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ARTICLE *in* JOURNAL OF APPLIED POLYMER SCIENCE · JUNE 2005

Impact Factor: 1.77 · DOI: 10.1002/app.21346

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Fourier Transform Raman and Fourier Transform Infrared Spectroscopy Studies of Silk Fibroin

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Received 24 October 2003; accepted 17 May 2004
DOI 10.1002/app.21346
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: The chemical and conformational structures of *Bombyx mori* silk were studied with the complementary techniques of Fourier transform Raman spectroscopy and Fourier transform infrared spectroscopy. The Fourier transform Raman spectrum of silk showed strong bands for the photosensitive aromatic amino acids tyrosine, tryptophan, and phenylalanine. Intensive UV/ozone irradiation reduced the Raman intensities of the amide III band and the tyrosine signals due to peptide chain scission of the silk polymer and the photochemical changes in the tyrosine residues on the silk molecules. However, the Raman spectroscopy changes for tryptophan were less clearly defined because of peak overlapping with other amino acid signals and the low concentration of tryptophan. The Fourier transform infrared (attenuated total reflectance) technique, coupled with second-derivative spectroscopy analysis, demonstrated a decrease in the crystallinity degree and tyrosine content of UV/ozone-irradiated silk, as indicated by changes in the Fourier transform infrared bands of amide III doublet and tyrosine signals. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 96: 1999–2004, 2005

Key words: FTIR; microstructure; radiation; Raman spectroscopy; silk

INTRODUCTION

Silk fibroin is a natural biological polymer composed of 18 α-amino acids. The exposure of silk to sunlight produces obvious photoyellowing followed by phototendering, which is a shortcoming of silk products. Previous studies have revealed that the absorption spectrum of silk has a maximum wavelength of about 280 nm because of the presence of the aromatic amino acids tryptophan, tyrosine, and phenylalanine.[1] Silk yellowing is caused by the photooxidation of tyrosine and tryptophan residues, which results in the formation of yellow chromophores.[2–4] Tyrosine is considered the most important source of silk yellowing because of its greater abundance (>10 wt %) with respect to tryptophan (<0.5%).[5] Silk phototendering is caused by peptide fission initially at the weaker C—N bond, which leads to a loss of fiber strength.[6] Photochemical reactions, including silk photoyellowing and phototendering, usually take place from the material surface to the bulk. In previous articles, we examined the relationship between silk surface tyrosine and silk photoyellowing.[7–9]

Fourier transform infrared (FTIR) spectroscopy in the attenuated total reflectance (ATR) mode is a nondestructive surface analysis technique. The technique coupling FTIR (ATR) spectroscopy with second-derivative spectroscopy analysis has been used in previous studies to qualitatively and quantitatively examine the oxidative damage of wool and hair fibers[10–12] and the secondary structures of wool and global proteins,[13,14] but little work has been reported on investigations into the structure of silk with this combination technique.

Raman spectroscopy is a vibrational technique providing information complementary to that of infrared spectroscopy. The Raman effect is a scattering process involving interactions between the incident photons and the sample molecules, the Raman peaks corresponding to inelastically scattered photons. In contrast, the peaks in an infrared spectrum correspond to energies in which infrared photons have been absorbed by the molecules.[15] For molecules to be Raman-active, the vibration must induce a change in the molecular polarizability, whereas for infrared activity, the vibration requires a change in the dipole moment

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Contract grant sponsor: Natural Science Foundation of China; contract grant number: 20075023.
Contract grant sponsor: Zhejiang Provincial Natural Science Foundation of China; contract grant number: ZC0207.
Contract grant sponsor: Key Laboratory of Advanced Textile Materials and Manufacturing Technology of the Chinese Ministry of Education.

