Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based biotyping of silk. I. Method development

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SUMMARY

A method for the analysis of silkworm (Bombyx mori L.) cocoons and silk fibers was established using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. This method was evaluated on the most popular four-way cross hybrid race Kinshu × Showa and its parent races Kinshu and Showa. Most of the peaks observed in the peptide mass fingerprints of the Kinshu × Showa cocoon were from the parent races Kinshu and Showa. Simultaneous acid cleavage of silk fibers at room temperature is a characteristic phenomenon that has not been observed in spider silk and animal hair.

Key words: acid fragmentation, cocoon, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), silk, silkworm (Bombyx mori)

INTRODUCTION

Most of the moths in Lepidoptera1) and some bees2) make cocoons for pupation. Similarly, the silkworm, Bombyx mori L., produces silk fibers that have been used in textiles for more than 2000 years. At that time, various silkworm races were collected3, 4) and investigated in detail5). Since then, the breeding of silkworms has continued to improve their economic value6). A hybrid race, Kinshu × Showa, is one of the most commonly reared races in Japan. This race was obtained from the cross-breeding of Kinshu and Showa7). Recently, silk has been used as a biomaterial8–10) and is therefore of increasing economic importance. Unfortunately, except for sericin, detailed biochemical analyses of silk proteins have been insufficient owing to their insolubilities11). However, recently, proteomic analyses of silk proteins were repeatedly reported following multi-step pre-treatments for solubilization of silk using several solvents12–14).

Peptide mass fingerprints (PMFs) obtained by MALDI-TOF MS have been used for the clinical diagnosis of infected microorganisms, namely the MALDI biotyping method15, 16). This method, which involves the direct analysis of biological samples, has expanded to non-clinical organisms, including spider mites17), silkworm microsporidia18), pollen19), and edible mushrooms20) for the identification of species. In the present study, we used the MALDI biotyping method to analyze the cocoons and silk fibers of silkworms. The developed method for the analysis of silk fibers and cocoons using MALDI-TOF MS was evaluated using the Kinshu, Showa, and Kinshu × Showa races.

MATERIALS AND METHODS

Reagents

Trifluoroacetic acid (TFA), formic acid (FA), sodium hydroxide, and sodium carbonate were purchased from Nacalai Tesque (Kyoto, Japan), while α-cyano-4-hydroxycinnamic acid (CAA) was obtained from Honeywell Fluka (Charlotte, NC, USA). Acetonitrile (ACN), acetic acid, and sodium hydrogensulfite were supplied by Fujifilm Wako Pure Chemical Co. (Osaka, Japan), and hydrogen peroxide was purchased from Santoku Chemical Industries Co. Ltd. (Tokyo, Japan). Additionally, Marseille soap was obtained from Miyoshi Soap Co. (Tokyo, Japan), while protein calibration standard I and bacterial standard (Bruker Daltonics, Billerica, MA, USA) for Autoflex III and Microflex were used for mass calibration.

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Materials

Silkworm (Bombyx mori L.) cocoons (Kinshu × Showa, Kinshu, and Showa) were generously provided by the Institute of Sericulture, Ibaraki, Japan. The other cocoons used (race Daizo p50 and race N137 × C146) were reared by the National Agriculture and Food Research Organization. Spider webs were collected in Tsukuba, and sheep wool from the National Agriculture and Food Research Organization. The obtained data were analyzed by Flex Analysis and on a MALDI biotyper 4.1.80 (Bruker Daltonics) with the default parameters, except for the mass range which was m/z –800–15,000.

RESULTS AND DISCUSSION

Acid hydrolysis of silk proteins

Silk textiles are known to weaken under acidic conditions and strengthen under alkaline conditions. In the present study, degumming was performed under alkaline conditions using sodium carbonate or Marseille soap. These agents were used for degumming, as they were believed to cause no damage to the silk fibers. The deterioration of silk by acids is well known and has been under investigation for several years through the use of Raman spectroscopy, NMR, and X-ray analysis. Fig. 1 shows the electrophoresis of the silk proteins. Silk mainly consists of three types of protein components: Glycosylated P25, sericin, and fibroin. The glycosylated P25 was believed to have been removed by degumming using sodium carbonate. Glycosylated P25 was observed as a band at 29 kDa (Fig. 1A and C).

