Biodegradation of *Bombyx mori* Silk Fibroin Fibers and Films

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ABSTRACT: The *in vitro* biodegradation of *Bombyx mori* silk fibroin was studied by incubating fibers and films with proteolytic enzymes (collagenase type F, α-chymotrypsin type I-S, protease type XXI), for times ranging from 1 to 17 days. The changes in sample weight and degree of polymerization of silk fibers exposed to proteolytic attack were negligible. However, tensile properties were significantly affected, as shown by the drop of strength and elongation as a function of the degradation time. Upon incubation with proteolytic enzymes, silk films exhibited a noticeable decrease of sample weight and degree of polymerization, the extent of which depended on the type of enzyme, on the enzyme-to-substrate ratio, and on the degradation time. Protease was more aggressive than α-chymotrypsin or collagenase. Film fragments resistant to enzymatic degradation were enriched in glycine and alanine. FT-IR measurements showed that the degree of crystallinity of biodegraded films increased. Soluble degradation products of silk films consisted of a range of peptides widely differing in size, deriving from the amorphous sequences of the silk fibroin chains. Biodegraded fibers showed an increase of surface roughness, while films displayed surface cracks and cavities with internal voids separated by fiber-like elements. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 91: 2383–2390, 2004

Key words: biopolymers; degradation; enzymes; fibers; films

INTRODUCTION

The silk fiber produced by the domesticated silkworm, *Bombyx mori*, is one of the most precious raw materials of natural origin employed in manufacturing textile products. In recent years, the unique chemical and mechanical properties of silk have made this protein polymer highly attractive for developing innovative applications, which mainly fall within the scope of technical textiles and devices for biomedical uses.[1] The use of silk for manufacturing surgical sutures has been known for years,[1–4] although their use has declined owing to competition by a wide range of similar products manufactured with synthetic polymers and to concerns about its biocompatibility. In fact, several cases of strong inflammatory response attributed to the use of silk sutures have been reported in the medical literature.[1,5] However, Altman et al.[1] have recently reviewed the scientific data available on this subject and concluded that sericin, the silk gum, is mostly responsible for the adverse biological response of silk sutures, while the behavior of fibroin is comparable to that of the most common synthetic polymers used as biomaterials.[6]

Tissue engineering is an emerging field of study focusing on the development of synthetic substitutes for damaged biological tissues and organs. The need for polymer matrices with different chemical, mechanical, and biological properties, versatile enough to meet various application requirements, has urged scientists to address more attention to natural fibrous polymers, such as silk. Silk fibers have been considered as starting material for the preparation of various kinds of medical devices, such as polymer–hydroxyapatite composites for bone regeneration,[7] wire ropes for the substitution of the anterior cruciate ligament,[8] and novel silk-based sutures and protective gauzes for the treatment of skin burns with improved blood compatibility.[9] Silk films, which can be prepared by casting an aqueous silk fibroin solution, are highly attractive for their permeability to oxygen and water vapor.[10,11] Similarly to fibers,[8] films exhibit the ability to support cell adhesion and growth,[12–15] and their use as scaffolds for skin[12] and bone regeneration[15] has been proposed.

As a protein, silk is susceptible to biological degradation by proteolytic enzymes.[10,16–19] The rate and extent of degradation may be highly variable, depending on a series of factors related to structural and morphological features of the polymer (fiber, film,

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sponge), processing conditions, as well as characteristics of the biological environment, and presence of different mechanical and chemical stresses.[1] It is generally accepted that biocompatibility is a prerequisite for designing tissue engineering scaffolds able to ensure optimum biological integration. An additional key factor is the degree of biostability of the polymeric matrix, which must match the functional needs and ensure optimum mechanical and physiological integration of the device. In this context, it is of chief importance to characterize the kinetics and mechanism of the biological degradation of different silk materials exploitable for the preparation of polymeric scaffolds for tissue engineering.

The aim of the present study is to investigate the degradation behavior of silk fibers and films exposed to different proteolytic enzymes for various times. Chemical, physical, and morphological features of biodegraded fibers and films were examined by means of different analytical techniques. Although *in vivo* degradation of biopolymers is a very complex process, involving various synergistic pathways of chemical, biochemical, physical, and mechanical origin, the study of the enzymatic degradation of silk fibroin *in vitro* is expected to contribute findings that may allow elucidation of the mechanism by which the material interacts with the biological environment and characterization of the functional properties of a polymer.