of the molecule. According to the rule of mutual exclusion, if a molecule has a center of symmetry, then a Raman-active vibration is infrared-inactive, and vice versa.[16] Polarizable, homopolar bonds such as C—C, S—S, N═N, and O—O give rise to intense Raman bands, whereas in the infrared spectrum, these bonds show either weak or undetectable bands. The advantages of Raman spectroscopy for studying protein fibers are that it is nondestructive and gives strong bands for aromatic structures such as the amino acids tryptophan, tyrosine, and phenylalanine. Structural information is provided by amide I and amide III vibrational bands in Raman spectra. Fourier transform Raman (FT-Raman) spectroscopy has been developed as a valuable analytical technique for research into wool fibers,[17–19] and until recent years, no FT-Raman study on the structure of silk had been reported.

In this study, FTIR and FT-Raman spectroscopy was used to investigate the structural changes in silk induced by UV/ozone irradiation, particularly with respect to the photosensitive amino acids tryptophan, tyrosine, and phenylalanine.

EXPERIMENTAL

Materials

A laboratory-degummed *Bombyx mori* twill fabric (80 g/m^2) was used in all the experiments in this research. An alkali–surfactant process was used for the silk fabric degumming, and the extent of degumming was checked with a picric acid/cochineal indicator solution.[8]

All the poly(amino acid)s [poly(L-phenylalanine), poly(L-tryptophan), and poly(L-tyrosine)] were obtained from Sigma Biochemicals (Poole, UK).

UV/ozone irradiation

UV/ozone irradiation was carried out with a model 42-220 laboratory UV/ozone instrument with a high-intensity, low-pressure mercury vapor grid lamp emitting UV radiation (mainly at 184.9 and 253.7 nm; Jelight Co., Inc., Irvine, CA). The silk samples were irradiated on both sides, with a distance of 1 cm between each sample and the lamp, for the specified time.

FTIR analysis

FTIR spectra were obtained with a Nicolet Magna 750 FTIR spectrometer (Madison, WI) in the ATR mode with a KRS5 crystal in a vertical holder. The spectra were the average of 200 scans at a 4-cm^{-1} resolution.

FT-Raman analysis

FT-Raman spectra were obtained with a Nicolet Raman spectrometer with a germanium detector. The samples were excited with a neodymium–YAG laser operating at 1064 nm. Each silk sample was mounted vertically on a stainless holder with double-sided adhesive tape, and the spectrum was accumulated from 1500 scans at a 4-cm^{-1} resolution with 0.55 W of laser power. The poly(amino acid) powder samples were measured with a capillary tube (1.1–1.2 i.d. $\times$100 mm), and the spectra were an average of 2000 scans at a 4-cm^{-1} resolution with 0.55 W of laser power.

To allow a comparative analysis of the band intensities, we normalized all spectra with the strong band at 1449 cm^{-1} assigned to CH_2 and CH_3 bending.

RESULTS AND DISCUSSION

FT-Raman spectroscopy and band assignments of silk

Preliminary studies on the laser power and scan numbers used in silk Raman measurements established that a moderate laser power of 550 mW and a scan number of 1500 were suitable for silk in terms of the spectral quality and an acceptable scan time. The Raman spectrum of nonirradiated silk (Fig. 1) showed well-resolved peaks, including strong bands for the photosensitive aromatic amino acids tryptophan, tyrosine, and phenylalanine and for the amide I and amide III bands.

The Raman band assignments for silk (Table I) were derived from our own analysis of Raman spectra of the poly(amino acid)s (polyphenylalanine, polytryptophan, and polytyrosine) and previous assignments for silk and related proteins.[20–23]

