Introduction

Silk is a proteinaceous fiber composed of two filaments of protein, fibroin, with the high-crystallinity and strong preferred orientation, which are bound together by a principally amorphous protein, sericin, which can be removed by degumming. Most historic silk has been made of degummed silk, namely, fibroin. Therefore, fibroin in fact involves the degradation of silk.

Fibroin is composed of the amino acids alanine, serine and glycine, which fold into anti-parallel/parallel $\beta$-sheet repeated motifs of Gly-Ala-Gly-Ala-Gly-Ser. They undergo aggregation into crystalline forming, which are considered to be responsible for their excellent mechanical properties because of their highly ordered structure. The crystallites are uniformly embedded in an amorphous matrix that is rich in residues with bulky, polar side-chains. The amorphous region is characterized by tyrosine residues that are considered to adopt distorted $\beta$-sheets, distorted $\beta$-turns, random coils and $\alpha$-helix structures, loose structures that are considered to confer the silk elastic property.

In the past, the degradation kinetics and mechanism of ancient silk and artificially aged silk were studied, which were aged by UV-irradiation, heating and modified humidity methods. However, research concerning the enzymatic degradation of silk fabrics was limited. After being buried in the ground for centuries, unearthed silk fabrics were bestrewed by various microorganisms. Furthermore, Forlani et al. had studied silk fibroin degradation by bacterial extracellular proteases. They cultured the bacterium Variovorax paradoxus, grown in a minimal medium in which silk fibroin represents the only source of carbon and nitrogen. The bacterium Variovorax paradoxus produces an extracellular protease that hydrolyzes fibroin. Therefore, it is essential to study the enzymatic degradation mechanism of silk textiles. In the present study, we used protease XIV, which was secreted from Aspergillus oryzae, to age modern silk fabrics so as to evaluate the enzymatic degradation mechanism.

Keywords Enzymatic degradation, protease XIV, $^{13}$C CP/MAS solid state NMR, EPR

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It is also very important to detect the lifetime and formation mechanism of the free radicals caused by external factors, including light, heat, pH, mechanic force and molecular structure, since they are revolved in many reactions, such as in the oxidation reactions of hydro-carbon and many organic chain reactions. Thus, we assumed that the enzymatic degradation of silk fibroin is also related to free radicals. Electron paramagnetic resonance (EPR), a powerful technique to trace the free radical of artificially aged protein, can be employed to investigate the enzymatic aging kinetics of silk fibroin at the electronic and atomic levels and to probe the radical chemistry.

In the present work, the enzymatic degradation of silk fabrics was studied mainly via EPR, $^{13}$C CP/MAS solid-state nuclear magnetic resonance (NMR), Fourier transfer infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). This study is expected to assist in understanding the structure-biodegradation relationship of silk fabrics at the molecular and atomic levels.

**Experimental**

**Preparation of artificially aged samples**

Modern silk fabrics, that were plain weaved, were bought from Hangzhou Fusi Textile Company and cut into $2 \times 10$ cm pieces. The degradation of silk fabrics was evaluated using protease XIV (protease from *Aspergillus oryzae*, Sigma China). Protease XIV was dissolved into pH 7.5, 0.1 M phosphate-buffers saline (PBS) with final enzyme concentration of 2 μL/mL. Prepared silk fabrics were then immersed into 200 mL of an enzyme solution, and incubated at 37°C. After aging for 5, 15, 25 days, eight samples were removed and were rinsed in deionized water five times, and then dried at ambient temperature. And also the enzyme solution was replaced every 5 days. Control samples were treated only in a PBS solution without any enzymes. Replacement of the PBS solution and the rinsing of control samples were carried out in the same manner as that for enzyme treated samples.

**Characterization**

The morphology of aged samples and the control sample were observed under a scanning electronic microscopy (JSM-5610LV, Japan) at a 5-kV working voltage and a 15-mm working distance. $^{13}$C CP-MAS NMR spectra were recorded on a Bruker DMX-500 spectrometer equipped with a CP-MAS accessory. The magic-angle spinning (MAS) was 4 mm, MAS BB/1H with a spinning frequency of 10 kHz. The test frequency was 400 MHz/1H, with a scanning time of 1800 times/s. The relaxation delay was 2 s. Using samples of 10 mg in the standard quartz EPR tube, a German Model EB200-SRC EPR spectroscope was used for an EPR measurement at room temperature. The parameters of the EPR were a microwave frequency of 9059.471 ± 2 MHz, a microwave power of 0.99800 mW, a modulation frequency of 100.00 kHz, a modulation amplitude of 2.000 × 1 mT, a time constant of 0.03 s and sweep time of 1 min.