EXPERIMENTAL

Materials

Raw silk yarn was degummed with an aqueous solution of 7 g/L Marseille soap, at 95°C, for 1 h, followed by thorough rinsing with warm distilled water. Degummed silk fibers were then extracted with petroleum ether to remove residual fatty matters, dried at room temperature, and kept under standard conditions (20°C and 65% RH). Silk fibroin films, about 50 μm thick, were prepared by dissolving the fibers with a saturated LiBr aqueous solution, at 60°C, for 3 h. The solution was filtered, dialyzed against water, and then cast on a polyethylene plate, at room temperature. Films were treated with a 50% v/v water–methanol solution to make them water-insoluble.

The following enzymes and buffer solutions were used: collagenase Type F (Sigma, St. Louis, MO, catalog number C7926), dissolved in 50 m*M* TES, 0.36 m*M* $CaCl_2$, pH 7.4; α-chymotrypsin Type I-S (Sigma, catalog number C7762), dissolved in 10 m*M* Tris-HCl, 5 m*M* $CaCl_2$, pH 7.8; protease Type XXI from *Streptomyces griseus* (Sigma, catalog number P0652), dissolved in 50 m*M* potassium phosphate, pH 7.5.

Measurements

To determine the weight loss, silk fibers and films were incubated in the following conditions: material-to-liquor ratio, 1:250; enzyme-to-substrate ratio, 1:8 or 1:20; temperature, 37°C; time, from 1 to 17 days. Blank samples were immersed in buffer alone, without enzyme. Decay of the enzymatic activity was determined on enzyme solutions kept at 37°C for different times, by using Sigma test procedures. At least 50% of the initial enzyme activity was retained after 2 weeks of incubation. At fixed times, fibers and films were taken out from the enzyme or buffer solution, extensively washed with water to remove any trace of absorbed enzyme, dried at 105°C, and weighed. Weight loss was expressed as percentage of the initial dry weight. Results are the average of triplicate tests.

High-performance–size exclusion chromatography (HP-SEC) runs were performed with a Waters (Milford, MA) chromatographic system controlled by a Maxima 820 Workstation, which included a GPC data-handling module.[20] Silk fibers and films (1–5 mg) were dissolved with a small volume of 50% w/v aqueous LiSCN, at room temperature, for 30 min. After dilution with 50 m*M* sodium phosphate buffer, pH 7.2, containing 0.15*M* KCl and 5*M* urea, the solution was extensively dialyzed in cellulose membrane tubing (Spectra/Por 6, MWCO = 3.5 kDa, Spectrum; Pharmacia, Uppsala, Sweden) against the same buffer. At the end of dialysis, the solution was diluted until 1 mg/mL, filtered, and analyzed with a Protein KW-804 column (Shodex, Showa Denko K.K., Tokyo, Japan). After removal of fibers or films, the solution containing the degradation products was immediately freeze-dried. For HP-SEC analysis, freeze-dried samples were dissolved in a small volume of 50 m*M* sodium phosphate buffer, pH 7.2, containing 0.15*M* KCl, filtered, and analyzed with a Protein Pak-60 column (Waters). Injection volumes ranged from 50 to 100 μL, flow rate was 0.5 mL/min. Eluate was detected at 254 nm. All samples were analyzed in duplicate. Molecular weight calibration was performed by using the HMW and LMW Gel Filtration Calibration Kits (Pharmacia Biotech, Piscataway, NJ).

Tensile properties of silk fibers were determined by using an Instron model 4500 tensile testing machine, at gauge length and speed of 10 mm and 10 mm/min, respectively. Data are the average of 15 measurements.

The amino acid composition was determined by acid hydrolysis with 6 *N* HCl, at 105°C, for 24 h, under vacuum. Free amino acids were derivatized with phenyl isothiocyanate (Sigma) and analyzed by HPLC with a PicoTag reversed-phase column (Waters), at a flow rate of 1 mL/min. Eluate was detected at 254 nm. Samples were analyzed in duplicate. The Amino Acid Standard H kit (Pierce, Rockford, IL) was used for calibration.

FT-IR spectra were measured with a Nicolet 150-P spectrometer (Nicolet Instruments, Madison, WI), equipped with an ATR diamond cell (Specac).

TABLE I
Weight Loss of Silk Fibroin Films*

Degradation time (days)	Weight loss (%)					
	Collagenase		α-Chymotrypsin		Protease	
	(a)	(b)	(a)	(b)	(a)	(b)
1	2.6	3.8	6.0	9.0	18.2	35.0
3	4.1	5.5	9.2	11.8	27.4	45.5
10	7.4	8.9	13.0	14.9	35.7	55.6
17	9.4	10.7	14.7	16.5	45.5	64.3

* Enzyme-to-substrate ratio: (a) 1:20; (b) 1:8.

Surface morphology was examined with a Stereoscan 440 (LEO Electronic Microscopy Ltd., Cambridge, England) scanning electron microscope at 10 kV acceleration voltage, after gold coating.