FT-Raman analysis of UV/ozone-irradiated silk

The most noticeable effect on the FT-Raman spectrum of UV/ozone-irradiated silk was the decrease in all the tyrosine signals at 855, 830, and 645 cm^{-1}. The doublet at 855 and 830 cm^{-1} arose from Fermi resonance between the symmetric ring-breathing vibration and the overtone of an out-of-plane ring-bending vibration of tyrosine.[24] The expanded spectra in the doublet region [Fig. 2(a)] clearly demonstrated that both the 855- and 830-cm^{-1} signals were reduced in intensity, and this reflected the reduction of tyrosine residues in silk. Other Raman studies of proteins have found that the relative intensity ratio of the doublet is sensitive to the nature of hydrogen bonding or the state of ionization of the phenolic hydroxyl group.[21,25] If the peak at 855 cm^{-1} is higher in intensity than the peak at 830 cm^{-1}, the tyrosine residues are moderately hydrogen-bonded or exposed in hydrophilic regions; Alternatively, if the intensity ratio is reversed, then the tyrosine is strongly hydrogen-bonded or buried within hydrophobic regions. Tyrosine in silk is located in the amorphous region,[26] which UV radiation and oxygen

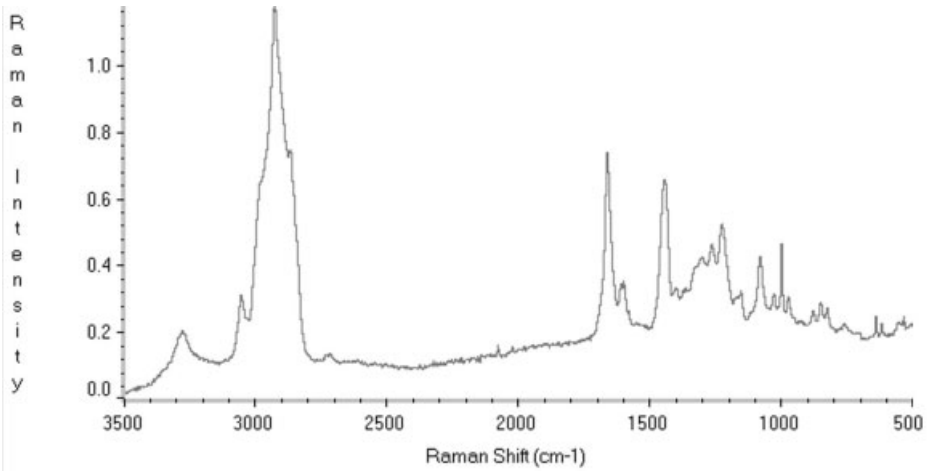

Figure 1 Raman spectrum of silk.

can penetrate more easily than the crystalline region. However, we observed no clear trend in the intensity ratio of the doublet signals. The Raman signal at 645 cm^{-1} was similarly due to the tyrosine ring-breathing mode and again showed a decrease in the peak intensity after UV/ozone irradiation; this supported the reduction in the tyrosine content [Fig. 2(b)].

The sharp signal at 1004 cm^{-1} was assigned to the C—C stretching vibration of the indole ring of tryptophan and the phenyl ring of phenylalanine. This

TABLE I
Raman Band Assignments for Silk

Raman band (cm^{-1})	Intensity	Assignment
622	w	Phenylalanine
645	m	Tyrosine
760	vw	Tryptophan
830	m	Tyrosine
855	m	Tyrosine
885	w	C—C skeletal stretch, tryptophan
979	w	C—C skeletal stretch
1004	s	Phenylalanine, tryptophan
1034	w	Phenylalanine
1087	m	C—N stretch
1159	w	C—N stretch
1232	m	Amide III (β sheet)
1269	m	Amide III (β sheet, disordered)
1308	m	C—H bend
1337	sh	C—H bend, phenylalanine
1449	vs	CH_2, CH_3 bending modes
1585	w	Phenylalanine
1605	m	Phenylalanine
1616	m	Tyrosine, tryptophan
1668	vs	Amide I (β sheet)
2875	s	CH_3 symmetric stretch
2934	vs	CH_3 asymmetric stretch
3063	m	C—N—H bend overtone
3286	m br	N—H stretch

s, strong; m, medium; w, weak; sh, shoulder; br, broad; v, very.