**Results and Discussion**

**Morphology observation**

The morphological differences between the four samples mentioned above were studied using the SEM. Images of aged samples and the control sample are shown in Figs. 1a - 1d. From Figs. 1b - 1d, silk fabrics are obviously destroyed after being immersed into a protease XIV solution, while there are no clear changes of the control sample, which was immersed into the PBS solution without any enzymes. An SEM image of the control sample (Fig. 1a) shows a smooth morphology, while the morphology of enzymatic degradation of silk fabrics (Figs. 1b - 1d) shows a corroded surface. Comparing the SEM images of samples aged different numbers of days Figs. 1b - 1d, with increased ageing days, the erosion of silk fabrics is more progressive. Furthermore, the silk fabrics were degraded progressively with increasing enzymatic aging times. In addition, the erosive areas seem to be stripped from silk fabrics, which is different from that of the silk fabrics aged by heating.
lighting and autoclaving. The surface of silk gradually became rough, and the silk fiber was broken down across the axis of the silk fiber eventually during heating, lighting and the autoclaving degradation process.10

Secondary structure changes

It is known that the infrared region of silk can be divided into three regions, amide I bands centered at 1700 - 1590 cm\(^{-1}\) were attributed to most C=O stretching vibrations in the protein backbone. Amide II bands in the 1590 - 1460 cm\(^{-1}\) region were assigned to N-H bending and C-N bending vibration, and amide III in the 1190 - 1280 cm\(^{-1}\) band was attributed to N-H bending vibration and C-N stretching vibrations. Figure 2 presents the three regions of aged samples and the control sample.13,14

As shown in Fig. 2, it is clear that there are obvious changes of aged samples, especially of the 5 days aged sample compared with the control sample. The band centered at 1699 cm\(^{-1}\) is monitored to the amide I band in section secondary structure-crystallinity estimator.15 The growth of that band was widely explained either by an increase of the anti \(\beta\)-sheet contribution or by the formation of new carboxyl groups (C=O).15 Compared with the control sample, for the 5 days aged sample, the intensity of that band decreased, while in case of 15, 25 days aged samples, that band increased. The result indicates that the number of carboxyl groups (C=O) on silk fibroins decreased after aging for 5 days, and upon a further degradation process, the number of carboxyl groups (C=O) increased. The band centered at 1336 cm\(^{-1}\) was assigned to C-H bending vibration of phenylalanine on the amorphous region.15 There is an obvious change of that band of a 5-aged sample compared with the control sample. With further degradation, that band gradually decreased. As shown in Fig. 3, the intensity of the Tyr peak centered at around 132 ppm decreased for the 5 day aged sample, while the intensity of that peak of the 15, 25-days aged samples gradually increased. Furthermore, there are slight changes of the peak of the random coil centered at 23 ppm. That peak decreased for the 5, 15-days aged samples, while it increased for the 25 day aged sample. In order to improve resolution and estimation of different conformation, the peaks between 24 and 15 ppm were deconvoluted and a curve fitted to yield a set of peaks from which the conformation could be determined. The peak simulations for the samples with different aging times and control sample are shown in Fig. 4.

Atomic level analysis

The atomic-level information concerning the silk structure provides answer concerning how the enzyme aging process occurred. Solid-state \(^{13}\)C NMR has basically become the inherent merit for such fiber structure determinations because solid-state NMR does not require single crystals, but also determination of the atomic level structure.16,17 The obvious morphological changes of silk fabrics imply that silk fabrics are damaged by enzymes. It is easy to study the enzymatic degradation process via characterizing the atomic-level information of the silk structure by using a high-resolution \(^{13}\)C solid-state NMR study. Figure 3 shows the \(^{13}\)C CP/MAS solid-state NMR spectra of aged-samples and the control sample.