RESULTS AND DISCUSSION

Weight loss

To study the weight loss induced by proteolytic degradation, silk fibers and films were incubated with collagenase, α-chymotrypsin, or protease, and the changes in sample weight were measured at different times. Silk fibers showed only random changes, not exceeding 2% as compared to the initial weight, irrespective of the incubation time and of the type of enzyme (data not shown). On the other hand, significant changes in sample weight were observed on silk films. The results listed in Table I indicate that the extent of weight loss depended on the type of enzyme, on the enzyme-to-substrate ratio, and on the treatment time. The weight loss of blank films was negligible ($\leq$ 3%), owing to the preliminary treatment with 50% v/v aqueous methanol which made the films water insoluble.[10] Among the enzymes used, protease was more aggressive than α-chymotrypsin or collagenase, and it always caused greater weight loss. With increasing the enzyme-to-substrate ratio, the weight loss tended to increase slightly with collagenase and α-chymotrypsin, but significantly with protease. The extent of proteolytic degradation also increased with treatment time, despite the decay in enzymatic activity, especially at long treatment times.

The amount and accessibility of the sites of proteolysis have probably played an important role in determining the extent of degradation of silk films. As concerns the sequence of the site of cleavage, protease from *S. griseus* is the least specific among the enzymes used. The bacterial collagenase (*Clostridium histolyticum*) hydrolyzes the X–Gly bond in the sequence X-Gly-Pro,[21] while the serine protease α-chymotrypsin (from bovine pancreas) cleaves X–Y bonds where X may be an amino acid with an aromatic (Tyr, Phe, Trp) or a large hydrophobic (Val, Ile, Leu) side chain.[22] The number of cleavage sites theoretically available in silk fibroin for α-chymotrypsin is quite large. In fact, this enzyme is used as a tool for preparing amorphous (Cs) and crystalline (Cp) peptides from an aqueous silk fibroin solution for chemical and structural studies.[16] The yield of Cs and Cp fractions usually accounts for about 45% and 55% by weight, respectively. The lower yield of soluble peptides observed with silk films can be attributed to steric hindrance, which limited diffusion of α-chymotrypsin within the dense film texture.

Referring to collagenase, although Gly is highly abundant in silk fibroin and the Gly-Y sequence forms almost 90% of the fibroin chain, the combination X-Gly-Pro is quite rare, accounting for about 11 positions present in the amorphous arrays.[23] Therefore, the low yield of proteolysis is probably attributable to the low number of available cleavage sites. On the other hand, the protease from *S. griseus* could degrade fibroin films more easily owing to the lower specificity for the chemical structure of the cleavage sites.

The different degradation behavior of fibers and films observed in this study is in good agreement with previously reported data[10] and confirms the influence of substrate structure and morphology on the rate and extent of the enzymatic attack. Although silk fibroin is susceptible to proteolytic degradation, enzymes do not readily attack fibers, whose resistance to proteolysis is probably due to a combination of factors, that is, smoothness of the fiber surface, which lacks any gap to the inside, close packing of the fibroin chains, high degree of order, and orientation of the fibrous structure, even in the amorphous regions.[24] Unlike fibers, silk films are isotropic in nature, with an amorphous phase more prone to swelling, which allows enzymes to reach a sufficiently close approach with the cleavage sites available on the substrate.[10]

Degree of polymerization

The degree of polymerization of silk fibroin from biodegraded fibers and films was studied by HP-SEC.[20] Silk fibers exposed to enzymatic degradation exhibited only slight changes in molecular weight distribution (data not shown). On the other hand, the HP-SEC pattern of biodegraded films was characterized by a shift to lower molecular weight ranges of the main chromatographic peak (Fig. 1). The following quantitative parameters of the molecular weight distribution were calculated from the raw chromatographic data: weight-average ($\bar{M}_w$) and number-average ($\bar{M}_n$) molecular weights, which provide a measure of the average chain weight and length; peak molecular weight (P_{mw}), which gives the weight of the most abundant polypeptide fraction in the sample; polydispersity index ($\bar{M}_w/\bar{M}_n$), which indicates the breadth of the mo-

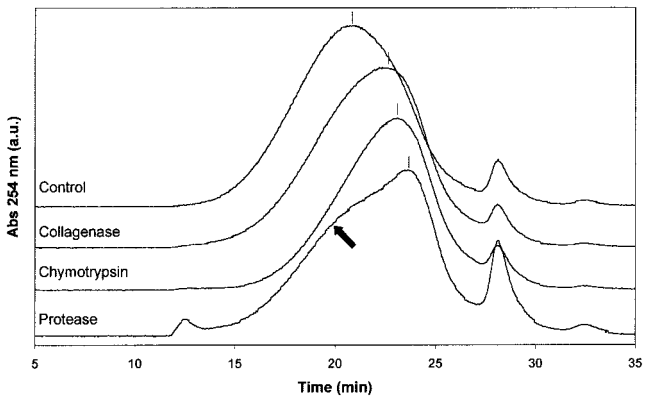

Figure 1 HP-SEC elution curves of silk fibroin from films incubated with proteolytic enzymes for 17 days. The arrow indicates the high molecular weight polypeptide fraction characteristic of silk fibroin after degradation with protease from *S. griseus*.

lecular weight distribution. The results are listed in Table II.