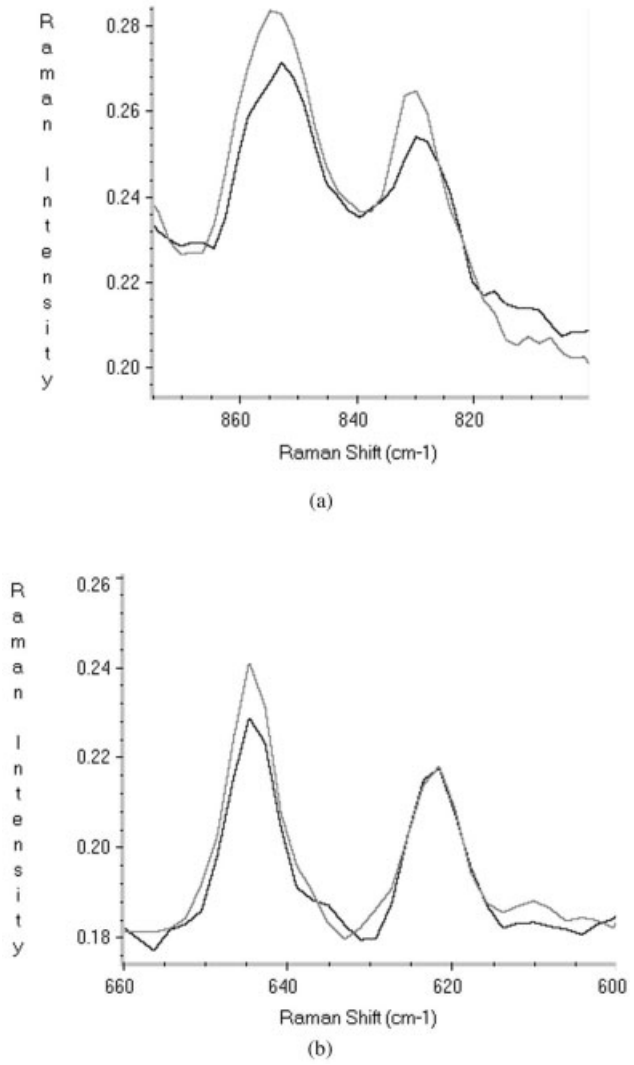

Figure 2 Raman spectra in tyrosine regions of nonirradiated silk fabrics (upper curves) and UV/ozone-irradiated (40 min) silk fabrics (lower curves): (a) 800–875 and (b) 600–660 cm^{-1}.

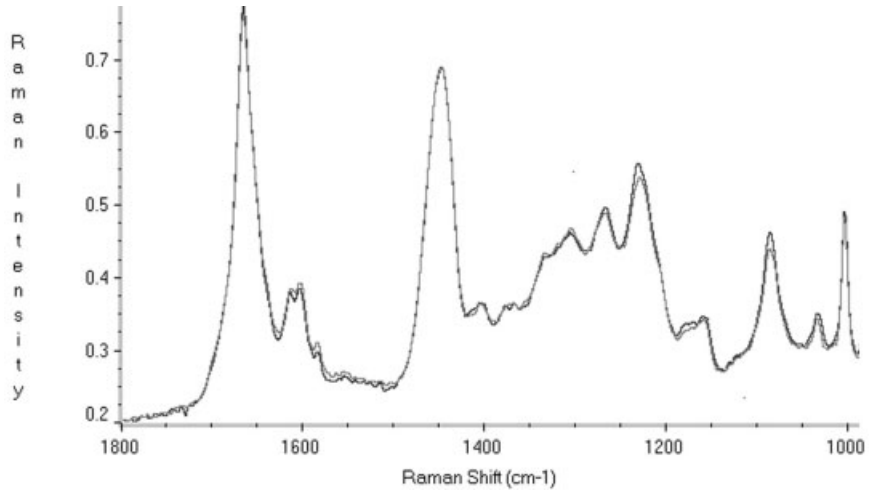

Figure 3 Raman spectra (amide-band region) of nonirradiated silk fabrics (upper curve) and UV/ozone-irradiated (40 min) silk fabrics (lower curve).

