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Photodegradative changes in chemical structures of silk studied by pyrolysis–gas chromatography with sulfur chemiluminescence detection

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Abstract

A novel approach to trace photodegradation processes of silk was developed by means of pyrolysis–gas chromatography (Py–GC) with sulfur chemiluminescence detection (Py–GC/SCD). A series of photodegraded silk fabric samples prepared by exposure to artificial sunlight were investigated in this work. Various sulfur-containing amino acid related compounds such as H_2S, COS, CH_3SH, CH_3SCH_3, CS_2 and $CH_2{=}CHSCH_3$ were detected on the pyrograms of samples, a weighing about 200 μg, pyrolyzed at 600°C under the flow of He carrier gas and separated using temperature programmed fused silica capillary column followed by selective SCD. The yields of the characteristic products proved to decrease as a function of irradiation time. This change was quantitatively evaluated by use of three calibration curves prepared for methionine (Met), cysteine (Cys) and cystine (Cys–Cys) residues where aliquot amounts of four different kinds of protein enzyme solutions with known residue distributions were used as standard reference materials, and CH_3SH, H_2S and CS_2 were used as the key components for Met, Cys and Cys–Cys residues in protein, respectively. Thus obtained results revealed that among three different sulfur-containing residues in silk fibroin, the S–S bonds in Cys–Cys residues were cleaved preferentially at the very initial stage and that Met residues in silk were slightly stabler than Cys residues.

Keywords: Silk; Photodegradation; Disulfide bond; Pyrolysis–gas chromatography; Sulfur chemiluminescence detection

1. Introduction

Silk is readily damaged and weakened by exposure to light, making the deterioration mechanism of interest not only to protein biochemists but also to the conservators in museums where many ceremonial garments and related items made of silk are displayed and/or stored. The deterioration of silk has been often assessed based on various chemical and physical changes of the fibrous protein, silk fibroin. For example, color, surface morphology, and loss of breaking strength of photodegraded silk fabric have been used to describe the initial degradation in terms of physical changes in a silk fibroin [1]. Moreover, degradation of silk fibroin induced by UV-irradiation was also studied by stress–strain measurements, thermal analysis and X-ray diffraction method [2].

Recently, the changes in amino acid composition of silk after irradiation have been investigated by amino acid analysis using hydrolysis of peptide chains followed by chromatographic separation [1–3]. So far, it was reported that selective destruction of amino acids accompanied by peptide bond cleavage took place during photoirradiation mainly at tyrosine [1,3] and serine [1–3] residues existing in the amorphous region in silk fibroin. Becker et al. also investigated the degradative changes in silk with amino acid analysis and solubility in order to assess the preservation of historic silks [1,4].

On the other hand, a sensitive method for the determination of amino acid residues in enzyme proteins was reported by using pyrolysis–gas chromatography (Py–GC) [5,6]. It was also proved by Py–GC coupled with sulfur selective flame photometric detection that the disulfide (S–S) bonds in the cross-linked cystine (Cys–Cys) residues, comprising two cysteine (Cys) residues, in the proteins were preferentially cleaved during the thermal denaturation at around 250°C [7].

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In addition, studies on photodegradation of wool protein fibers suggested that the cleavage of S–S bonds in Cys–Cys residues caused by irradiation would contribute significantly to its yellowing [8,9]. In these studies, Lennox estimated the amount of Cys and Cys–Cys residues in the photo-yellowed wool by means of amino acid analysis and polarography, and pointed out that the main effect of irradiation attributed to conversion of –S–S– to –SH groups, resulted in the significant decrease of Cys–Cys residues in the degraded peptide chain [8]. Furthermore, Maclaren demonstrated that photo-yellowing of wool was accompanied by decrease of S–S bonds (Cys–Cys residues) and marked increase of –SH groups [9].

These studies mentioned above strongly suggest that the decomposition of Cys–Cys residues in silk fibroin should occur during photodegradation of silk. However, the decomposition of Cys–Cys residues in silk fibroin during photodegradation has not been proved yet mainly because the amount of sulfur-containing amino acid residues in silk fibroin is as little as 0.5% [10]. In this work, photodegradative changes in the chemical structures of silk were examined by focusing on the changes in the sulfur-containing amino acid residues such as methionine (Met) and Cys and Cys–Cys residues by means of Py–GC with sulfur chemiluminescence detection (Py–GC/SCD).