The average size of the fibroin chains decreased to a different extent, depending on the enzyme used, on the extent of weight loss, and on the degradation time. Despite the extensive weight loss, the films exposed to protease exhibited $\bar{M}_w$ values higher than those degraded with α-chymotrypsin or collagenase. This result is attributable to a peculiar feature appearing in the chromatographic profiles of these samples, that is, the presence of two polypeptide fractions, one falling in the low and the other in the high molecular weight range (the latter is indicated by an arrow in Fig. 1). The higher degree of heterogeneity of these samples is also reflected by the increase of the corresponding values of polydispersity index.

The values of P_{mw} of biodegraded silk fibroin films were in the following order: collagenase > α-chymotrypsin > protease, indicating that this parameter was related to weight loss, that is, the higher the weight loss the lower the molecular weight of the most abundant polypeptide fraction in the sample. The values of $\bar{M}_w/\bar{M}_n$ decreased after incubation with α-chymotrypsin, increased with protease, and remained essentially unchanged with collagenase. All of these features, which characterize the molecular weight distributions of the different biodegraded films, can be related to the specific mechanism of the enzymatic attack, as well as to the number and accessibility of the available cleavage sites in the silk fibroin chains.

TABLE II
Molecular Weight Distribution Parameters of Silk Fibroin from Biodegraded Films

Sample	$\bar{M}_w$ (kDa)	$\bar{M}_n$ (kDa)	P_{mw} (kDa)	$\bar{M}_w/\bar{M}_n$
Control	119.8	57.9	79.8	2.07
Collagenase				
Day 3	98.5	47.5	53.9	2.07
Day 10	96.8	48.5	52.9	2.00
Day 17	94.3	45.2	45.8	2.09
α-Chymotrypsin				
Day 3	77.4	42.0	41.1	1.84
Day 10	65.7	37.7	37.6	1.74
Day 17	53.7	34.0	34.8	1.58
Protease				
Day 3	109.6	44.1	36.4	2.49
Day 10	105.6	41.9	33.4	2.52
Day 17	102.4	42.6	31.4	2.40

TABLE III
Tensile Properties of Biodegraded Silk Fibers

Sample	Breaking load (N)	Elongation at break (%)
Control	4.68 ± 0.10	32.7 ± 1.6
Collagenase		
Day 1	4.13 ± 0.13	26.3 ± 1.0
Day 3	3.86 ± 0.14	25.1 ± 1.6
Day 10	3.64 ± 0.13	25.7 ± 1.0
Day 17	3.60 ± 0.12	23.5 ± 1.0
α-Chymotrypsin		
Day 1	3.84 ± 0.07	28.1 ± 1.1
Day 3	3.72 ± 0.11	27.8 ± 1.4
Day 10	3.98 ± 0.09	26.4 ± 0.8
Day 17	3.78 ± 0.09	25.8 ± 0.7
Protease		
Day 1	3.74 ± 0.10	21.6 ± 1.2
Day 3	3.52 ± 0.16	20.5 ± 1.2
Day 17	3.14 ± 0.12	18.1 ± 1.2

Tensile properties of silk fibers

To elucidate the effect of proteolytic enzymes on the physical properties of silk fibers, tensile measurements were carried out on biodegraded fibers. The results listed in Table III indicate that both breaking load and elongation at break decreased. Protease was more aggressive than collagenase or α-chymotrypsin, resulting in loss of strength and elongation of 33% and 45%, respectively, after 17 days of incubation.

The tensile measurements confirm that silk fibers are susceptible to proteolytic attack.[18,19] It could not be as extensive as that observed for films because of structural and morphological reasons, as demonstrated by the negligible changes in fiber weight and degree of polymerization. However, it is likely that enzymes caused localized degradations along the fiber axis, corresponding to more accessible sites where proteolysis could occur due to closer enzyme–fiber interaction. These randomly distributed degradation sites became the weak points responsible for the observed drop of strength and elongation.