strong band was expected to be sensitive to changes in tryptophan, but there was little apparent change in the peak intensity with UV/ozone irradiation (Fig. 3). Previous research[5] with amino acid analysis has demonstrated the significant loss in the amount of tryptophan with UV irradiation, but this was not reflected in the Raman spectroscopy analysis because of the much higher concentration of phenylalanine in comparison with tryptophan (ca. 3:1 in silk) and the relatively stronger and broader Raman scattering signal of phenylalanine in comparison with tryptophan, which masked the changes in tryptophan.[27] The band at 760 cm^{-1}, assigned specifically to tryptophan, was very weak, but a reduction in the signal intensity was observed. Other bands for tryptophan were obscured in the silk spectra by overlapping signals from other amino acids, which resulted in difficulty in detecting the changes in tryptophan signals.

The Raman bands at 622, 1034, and 1605 cm^{-1} were specifically associated with phenylalanine residues. These bands appeared little changed after UV/ozone irradiation, and this suggested that phenylalanine was more stable under the UV/ozone irradiation.

The amide I and amide III Raman vibrations were sensitive to the conformational structure of the fibrous protein.[20] The amide I signal, indicative of the antiparallel β-sheet conformation, of silk occurred at 1668 cm^{-1}, differing from the 1655-cm^{-1} signal for the α-helical conformation of wool. The strong amide I mode consisted predominantly of a carbonyl (C═O) stretching vibration with a small contribution from the C—N—H in-plane bending and C—N stretching vibration.[20] The amide III signal for the antiparallel β-sheet conformation of silk appeared at 1232 cm^{-1} and was much stronger than that at 1245 cm^{-1} for the α-helical conformation of wool.[19] The amide III band was due mainly to the C—N—H in-plane bending and C—N stretching vibration and was more directly sensitive to the conformation of the polypeptide chain.[20] There was a decrease in the signal intensity at 1232 cm^{-1} after UV/ozone irradiation (Fig. 3), and this indicated that extended UV/ozone irradiation caused peptide chain scission. In addition, a small reduction in the amide I band of UV/ozone-irradiated (40 min) silk appeared, further supporting the proposed cleavage of the peptide chain. The Raman signals around 1087 cm^{-1}, corresponding to the C—N bond vibration, also showed a reduction in the intensity, further confirming that the photodegradation of silk occurred at C—N bonds.

FTIR analysis of UV/ozone-irradiated silk

The FTIR (ATR) absorbance spectra of untreated silk and UV/ozone-exposed (40 min) silk are shown in Figure 4. The doublets at 1230 and 1263 cm^{-1} were assigned to amide III; the signal at 1263 cm^{-1} was associated with β-pleated-sheet conformation, and the

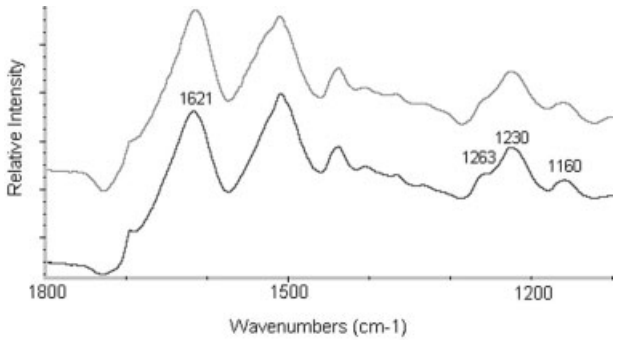

Figure 4 FTIR (ATR) spectra of nonirradiated silk fabrics (lower curve) and UV/ozone-irradiated (40 min) silk fabrics (upper curve) at 1100–1800 cm^{-1}.