NMR signals arising from the major amino acid residues were well resolved and easily assigned to the peaks of Gly, Ala, Ser, Tyr and the amorphous region (including random coil and \(\alpha\)-helix),18 as shown in Fig. 3. The chemical shifts centered at 172 ppm was assigned to the peak of Gly on the crystalline region.18,19 That peak intensity of the 5-day aged sample increased, while with the increasing degrading times, it gradually decreased. As shown in Fig. 3, the intensity of the Tyr peak centered at around 132 ppm decreased for the 5 day aged sample, while the intensity of that peak of the 15, 25-days aged samples gradually increased. Furthermore, there are slight changes of the peak of the random coil centered at 23 ppm. That peak decreased for the 5, 15-days aged samples, while it increased for the 25 day aged sample. In order to improve resolution and estimation of different conformation, the peaks between 24 and 15 ppm were deconvoluted and a curve fitted to yield a set of peaks from which the conformation could be determined. The peak simulations for the samples with different aging times and control sample are shown in Fig. 4.
Hashimoto et al.\textsuperscript{20} reported that Ala residues of Cβ were around 24 to 15 ppm. In their study, they found that the Ala Cβ peak was deconvoluted by assuming five peaks, the Ala Cβ peak in the $^{13}$C CP/MAS NMR spectrum of the Cp fraction (chymotripsin cleavage) (56%) was independently observed and deconvoluted to three peaks at 21.7, 19.6 (β-sheet) and 16.5 ppm (distorted β-turn/random coil). The Ala Cβ peak in the $^{13}$C CP/MAS NMR spectrum of the other fraction (44%) was deconvoluted into two peaks at 21.3 (distorted β-sheet) and 16.5 ppm (random coil).\textsuperscript{21} Thus, in the curve-fitted spectra, we must consider two kinds of domains, crystalline and non-crystalline. Compared to the curve-fitted peak of the control sample, the non-crystalline region of the 5-day aged sample is degraded (overlapping of the peaks centered at 21.32, 18.51 and 16.14 ppm). What is more, upon the further degradation process, the curve-fitted peak centered at 15.85 ppm gradually disappeared. These results indicate that the amorphous region of silk fabrics is degraded primarily, and with the enzymatic degradation process going on, the crystalline region is gradually degraded. The results are consistent with the result of FTIR. Based on the results of the above studies and discussion, we proposed that the amorphous region of silk is mainly attacked by enzymes at the beginning. Also with enzymatic degradation occurring, the crystalline region of silk is gradually degraded.

**EPR spectroscopy analysis**

In our previous study,\textsuperscript{11} it turned out that the carbonyl radicals were found in lighting aging wool fibers, where the g factor of the EPR spectra was 2.0029 ± 0.0005. The EPR spectra of enzyme-aged samples and the control sample are shown in Fig. 5.

All of the EPR spectra have the same resolved characteristic absorption peak centered at the highly isotropic g-factor, whose value is 2.0043, indicating that the signals originate from the cubic symmetry of the radical center.\textsuperscript{22} After a study using Raman spectroscopy, Oliva et al.\textsuperscript{23} pointed out that the deterioration of ancient silk fabrics is related to the formation of carbonation. There are carbonyl radicals formed during the aging process. What is more, these single caves without any hyperfine structure indicate that the spin density is localized at the atom(s) possessing zero nuclear spin, and the common stable paramagnetic metal species, e.g. Fe$^{3+}$, Mn$^{2+}$ and Cu$^{2+}$, are excluded judging from the peak-to-trough width (~1 mT) of the
curves. Furthermore, thanks to the narrow peak-to-trough width, and the light asymmetry of the spectra, it turns out that the unpaired electron is localized in the carbon radicals in the silk fiber matrix. Accordingly, the value of $g = 2.0043$ means the EPR spectra are assigned to be the carboxyl radical.

Compared with the control sample, we can find that the EPR spectrum intensity of the 5-day aged sample is lowest, while with increasing degradation time (aged 15 days and aged 25 days samples), the intensity is gradually increased. Also at the end of the degradation procedure (25 day aged sample), the intensity is higher than the control sample. As discussed above, EPR absorption peaks are assigned to be carboxyl radical. EPR data show that the EPR absorption peak intensity of the 5-day aged sample is the weakest, and upon a further degradation process, that intensity of aged 15, 25 days' samples is gradually increased. FTIR spectra show that the carboxyl groups (C=O) decreased after aging for 5 days, and on the further degradation process, the carboxyl groups (C=O) increased. The results of FTIR and EPR indicate that the enzymatic degradation may be related to the free radical. Therefore, we hypothesis that at the first degradation stage that the degradation mainly occurred in the non-crystalline region for the 5-day aged sample; the free radicals were apt to lose activities due to the loose structure of the non-crystalline region; however, at the second degradation stage, the degradation mainly occurred in the crystalline region; the free radicals produced in this region were not easy to lose activities and tended to be stored.

Conclusions

The morphological change is different from the heating and lighting aged samples. The micro-filament of aged samples seemed to be stripped from the silk fabrics. According to the results of FTIR and $^{13}$C CP/MAS solid-state NMR, we propose that the enzymatic degradation under protease XIV could be divided into two stages, at the first degradation stage, the amorphous region of silk is degraded. At the second degradation process, the crystalline region of silk is degraded slowly. The EPR spectra indicate that the enzymatic degradation process was related to the free radical with a g-factor value of 2.0043. With changes of the intensity of the EPR peaks, we proposed that at the first degradation stage, the free radicals were apt to lose activities due to the loose structure of the non-crystalline region; however, at the second degradation stage, the free radicals produced in crystalline region were not easy to lose activities and tended to be stored. These findings would contribute to the explanation concerning the deterioration mechanism of ancient silk and pave the way for better preservation of these textiles heritages. The results were thought to be helpful in other research that involves fibroin degradation, not only for the conservation purposes.

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