It is worth noting that tensile measurements were more sensitive in detecting the onset of biodegradation of silk fibers than other analytical tools, such as

TABLE IV
Amino Acid Composition of Silk Fibroin from Films Biodegraded with Protease *(Streptomices griseus)*

AA (mol%)	Control	3 days	10 days	17 days
Gly	45.0	45.9	46.5	46.7
Ala	29.4	30.4	31.5	31.7
Ser	10.9	10.5	10.0	10.7
Total	**85.3**	**86.8**	**88.0**	**89.1**
Tyr	5.8	5.8	5.7	5.0
Val	2.3	2.2	2.2	1.8
Acidic AA	2.4	1.7	1.2	1.1
Basic AA	1.0	0.7	0.6	0.6
Other AA	3.2	2.8	2.3	2.4
Total	**14.7**	**13.2**	**12.0**	**10.9**

determination of the weight loss and of the degree of polymerization of silk fibroin. In fact, it is reasonable to assume that, in the case of fibers, biodegradation can be triggered without any noticeable effect on the weight of fibers and on the molecular weight distribution of the constituent fibroin chains, especially at relatively short treatment times as those used in the present work.

Chemical and spectroscopic characterization of silk films

The weight loss of silk films exposed to proteolytic enzymes is attributable to cleavage of the fibroin chains and formation of a range of soluble peptides. The analysis of residual silk films recovered after enzyme treatment may provide deeper insight into the kinetics and mechanism of the enzymatic attack. Table IV lists the amino acid composition of silk films incubated with protease for different times. The total content of glycine, alanine, and serine increased gradually with increasing the contact time with the enzyme. Accordingly, tyrosine, valine, and other polar and bulky side-chain amino acids decreased. As is well known, the three simple amino acids glycine, alanine, and serine form the crystalline regions of silk fibroin, while the amorphous regions are highly enriched in amino acids with bulky and polar side chains.[23] The decrease of the latter is therefore associated with preferential degradation of the amorphous film domains, where the enzyme could penetrate more easily, cleave the sensitive peptide bonds, and release free soluble peptides.

The cleavage of amorphous peptide sequences is expected to enhance the crystalline character of biodegraded films. This hypothesis was verified by measuring the FT-IR spectra of silk films after incubation with proteolytic enzymes for different times. The spectra of the films incubated for 17 days with collagenase, α-chymotrypsin, and protease are shown in Figure 2. The intense amide I and II bands at 1620 and 1512 cm^{-1} are typical of crystalline silk fibroin films with prevailing α-sheet molecular conformation.[25] Interesting features can be observed in the range of the skeletal vibrations around 1000 cm^{-1}. The control sample showed two weak bands at 1001 and 976 cm^{-1}, attributed to the "Gly-Gly" and "Gly-Ala" sequences, respectively.[26] The intensity of these bands increased significantly in biodegraded films, indicating an enrichment in amino acid sequences characteristic of the crystalline domains. This observation is confirmed by the behavior of the two amide III vibrations at 1264 and 1231 cm^{-1}, which are considered markers for the crystalline and amorphous structure of silk fibroin, respectively. The "I1264/I1231" intensity ratio is often used to estimate the degree of crystallinity of regenerated silk fibroin materials.[27] Figure 3 shows the plot of "I1264/I1231" as a function of the weight loss of silk films. The degree of crystallinity of biodegraded films increased to an extent directly related to the weight loss, that is, the higher the weight loss the higher the degree of crystallinity.

Characterization of degradation products

The soluble peptides formed during proteolytic degradation of fibers and films with protease were recovered and analyzed by HP-SEC. Figure 4 shows the chromatographic curves of the degradation products obtained from films at different degradation times. The pattern is typical of a complex mixture of peptides widely differing in size, with molecular weight values ranging from about 24,000 to 3,000. With increasing the time of degradation, the intensity of the peaks falling in the low molecular weight range increased, but medium-to-high molecular weight peptide fractions were still present even at long treatment times. This suggests that protease could attack both the film and the soluble peptides already released into the solution, splitting them into smaller fragments.

The amount of soluble peptides released from fibers and films incubated with protease was calculated from the intensity of the chromatographic peaks recorded at 254 nm and plotted in Figure 5. The results indicate that films are readily degraded by protease while fibers are more resistant to proteolytic attack, as evidenced by the extremely low amount of soluble degradation products detected. Moreover, the amount of soluble peptides released by silk films increased steadily at short incubation times, in good agreement with the weight loss data (Table I).