2. Experimental

2.1. Materials

Degummed silk cloth woven as a test fabric at National Research Institute of Sericultural and Entomological Science, Tsukuba, Japan was used as the control sample. Photodegradation of silk samples was performed by exposure to simulated sunlight in a weather-meter [Suga, (Tokyo, Japan) Wel-24AX-HC-EC] at 46°C and 60±5% relative humidity for 5, 27 and 52 days. Four reference protein enzymes with known residue distributions were used as calibration standards for the determination of amino acid residues; egg white lysozyme, horse heart myoglobin and bovine hemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and bovine pancreas trypsin from Wathington Biochemical Co (Lakewood, NJ, USA). Their molecular weight and the number of sulfur-containing amino acid residues are listed in Table 1 together with their total residue numbers.

Table 1
Protein enzymes used for calibration

Protein (origin)	MW	Number of residues			
		Sulfur containing amino acids [amino acid composition (wt %)]			Total
		Cys	(Cys–Cys)[a]	Met	
Lysozyme (egg white)	14,300	8 (5.7)	(4)	2 (1.8)	129
Trypsin (bovine pancreas)	23,300	12 (5.3)	(6)	2 (1.1)	229
Myoglobin (horse heart)	17,000	0 –	(0)	2 (1.5)	153
Hemoglobin (bovine blood)	64,500	2 (0.32)	(1)	8 (1.6)	572

[a] All Cys residues in the enzyme proteins utilized have the Cys–Cys structure.

2.2. Py–GC/SCD

A vertical microfurnace pyrolyzer [Frontier Lab (Koriyama, Japan) PY-2020D], which enables pre-heating free pyrolysis at desired temperatures for thermally labile samples such as proteins, was directly attached to a gas chromatograph [Hewlett-Packard (Avondale, PA, USA) HP 6890] with a fused-silica capillary column [J&W (Folsom, CA, USA) DB-1; 30 m long×0.32 μm i.d.] coated with thick film (5.0 μm) of polydimethylsiloxane immobilized through chemical cross-linking which is suitable for separation of lower boiling-point compounds. The column effluents were selectively detected by a sulfur chemiluminescence detector (SCD) [Sievers (Boulder, CO, USA) Model 355 SCD] [11,12] using the chemiluminescence from the reaction of ozone with SO species produced during combustion of the sulfur-containing analyte. Flame ionization detector (FID) was also used to observe reference pyrograms.

About 200 μg of the silk fiber sample chopped into about 0.5 mm long, or 1 μl of aqueous solution of the protein enzymes in a concentration of 0.25–2.0% (w/v), taken in an inactivated disposable stainless steel sample cup [Frontier Lab PY1-EC50; 5.0 mm height×3.7 mm i.d.×4.0 mm o.d.] was mounted in the vertical pyrolyzer, and then dropped into the heated center to initiate pyrolyzation of the sample under a flow of He carrier gas. The optimum pyrolysis temperature of 600°C to attain the highest yield of peaks derived from sulfur containing amino acid residues was empirically determined [6]. The 50 ml/min He carrier gas flow at the pyrolyzer was reduced to 1.0 ml/min at the capillary column by means of a splitter. The column temperature was first held at 30°C for 5 min, then increased to 100°C at a rate of 5°C/min.

3. Results and discussions

Fig. 1 shows the pyrograms of the control silk sample observed with (a) FID and (b) SCD at 600°C. Most of

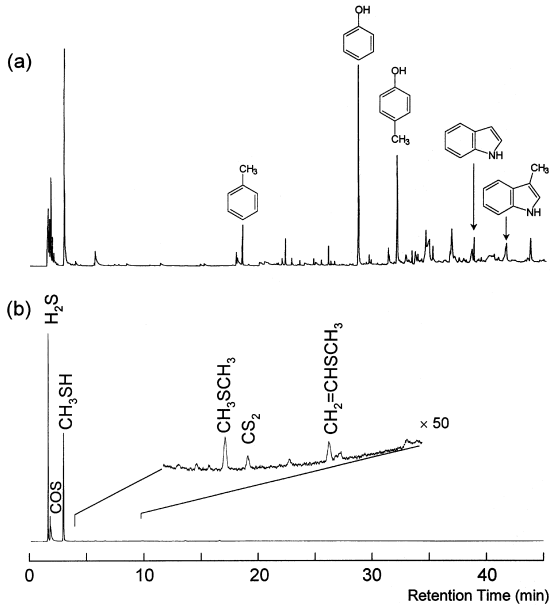

Fig. 1. Pyrograms of silk at 600°C observed by (a) FID and (b) SCD.

