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Short communication

Changes in the properties and protein structure of silk fibroin molecules in autoclaved fabrics

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ABSTRACT

The influence of autoclaving on the properties of silk fibroin fabrics (SF_{fab}s) was evaluated. High-performance liquid chromatography revealed that the molecular weight (Mw) of the fibroin molecule in SF_{fab}s decreased as the treatment temperature and time increased, which suggests that the fibroin molecule in the fabrics was thermal degraded. Additionally, differential scanning calorimetry experiments revealed a shift of the endothermic peak to higher temperature with increasing treatment temperature and time, which indicates an improvement in the thermal stability of the fibroin molecule in the autoclaved fabrics. Furthermore, ^{13}C cross-polarization/magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) analysis revealed that the intensity ratios for the β-sheet and random-coil peaks fluctuated during autoclaving. The NMR analysis also revealed the presence of an unknown structure in the autoclaved SF_{fab}s under certain conditions. The changes in the Mws and degradation temperatures for the autoclaved SF_{fab}s are assumed to be induced by the structural changes observed via NMR. Consequently, autoclaving is considered to be an effective technique for modifying the secondary structure and properties of SF_{fab}s with high β-sheet contents using only water and heat without added reagents.

1. Introduction

Silk fibroin produced by *Bombyx mori* is a protein-based natural polymer that has been commercially used as a textile fibre for a very long time because of its excellent lustre, softness, hygroscopicity and mechanical strength. Silk fibroin fibres have also been utilized as surgical sutures in clinical settings without any serious side effects. Due to these properties, silk fibroins are useful materials not only in the textile field but also in biomedical applications [1–7].

Silk fibroin heavy chains comprise crystalline regions with repeating sequences such as Gly-Ala-Gly-Ala-Gly-Ser, and non-repetitive amorphous regions. Silk fibroin is known to possess three crystal structures referred to as silk I, silk II and silk III. Silk I structures are observed in the glands of the silkworm, whereas silk II is an ordered, β-sheet structure, and silk III has a threefold structure and is observed at air/water interfaces [8–10]. During spinning of cocoons by silkworms, silk I structures are transformed to silk II (β-sheet) structures. Other various external stimuli, such as pH, metal ions and alcohols, are also known to induce the transformation from silk I to silk II (β-sheet) [10–12].

Magic angle spinning (MAS) solid-state nuclear magnetic resonance (NMR) studies are useful for obtaining information about the structure and mobility of molecules in silk materials [13]. In our previous structural study using solid-state ^{13}C cross-polarization (CP)/MAS NMR, structural changes in the fibroin molecules in silk fibroin sponges were observed following wet heating and autoclaving. In contrast, silk fibroin sponges heated without water exhibited no structural changes. *In vitro* experiments using these treated silk fibroin sponges and films with different structures suggested that secondary structures affect cell proliferation and cell motility. These results therefore suggested that heating in the presence or absence of water may regulate the structure of silk fibroin molecule. Such a discovery is important because controlling

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the higher-order structure of fibroin molecule is thought to be necessary for regulating the various properties of silk fibroin materials.

Autoclaving is a wet-heating treatment at higher temperature under high pressures and is used as a sterilization method for various materials and foods. In the biomedical field, many materials, such as metals [15,16], are sterilized via autoclaving, although many polymer-based biomaterials are not suited to autoclaving because of their low thermal resistance [17,18]. Generally speaking, natural polymer-based biomaterials, such as collagens and chitosans, also have lower thermal resistances similar to those of synthetic polymer-based biomaterials [19–21]. In the case of silk fibroin, the effect of wet heating on its protein structure has been investigated using films, hydrogels and sponges [12,22–25], and it has been reported that wet heating induces significant structural transformations from silk I or random coils to β-sheets due to the lower initial β-sheet content in these silk materials. In contrast, such a structural transformation should be less likely for silk fibroin fibres and fabrics because of their high β-sheet content. Although it is known that silk fibroin fibres and fabrics have higher thermal resistances [26], to the best of our knowledge, no detailed investigation of the influence of heat treatment on the molecular structure of these materials has yet been reported.

Therefore, changes in the structure of the fibroin molecule in silk fibroin fabrics (SF_{fab}s) with an initial high β-sheet content following heat treatment via autoclaving at 121 and 132 °C are described in this study. Scanning electron microscopy (SEM) imaging, high-performance liquid chromatography (HPLC), differential scanning calorimetry (DSC) and solid-state ^{13}C NMR analyses were performed to investigate the changes in the appearance of the SF_{fab}s and fibroin protein, and the influence of autoclaving at the molecular level of these materials was evaluated based on the results.