Surface morphology

Morphological changes induced by enzymatic treatment were studied by scanning electron microscopy. It is well known that the surface of degummed silk fibers is highly smooth, showing only very fine longitudinal

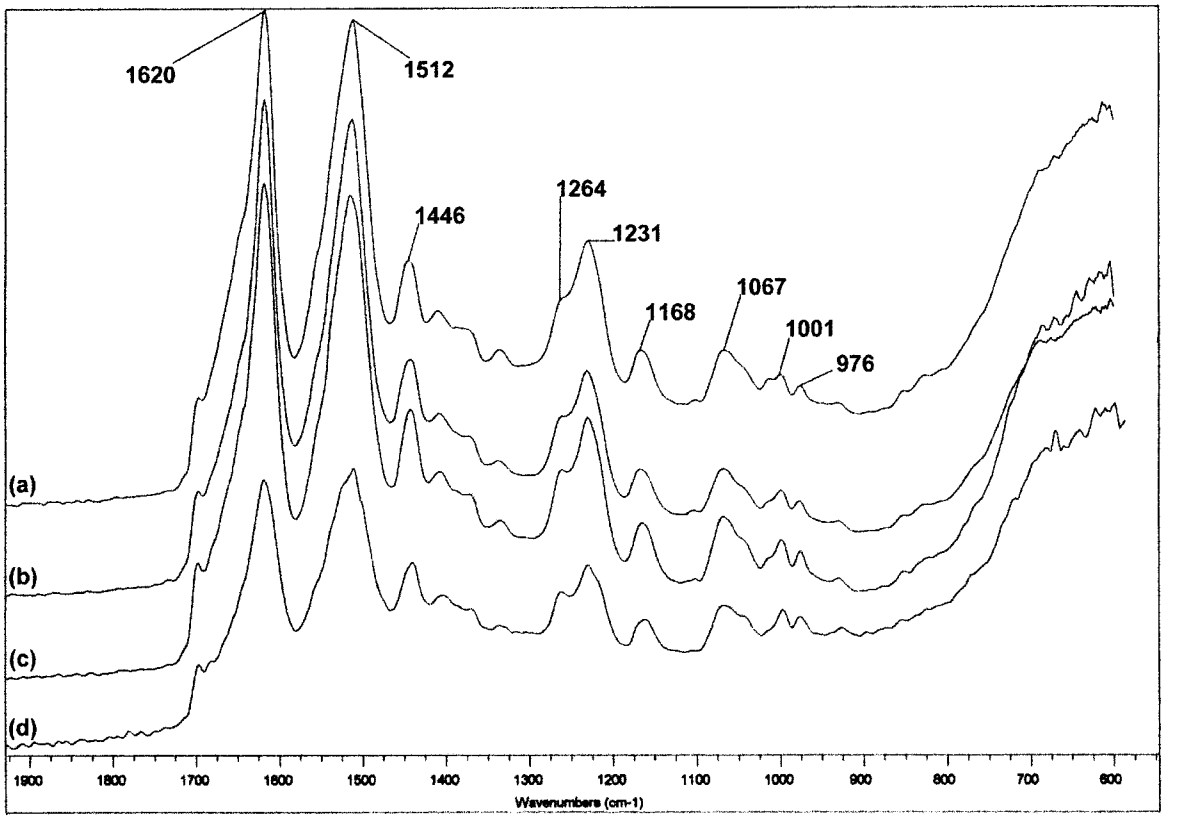

Figure 2 FT-IR spectra of silk films untreated (a) and incubated with collagenase (b), α-chymotrypsin (c), and protease (d) for 17 days.

striation attributable to the fibrillar structure of the fiber [Fig. 6(a)]. Following exposition to protease, silk fibers showed a higher degree of surface roughness and the longitudinal arrangement of fibrils became more visible [Fig. 6(b)]. No evidence of stronger surface degradation was found.

The morphology of silk films was drastically influenced by the enzymatic degradation. The surface of air-cast films, usually very even [Fig. 7(a)], showed the presence of extensive degradation, which appeared in form of cracks and surface stripping of the most external film layers [Fig. 7(b)]. The presence of several cavities, with a characteristic internal morphology formed by interconnected voids separated by fiber-like elements [Fig. 7(c)], confirms that the swollen film allowed the enzyme molecules to penetrate and diffuse inside the film matrix toward the available cleavage sites.

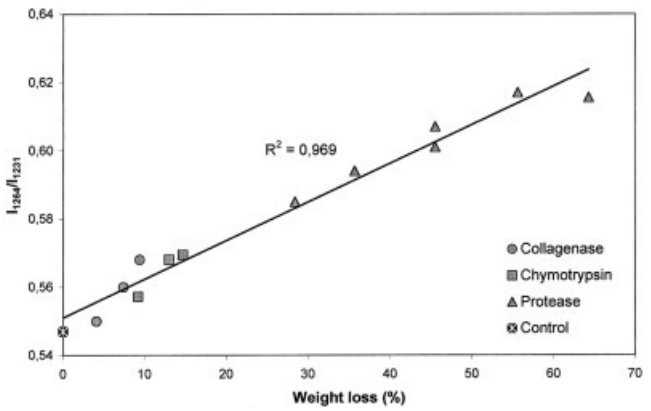

Figure 3 Degree of crystallinity versus weight loss of silk films incubated with proteolytic enzymes. The degree of crystallinity is expressed as intensity ratio between the IR bands at 1264 and 1231 cm-1 (amide III).