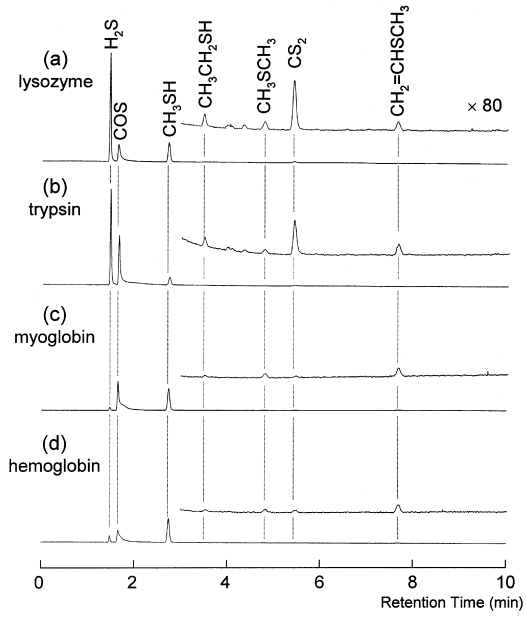

Fig. 2. Pyrograms of the standard protein enzymes observed by SCD:

the large peaks on the pyrogram observed with FID (a) are aromatic products derived from aromatic amino acid residues such as phenylalanine, tyrosine and tryptophan [5]. In this pyrogram, however, it is almost impossible to discriminate the sulfur-containing products formed from trace contents of Cys and Met residues in silk. On the other hand, the peaks of sulfur-containing products such as H_2S, COS and CH_3SH, derived from the sulfur-containing amino acid residues in silk are discriminatively detected on the pyrogram observed with SCD (b). Here, it was reported that CH_3SH, CH_3SCH_3 and $CH_2{=}CHSCH_3$ were mainly produced from Met residues, H_2S was mainly produced from Cys residues, and CS_2 from Cys–Cys residues reflecting S–S bonds, respectively [6]. In addition, although carbonyl sulfide (COS) was also detected in the pyrogram, its formation mechanism has not been clarified yet. Therefore, CH_3SH, H_2S and CS_2 were used in the following study as the representative key products for the determination of Met and Cys and Cys–Cys residues, respectively.

Here, the four standard protein enzymes were measured by Py–GC/SCD under the same conditions as for the silk samples to make calibration curves for the determination of the sulfur-containing amino acid residues. Fig. 2 shows the pyrograms of these enzymes; (a) lysozyme, (b) trypsin, (c) myoglobin and (d) hemoglobin. In the pyrograms of lysozyme (a) and trypsin (b), which contain many Cys and some Met residues, all the sulfur-containing key products such as H_2S, CH_3SH and CS_2 are clearly observed, while in the pyrogram of myoglobin (c), which contains no Cys residue, and that of hemoglobin (d), of which Cys content is significantly low, the peak intensities of H_2S and CS_2 are much weaker than those observed in the pyrograms (a) and (b). These results observed for standard protein enzymes suggest that the peak intensity of the key products are reflecting the amounts of the corresponding sulfur-containing amino acid residues in a given protein sample.

Fig. 3 shows the relationship between the intensity of each characteristic peak on the pyrograms for (a) Met, (b) Cys and (c) Cys–Cys residues and the content (*n* mol) of the corresponding amino acid residues based on pyrolyzed aliquots of reference aqueous solution. The fact that almost linear relationships are observed in every case demonstrates that the content of the sulfur containing amino acid residues in a given protein sample can be estimated from the intensities of the characteristic peaks on the pyrograms using such calibration curves. It is interesting to note, however, that slightly systematic shifts of the plots are observed depending on the type of the reference protein enzymes, suggesting that the sequence structures of the amino acid residues in the peptide chain might affect the pyrolysis reactions of the sulfur-containing amino acid residues contained in it to some extent.

Fig. 4 shows the pyrograms of (a) the unirradiated silk sample, and the silk samples exposed for (b) 5 days, (c) 27 days and (d) 52 days. The intensities of most of the key peaks apparently decrease with an increase in the irradiation time. The contents of Met, Cys and Cys–Cys residues in the silk samples were then estimated from the corresponding key peak intensities using the calibration curves shown in Fig. 3. The quantitative results thus obtained for the silk samples are summarized in Table 2 along with the potential errors evaluated

on the basis of the systematic variations in the calibration lines shown in Fig. 3. The observed amino acid compositions for both Cys and Met residues of the control sample were about 0.2%, respectively, which are in a good agreement with those inferred from DNA sequencing [13,14]. Moreover, the Cys–Cys residues constructing the S–S bridges was estimated to be 0.036%, which corresponds to approximately 20% of total Cys residues (0.18%) suggesting that about 80% of Cys residues does not form S–S bonds but remains as the free –SH groups in the original silk fibroin. The relative standard deviation (RSD) for the determination of the Cys residue was ca. 5% for three repeated runs for the control silk sample, suggesting adequate reproducibility to trace the photodegradative changes of silk.