2. Materials and methods

2.1. Autoclaving treatment

The SF_{fab}s (thickness = 160 μm) were cut into 2 × 2 mm squares. The square samples were then immersed in ion-exchanged water and heated at 121 °C and a pressure of 0.11 MPa or 132 °C and a pressure of 0.20 MPa for a given time using an autoclave apparatus (NCC-1701, AS ONE, Tokyo, Japan).

2.2. Field-emission scanning electron microscopy (FE-SEM)

The surface morphologies of the fibroin samples were observed via field-emission scanning electron microscopy (FE-SEM) (JSM-6700F, JEOL, Tokyo, Japan) at an acceleration voltage of 1.0 kV and magnifications of 250 and 1000. Samples (10 × 10 mm) were fixed on the support using a conductive carbon tape.

2.3. High-performance liquid chromatography (HPLC)

The autoclaved SF_{fab}s were each dissolved in 9.0 M LiBr and diluted with an elution buffer (1/15 M pH 7.0 phosphate buffer system containing 2 M Urea and 0.1 M Na_2SO_4). After dilution, the solutions were filtered through a 0.45 μm hydrophilic PTFE membrane (Merck KGaA, Darmstadt, Germany). Shodex PROTEIN KW804 (8.0 mm I.D. × 300 mm, Showa Denko K.K., Tokyo, Japan) and PROTEIN KW-G columns were used in the HPLC system (SHIMADZU Corporation, Kyoto, Japan) equipped with an SPD-M10A UV-VIS detector. The system was operated at a flow rate of 0.5 ml/min. The temperature of the columns was maintained at 40 °C. Standard proteins (29,000–700,000 Da) from a Gel Filtration Markers Kit for Protein (Sigma–Aldrich Co. LLC., MO, USA) were used to calibrate the Mws.

2.4. Differential scanning calorimetry (DSC)

DSC measurements were performed on 10 mg samples of autoclaved SF_{fab}s using a DSC6200 (Seiko Instruments Inc., Chiba, Japan) at a heating rate of 15 °C/min under a nitrogen gas flow.

2.5. Solid-state ^{13}C NMR analysis

High-resolution solid-state ^{13}C NMR spectra were obtained using an EX-270 with a magnetic field of 6.34 T (JEOL, Tokyo, Japan). The ^{13}C cross-polarization/magic angle spinning (CP/MAS) NMR spectra were obtained at a frequency of 67.9 MHz. Each sample was placed in a zirconia rotor and spun at speeds up to 4.1 kHz. The contact time was 2 ms and the repetition time was 5 s. The ^{1}H radio frequency field strength was approximately 50 kHz for Hartmann–Hann conditions and proton decoupling. Approximately 16,000 scans were accumulated for each spectrum to attain a reasonable signal-to-noise ratio. All spectra were calibrated using adamantane as the standard, and the chemical shift of the adamantane CH_2 peak appearing at 29.5 ppm was referenced to the peak for tetramethylsilane appearing at 0 ppm.

3. Results and discussion

3.1. Macroscopic observation

The morphological changes in the SF_{fab}s investigated using FE-SEM are shown in Fig. 1. Note that colour measurements obtained using a colorimeter indicated that no colour change, such as yellowing, occurred in any of the autoclaved SF_{fab}s (Fig. 1(a)). Notably, no morphological changes at either magnification, such as the fibrillation of the fibres, were observed for the autoclaved SF_{fab}s. Heat treatment with water under high pressure therefore did not affect the morphologies of the SF_{fab}s.

3.2. Molecular weight (Mw)

To evaluate the changes in the Mw of the silk fibroin molecule in the SF_{fab}s after heat treatment, each autoclaved sample was dissolved in 9.0 M LiBr and then diluted with a phosphate elution buffer for HPLC analysis. The elution curve for each fibroin sample was obtained by detecting the UV absorption at 280 nm (Fig. 2(a)). These results indicate that the molecular size of the silk fibroin was distributed over a range and some molecular decomposition occurred as a result of autoclaving. The retention time for the peak of the non-treated SF_{fab} appeared at 21.3 min and the molecular weight of the fibroin molecule in this sample was estimated to be 325,300 Da using protein standards. The retention times for the peak tops of the autoclaved SF_{fab}s were shifted to longer elution times with increasing treatment time at both 121 and 132 °C. Additionally, peaks that seemed to be decomposition products appeared at approximately 26 and 27 min, and also, the peak width become narrow as the treatment time increased (Fig. 2(a)(b)). These results indicate that the average molecular size of the fibroin molecule after autoclaving was reduced. The estimated Mw for each peak in the chromatographs of the autoclaved SF_{fab}s was calculated using protein standards and the change in the Mw as a function of the autoclaving time at each temperature is presented in Fig. 2(c). It can be observed in the figure that the Mw of the fibroin molecule in the SF_{fab}s decreased as the treatment temperature and time increased. These results indicate that longer autoclaving induces molecular breakdown of the fibroin molecule in