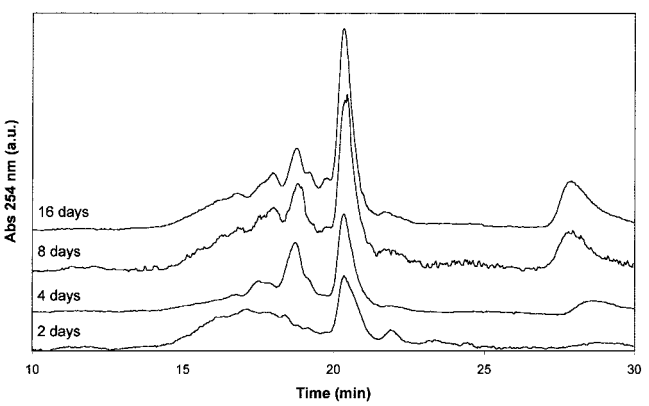

Figure 4 HP-SEC elution curves of soluble polypeptides released from silk films incubated with protease from *S. griseus* for different times.

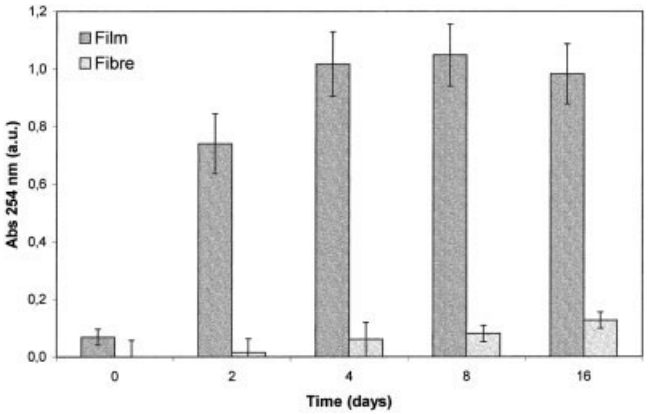

Figure 5 Amount of soluble polypeptides released from silk fibers and films incubated with protease from *S. griseus* for different times. The amount of polypeptides is expressed as intensity of the HP-SEC curves recorded at 254 nm.

CONCLUSIONS

The whole accumulated results on the biodegradation of silk fibers and films allow to conclude that silk fibroin is susceptible to proteolytic attack, but the extent of degradation depends on the structure and morphology of the substrate, as well as on the kind of enzyme used, that is, on the cleavage site specificity.

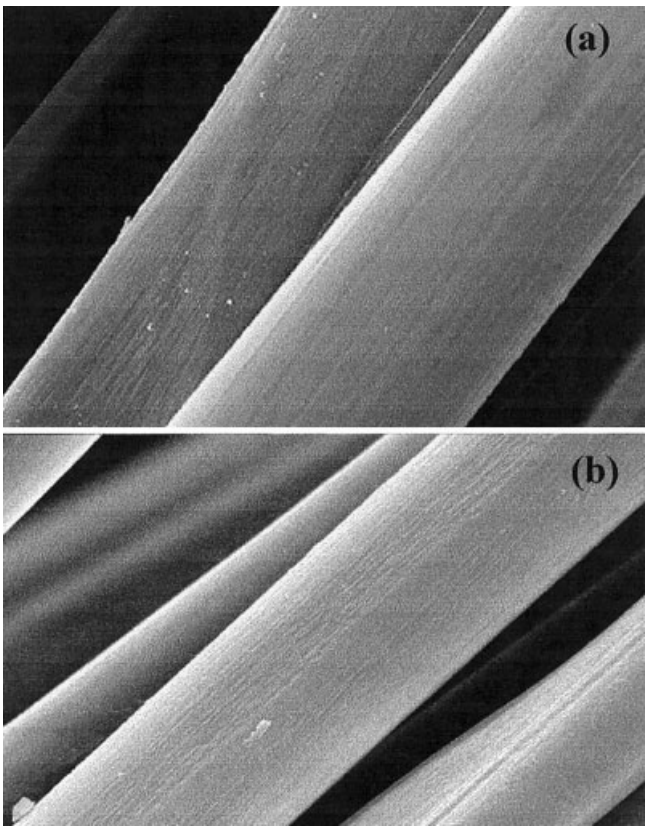

Figure 6 SEM photographs of silk fibers untreated (a) and incubated with protease from *S. griseus* for 17 days (b).