Degumming

Before degumming, the raw cocoons were heat-treated at 120°C for 10 min and 60°C for 6 h. The heated cocoons (120 mg) were then washed in 30–40°C distilled water for 10 min before being stirred in 98–100°C water, containing 120 mg of sodium carbonate or 120 mg of Marseille soap, in a 50 μL beaker for 40 min (boiling water was added to maintain the initial volume). The fibers were then washed in water, followed by a sodium hydrosulfite solution (120 mg), and then again in water (all solutions were at 30–40°C) for 5 min each.

Electrophoresis

SDS-PAGE was performed using an 18.3% acrylamide gel. For native silk proteins, 10 μL aliquots of extracts from 2 mg of silk proteins in 50 μL of SDS sample buffer were heated at 100°C for 10 min before being applied to the electrophoresis gel. For 70% FA-treated cocoons, proteins were extracted from a mixture of 2 mg of silk fibers/50 μL of 70% FA that was vortexed for 10 min and re-extracted with the same volume of ACN. Dried extracts were dissolved in SDS sample buffer and placed in an electrophoresis gel, which was visualized by Coomassie brilliant blue stain.

Tris-Tricine PAGE was performed with 32.4% polyacrylamide gel without a spacer gel. For 2.5% FA-treated silk fibers, the extracts used were taken from 1 mg of silk fibers immersed in 100 μL of 2.5% FA containing 50% ACN for 5 min at room temperature (26°C). Dried extracts were dissolved in sample buffer for Tris-Tricine electrophoresis. The gel was visualized using silver staining.

Mass spectrometry

Silk fibers (1 mg of silk/100 μL of 2.5% FA containing 50% ACN) were vortexed for 5 min and used for analysis. One microliter of saturated CAA containing 2.5% TFA and 50% ACN was added to 1 μL of silk extract. The resulting mass spectra (1,200 shots, n=2) were obtained from m/z 800 to 20,000 in linear positive mode (Microflex and Autoflex III, Bruker Daltonics). In some cases, half volumes of 3% hydrogen peroxide were added to the Marseille soap-treated silk and dried before acid extraction. Otherwise, a single thread of silk fiber was placed on double-sided conductive carbon tape attached to the MALDI target (MTB 384 massive, Bruker Daltonics). The silk thread was then immersed in the matrix solution.

Fig. 1. Electrophoresis of silk proteins.

(A) SDS-PAGE of silk proteins (N137 × C146), M, molecular marker. 1, Heat-treated cocoons. 2, Sodium carbonate-treated cocoons. 3, Marseille soap-treated cocoons. (B) SDS-PAGE of silk proteins and 70% formic acid (FA)-treated extracts. The dried sample was dissolved in the SDS sample buffer. M, molecular marker. 1, silk fibers from outside of cocoons (Daizo). 1’, silk fibers from inside of cocoons (Daizo). 2, Marseille soap-treated cocoons (Kinshu × Showa) were hydrolyzed by FA. 3, Sodium carbonate-treated cocoons (Kinshu × Showa) were hydrolyzed by FA. (C) Tris-Tricine PAGE of FA-treated silk proteins (Kinshu × Showa). Silk proteins were extracted by being submerged in 2.5% FA containing 50% acetonitrile for 5 min. M, molecular marker. 1, Heat-treated cocoons. Concentrated extract (300 μL) was applied. 2, Heat-treated cocoons. Concentrated extract (600 μL) was applied. 3, Sodium carbonate-treated cocoons. Concentrated extract (600 μL) was applied.
P25 bands in Fig. 1B were slightly smaller than those in the band in Fig. 1A. However, the degradation of glycosylated P25 based on electrophoresis is not conclusive.

Acid hydrolysis was attempted at a lower concentration of acid (Fig. 1C), and several bands were observed in the extracts from 2.5% FA containing 50% ACN. Clear bands were observed with no smear bands, indicating that controlled acid hydrolysis was successful under these conditions. Additionally, no band was observed in the acid-treated and Marseille soap-treated silk fibers in Tris-Tricine PAGE (data not shown).