In order to visualize the changes in contents of the sulfur-containing amino acid residues, the quantified amino acid values shown in Table 2 are illustrated in Fig. 5 as a function of irradiation time. The fact that all the sulfur-containing amino acid residues in silk decreased with the exposed time suggests that the sulfur-containing amino acid residues in silk decompose during the irradiation through releasing sulfur-containing compounds such as sulfur, hydrogen sulfide and/or carbon disulfide. The observed result that Cys residues decreased more rapidly than Met residues indicates that Cys residues in silk are much more susceptible to photodegradation. Moreover, the fact that the Cys–Cys residues decreased drastically down to ca. 50% at the initial stage of irradiation compared with slower decrease for Cys residues clearly suggests that the S–S bond cleavages in the silk sample might occur preferentially during its photodegradation.

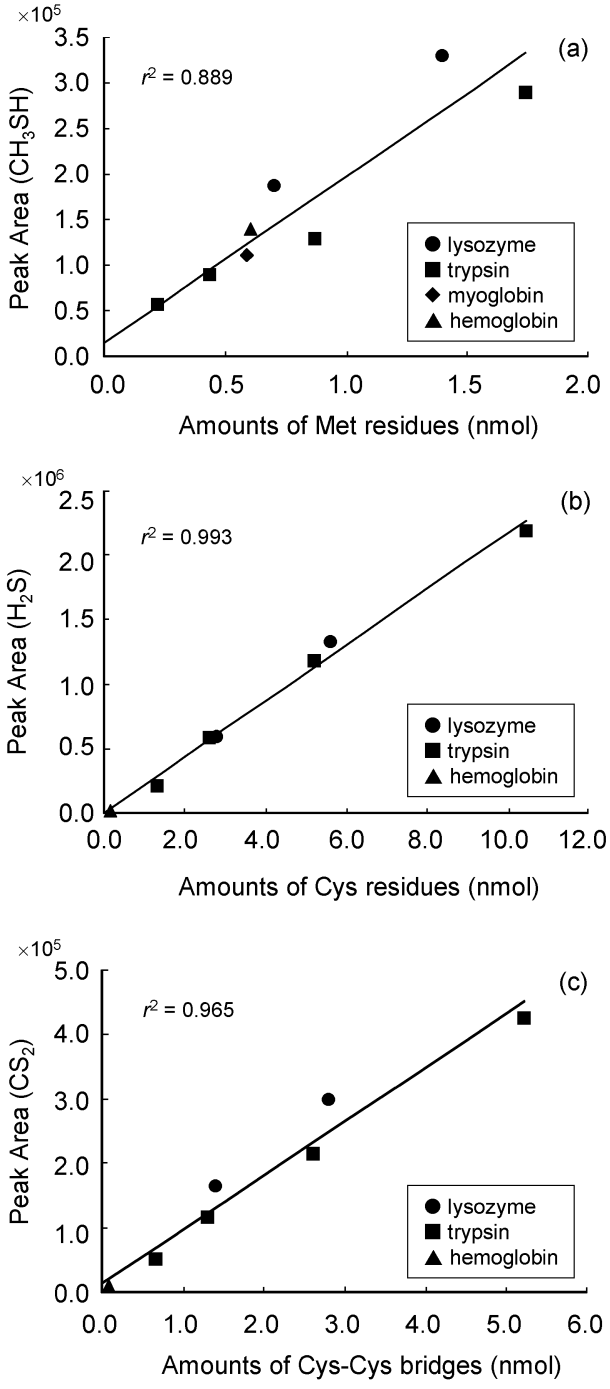

Fig. 3. Calibration curves for (a) Met residue, (b) Cys residue and (c) Cys–Cys bridges observed by use of known amounts of standard protein enzymes; lysozyme, myoglobin, trypsin and hemoglobin.