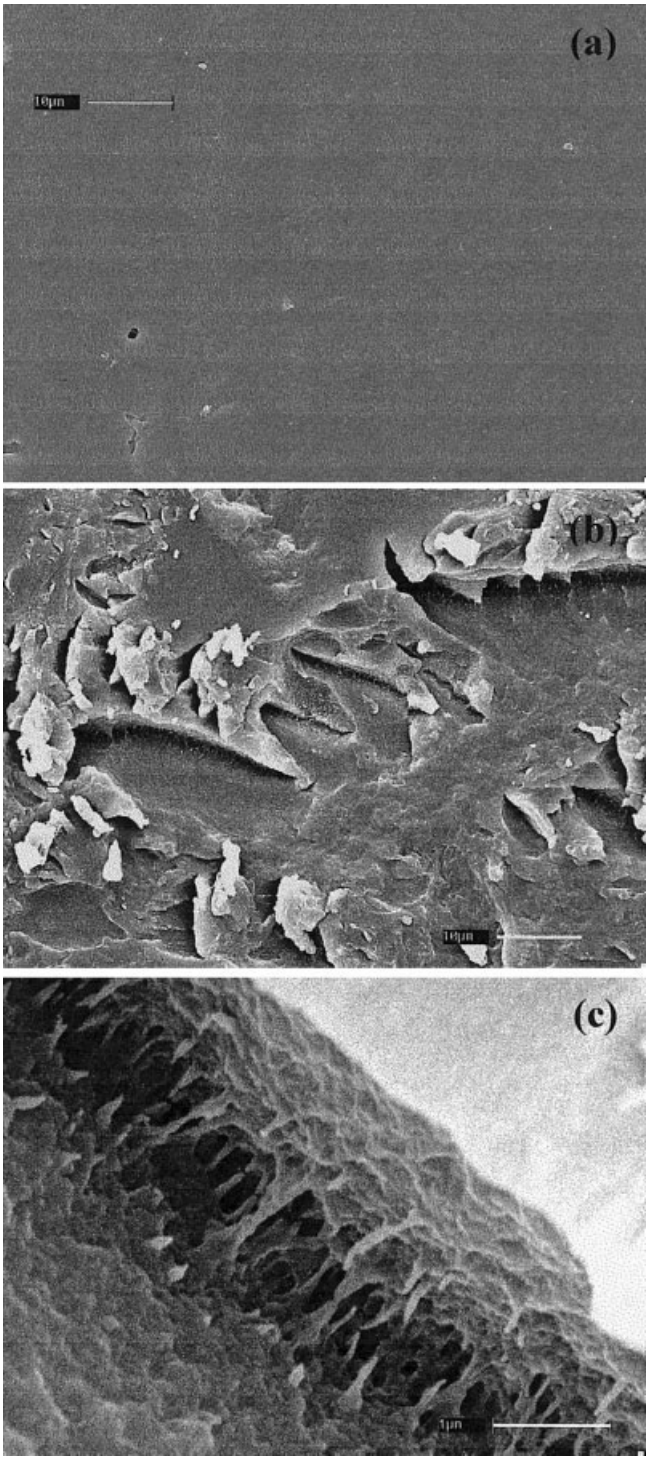

Figure 7 SEM photographs of silk films untreated (a) and incubated with protease from *S. griseus* for 17 days (b); (c), detail of (b).

Silk fibers were attacked at a very low extent by proteolytic enzymes. The onset of enzymatic degradation was detected by tensile measurements, which revealed a drop of strength and elongation attributed to local degradation randomly distributed along the fibers.

Films were degraded more readily by the different proteolytic enzymes used in this study, although to a different extent. The protease from *S. griseus* was more aggressive toward silk films. Proteolysis resulted in extensive weight loss and formation of a range of soluble peptides derived from selective cleavage of the amorphous sequences of the fibroin chains. As a result, the average molecular weight of silk fibroin in the residual films decreased, and the crystalline character of the films increased, as evidenced by chemical and spectroscopic analyses.

The morphological characterization of biodegraded films confirmed that the enzyme molecules could penetrate into the swollen film matrix and come into close contact with the available cleavage sites distributed along the amorphous arrays of the silk fibroin chains.

In conclusion, we think that the *in vitro* approach used in the present study may represent a useful tool for studying the rate and mechanism of the biodegradation of different silk fibroin materials as well as for predicting their interaction with the biological environment *in vivo*.

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