Based on the electrophoretic analysis, 2.5% FA containing 50% ACN was used for MALDI-TOF MS analysis. Precise identification of polypeptide fragments in the electrophoresis was not yet determined using a proteome technique (Fig. 1C), and they remained for further analysis.

**Analysis scheme**

For detailed proteomic analysis of silk, several solvents must be used for the solubilization of fibroin prior to analysis\(^\text{12-14}\). Approximately 1 week was required for solubilization, including dialysis, 1 d for trypsinization, and 1 h for MS analysis. Although much information would be expected from the protein chemical analysis, skilled techniques are needed to use the instruments in addition to significant time and cost. The procedure used in the present study required only 10 min in total, and only a small portion of

![Acid hydrolysis analysis scheme of silkworm cocoons by liquid extraction and solid analysis.](image)

(a) Silk fibers (raw, heat-treated, degummed, and silk textile) could be analyzed by MALDI-TOF MS. (b) Proteins in silk materials were fragmentized by acid. (c) One microliter of extract (1 mg of silk fibers/100 μL of 2.5% formic acid containing 50% acetonitrile) was placed on the MALDI target. Laser (lightning mark) were applied to the sample and analyzed in a linear positive mode in MALDI biotyping. Otherwise, silk fibers or part of silk textiles (approximately 1 mm\(^2\)) were placed on double-sided conductive carbon tape (black) and submerged in 1 μL of the matrix solution after drying at room temperature on solid analysis. (d) Mass spectrum was obtained in 10 min. Inset, (S) standard peptide mass fingerprint (PMF) data preserved in the database. (M) Unknown sample which were matched with a PMF in the database. All peaks were matched within m/z 5.0. (U) Unknown sample which were unmatched comparing with a PMF in the database. In this PMF, one peak was missing, and another peak was shifted.
FA and ACN for the analysis (Fig. 2). Prior to the MALDI biotyping analysis of silk proteins, several matrices were tested, although CAA was routinely used for the analysis (Appendix Fig. 1). Color pigments in Daizo cocoons were out of the mass range and were not observed in the PMFs. CAA was the best matrix for MALDI biotyping analysis of silk proteins.

There are two methods for the analysis of silk: liquid analysis and solid analysis. The MALDI biotyping analysis of silk fibers in liquid analysis is shown in Fig. 2. (a) Small pieces of silk fiber (1–2 mg) were excised from cocoons or textiles. (b) Silk fibers were extracted in 2.5% FA containing 50% ACN (1 mg/100 µL) for 5 min using a vortex at room temperature. TFA and acetic acid showed the same fragmentation of silk proteins under diluted conditions and at room temperature (data not shown). Centrifugation is not essential. (c) One microliter of the extract was placed on the MALDI target. After drying at room temperature, the same amount of matrix solution was applied to the sample. MALDI biotyping analysis is usually performed between m/z 2,000–20,000.15,16 However, because several peaks were observed in the low mass range of silk proteins, we decided to analyze from m/z 800. Polypeptides ionized by a laser assisted by a matrix were flighted in a vacuum tube and separated depending on their mass. Masses of analytes were calculated based on the time of flight from the target to the detector. Both N2 and YAG lasers were available for silk protein analysis. (d) Analysis of silk proteins using MALDI-TOF MS was completed in less than 10 min, including extraction. If a data set of PMFs on silk proteins was available, it would be possible to identify the races of cocoons by data matching using MALDI Biotyper or Speclust29 via the web (http://co.bmc.lu.se/speclust/cluster.pl) without DNA analysis. Nevertheless, DNA analysis of silk fibers was impossible if there were no pupae inside the cocoon.

When the solid analysis was applied to silk fibers using a double-sided conductive carbon tape, (a) a small silk piece (approximately 1 mm²) or a single string was fixed on the carbon tape (black) using forceps. (c) The matrix solution was directly applied to the silk fibers. In liquid analysis, fragmentation occurred during extraction using FA and ACN in a tube. Therefore, TFA in a matrix solution fragmented the silk proteins during solid target extraction. FA treatment can be omitted from the solid analysis. Proteins in the silk fibers were fragmented by acid during evaporation at room temperature (1–2 min). (d) The peak intensities derived from fibroin on target acid hydrolysis were smaller than those in the liquid reaction, and proteins in the silk fibers were fragmented by the acid in the matrix solution on the MALDI target during evaporation. There were two tips for the on-target solid analysis of silk fibers. First, a protein standard marker was placed on the carbon tape because the thickness of the carbon tape can cause mass shifts. Slippage of mass values is caused by the rough surface of the carbon tape. Second, when carbon tape was used for the analysis, pre-vacuuming was recommended to remove the air inside the carbon tape. Sometimes, a significant amount of time was required to reach the appropriate vacuum conditions for the analysis.