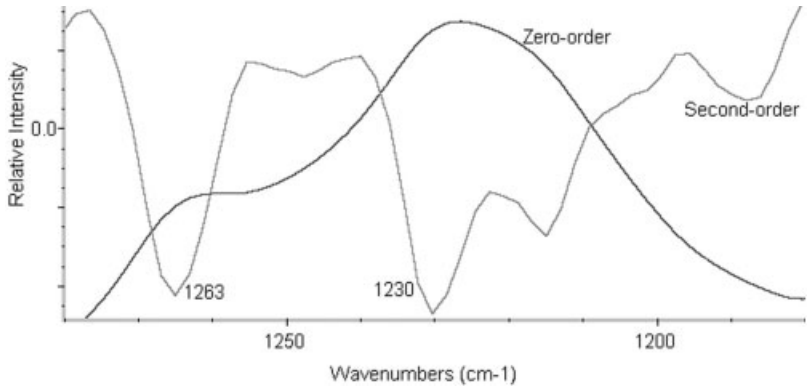

Figure 5 FTIR absorbance and second-derivative spectra of nonirradiated silk (amide III region).

signal at 1230 cm^{-1} was associated with random-coil conformation.[28] By comparing the intensities of the pair of component bands at 1263 and 1230 cm^{-1}, we could both qualitatively and quantitatively analyze the crystallinity degree of *B. mori* silk.[28,29] Second-derivative spectroscopy allowed the overlapping doublet bands to be resolved, as illustrated in Figure 5, in which the second-derivative spectrum is superimposed on the original absorbance spectrum of silk. Table II indicates that the crystallinity degree of silk obtained by FTIR analysis was around 48%, and intensive UV/ozone irradiation caused a considerable decrease in the silk crystallinity degree, probably because of the molecular rearrangement accompanied by peptide fission, as indicated by FT-Raman signal changes.

The signal at 1160 cm^{-1} was attributed to the vibrational absorption of phenol groups in the tyrosine residue on the silk polymer.[30] To probe the changes in the tyrosine content after UV/ozone irradiation, we took the amide I band at 1621 cm^{-1} as an internal standard peak that was relatively stable in its signal intensity. The absorbance band area ratio of the 1160-cm^{-1} signal to the 1621-cm^{-1} signal reflected the relative tyrosine content. Table II shows that UV/ozone irradiation resulted in a significant reduction in surface tyrosine, and this agreed well with Shao et al.'s[31] X-ray photoelectron spectroscopy analysis results.

TABLE II
FTIR Analysis of Silk Fibroin

Sample	Crystallinity degree [%, $A_{1263}/(A_{1230} + A_{1263})$]	Relative content of tyrosine (A_{1160}/A_{1621})
Untreated	47.7	0.56
UV/ozone-irradiated[a]	41.8	0.48

A, area.
[a] 40 min.

CONCLUSIONS

Complementary FT-Raman and FTIR techniques have been applied to probe the nature of silk fibroin. The predominant advantage of FT-Raman spectroscopy for silk fibroin study is that the photosensitive aromatic amino acids tryptophan, tyrosine, and phenylalanine present strong signals. In addition, information on the secondary structure can be provided by the amide I and amide III Raman vibrations. The FTIR (ATR) technique, coupled with second-derivative spectroscopy analysis, allows the structural changes in the relative tyrosine content and the crystallinity degree of silk fibroin to be quantitatively determined.

Both the FT-Raman and FTIR vibrational spectroscopy studies demonstrated that intensive UV/ozone irradiation caused significant photochemical changes in silk tyrosine residues, which accounted for silk photoyellowing.

The FT-Raman spectroscopy study indicated the peptide scission of UV/ozone-irradiated silk and the photodegradation of the silk polymer at C—N bonds, whereas FTIR spectroscopy showed a reduction in the silk crystallinity degree after UV/ozone irradiation due to molecular rearrangement with peptide scission, which accounted for silk phototendering.

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