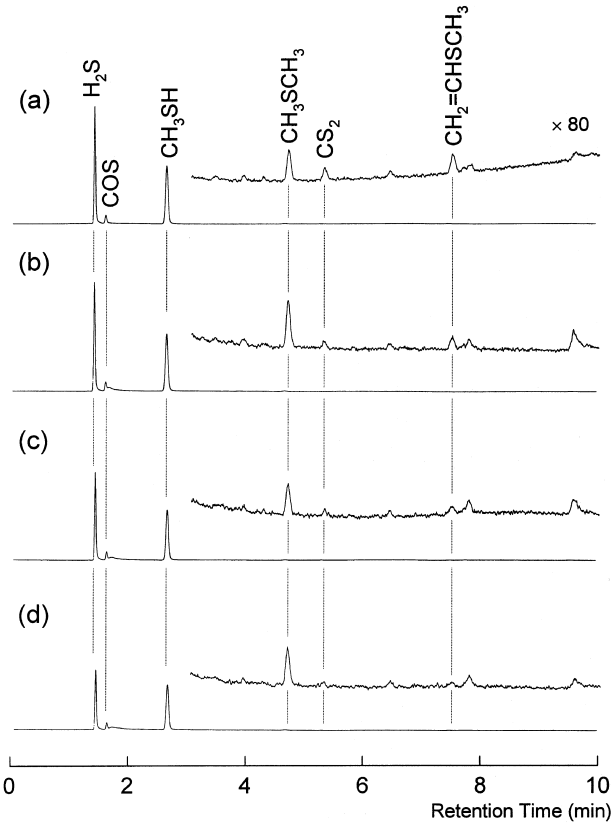

Fig. 4. Pyrograms of photodegraded silk samples at 600°C observed by SCD: (a) not exposed; (b) exposed for 5 days; (c) exposed for 27 days; (d) exposed for 52 days.

Table 2
Content of the sulfur-containing amino acid residues contained in exposed silk samples determined by Py–GC with SCD

Amino acid residue	Amount of the amino acid residue (μmol/g) [amino acid composition (wt %)]			
	Control (not exposed)	Exposed days		
		5	27	52
Met[a]	14.8±2.9 (0.22)	13.7±2.7 (0.20)	12.4±2.4 (0.18)	10.6±2.0 (0.16)
Cys[b]	14.6±0.8 (0.18)	12.3±0.5 (0.15)	10.3±0.3 (0.13)	7.0±0.2 (0.08)
Cys–Cys[c]	1.48±0.19 (0.036)	0.82±0.22 (0.020)	0.63±0.23 (0.015)	0.54±0.23 (0.013)

[a] From peak intensity of CH_3SH.
[b] From peak intensities of H_2S.
[c] From peak intensities of CS_2.

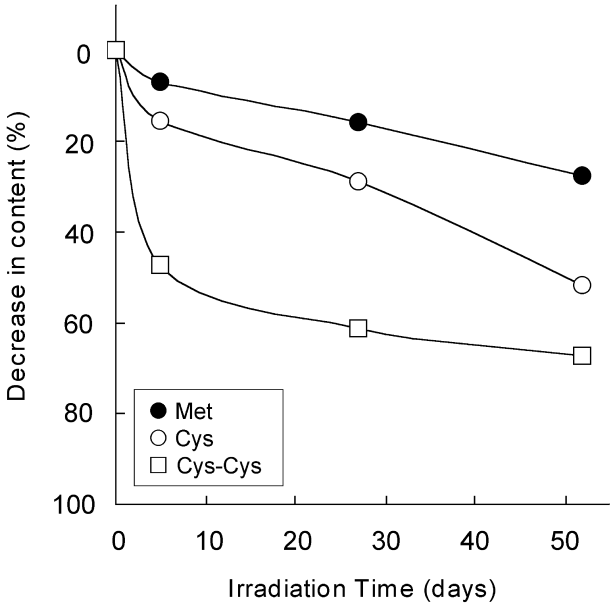

Fig. 5. Relationships between the decrease in content of sulfur-containing amino acid residues and irradiation time.

In conclusion, photodegradative changes in the chemical structures of silk were sensitively evaluated by tracing the sulfur-containing amino acid residues by means of Py–GC/SCD. The obtained results suggested that the S–S bonds in Cys–Cys residues in silk were cleaved in preference to the decomposition of the Cys residues at the very initial stage of irradiation. Since the developed method has both extremely high sensitivity and selectivity to sulfur-containing components, it would have potentially wider applicability not only to study structural changes in silk but also to trace minor sulfur-containing components in microorganisms and even cellular-level biological tissues.

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