MALDI-TOF MS was applied to acid-treated silk proteins in the present study. There have been several approaches to treating acid-treated silk, especially fibroin, e.g., Raman spectroscopy26), NMR27), and X-ray analysis28). In these analyses, a larger protein fragment called the repetitive region or crystal region in fibroin was analyzed physiochemically. In contrast, we focused on smaller peptides that were removed in these experiments.

**Acid hydrolysis of other fibrous proteins**

Generally, most proteins are not simultaneously fragmented under diluted acidic conditions at room temperature, even in 70% FA. In the present study, fibrous proteins, spider webs, and sheep wool were analyzed (Fig. 3). In this experiment, a stronger acid than FA – TFA - was used to determine the effects of acid in the fragmentation. Spider webs and sheep wool mainly consist of spidroin30) and keratin31), respectively. Based on the mass data obtained by MALDI-TOF MS, no fragmentation occurred in spider spidroin or sheep keratin, even in stronger acids. A small piece of Daizo cocoon showed PMFs in 2.5% FA. Therefore, acid peptide fragmentation is considered to be unique in silk proteins.

**Observation of differences**

*Kinshu × Showa* is one of the main silkworm races ob-

![Fig. 3. Analysis of sheep wool, spider silk, and silkworm cocoon.](image-url)

The acid hydrolysis method using 2.5% trifluoroacetic acid (TFA) was applied to animal-oriented fibers. (A) Sheep wool treated with 2.5% TFA. (B) Spider silk treated with 2.5% TFA. (C) Silkworm (Daizo) cocoons treated with 2.5% formic acid.
tained by four-way crossbreeding in Japan. Parent races of
the cocoons Kinshu and Showa were compared with Kinshu × Showa. The materials used in the experiment were not
direct lines of the parent and offspring. Therefore, this was
not an experiment of heredity. As shown in Figs. 4–6, clear
differences were observed between the Kinshu and Showa
races. PMFs of females and males in both Kinshu and Showa
were not different from each other, and PMFs of Kinshu male and Showa female are not shown in the figure except
Fig. 4A. Roughly, the PMF of Kinshu × Showa becomes the
sum of Kinshu and Showa in outline in heat-treated cocoons
(Fig. 4A), sodium carbonate-treated cocoons (Fig. 5A),
Marseille soap-treated cocoons (Fig. 6A), and hydrogen
peroxide-treated cocoons after the Marseille soap treat-
ments (Fig. 6C). Both parents’ characteristics and peaks
were observed in the Kinshu × Showa offspring, suggesting
that PMFs obtained by acid treatment of silk could be used
for the discrimination between cocoons and silk races.

However, several peaks in Kinshu × Showa were either
present or absent when compared to the PMFs of the par-
ents. Two peaks (m/z 1,720 and m/z 1,788) in the heat-treated
cocoons disappeared in Fig. 4B. Although a peak at m/z
1,720 was observed for both Kinshu and Showa, there was

![Fig. 4. Analysis of heat-treated cocoons.](image)

(A) m/z 800–8,000. (B) m/z 1,660–1,860. (C) m/z 5,500–
7,600. Kf, Kinshu female. KS, Kinshu × Showa. Sm,
Showa male. Disappeared peak was designated as delta
(Δ).

![Fig. 5. Analysis of sodium carbonate-treated cocoons.](image)

(A) m/z 800–7,500. (B) m/z 1,020–1,220. (C) m/z 1,220–
1,420. Kf, Kinshu female. KS, Kinshu × Showa. Sm,
Showa male. Disappeared peak was designated as delta
(Δ).
1,264 observed for both lapped peaks around Kinshu served in Kinshu, respectively (Fig. 5B). Over-and Showa Kinshu and (Fig. 5A). Two peaks (m/z 1,723 and m/z 1,738 could not be found in Kinshu × Showa. However, a new peak was observed at m/z 1,811 (Fig. 6B). Hydrogen peroxide-treated cocoons after the Marseille soap treatments also showed that the sum of Kinshu and Showa was Kinshu × Showa (Fig. 6C).

The reasons for offspring race-specific peak appearances and disappearances are not known. We could not use direct individual lines of Kinshu, Showa, and Kinshu × Showa; therefore, it was not possible to conclude whether the observed peak differences were the phenomena of heredity such as atavism or heterosis. This will require further analysis.

Single thread analysis
Cocoons and textiles are bundles of silk fibers. Therefore, the above analyses were the sum of several different silk threads. A method must be established to analyze a single silk thread. We attempted to place a single thread in a tube; however, we found that it was very difficult to do so. A single silk thread was placed in a tube and 100 μL of extraction solution was added; however, the obtained extracts did not show any peaks because of the low concentration (data not shown). Therefore, the silk fiber was anchored on a double-sided conductive carbon tape, which was used with an electron microscope, and on it, we applied the matrix solution dissolved in TFA and ACN. Fig. 7 inset showed the picture of a single thread of silk (arrowed) on carbon tape (black) which were applied the matrix (white particles).

A single thread of heated, sodium carbonate-treated, and Marseille soap-treated silk was analyzed on the carbon tape (Fig. 7). Fragmentations, which occurred during liquid extraction, were also observed in the solid analysis of heated (Fig. 7A), sodium carbonate-treated (Fig. 7B), and Marseille soap-treated (Fig. 7C) threads. Strictly speaking, the peak mass values obtained in solid analysis on carbon tape sometimes did not show accurate measured values because the thickness and the rough surface of the carbon tape and sample affected the mass data. The distance from the source to the detector was varied. These slippages of mass values were previously discussed in the MALDI biotyping analysis of a single spider-mite to identify the species using carbon tape[17]. The internal standard contained in the sample was used to obtain accurate mass values when carbon tape was applied to the analysis.

Solid analysis of the carbon tape was performed in a small section of a single silk thread, which might have been less than 1 mm in length. If 1 mm of silk thread was available from cocoons or textiles, it would be possible to identify the origin of silkworm races by MALDI-TOF MS in 10 min

![Fig. 6. Analysis of Marseille soap-treated cocoons.](image)

(A) m/z 800–3,500. (B) m/z 1,680–1,880. (C) Marseille soap-treated cocoons were also treated with hydrogen peroxide. m/z 800–3,500. Kf, Kinshu female. KS, Kinshu × Showa. Sm, Showa male. Disappeared peak was designated as delta (Δ) and newly appeared peak was designated as plus (+).

no peak at the same position in Kinshu × Showa. In contrast, the peak at m/z 1,731, which was not observed in Kinshu, became prominent on Kinshu × Showa. Two peaks at m/z 6,077 on Kinshu and m/z 7,045 on Showa disappeared in the Kinshu × Showa offspring (Fig. 4C).

Most of the peaks observed in Kinshu × Showa in the sodium carbonated-treated cocoons were also the sum of Kinshu and Showa (Fig. 5A). Two peaks (m/z 1,036 and m/z 1,106) disappeared in Kinshu × Showa, which were observed in Kinshu and Showa, respectively (Fig. 5B). Overlapped peaks around m/z 1,264 observed for both Kinshu and Showa were not observed in Kinshu × Showa (Fig. 5C).

In Marseille soap-treated cocoons, which were thought to consist of fibroin, showed differences between parent and offspring races, although most of the peaks were the sum of the parent races in the offspring race (Fig. 6A). Peaks at m/z 1,723 and m/z 1,738 could not be found in Kinshu × Showa. However, a new peak was observed at m/z 1,811 (Fig. 6B). Hydrogen peroxide-treated cocoons after the Marseille soap treatments also showed that the sum of Kinshu and Showa was Kinshu × Showa (Fig. 6C).
extraction method, at least 1–2 mg of silk was required for the extraction. In contrast, it was possible to analyze a silk thread with a length of approximately 1 mm on a solid analysis.

There are distinct differences in the commercial prices of cocoons among their countries of production, such as Japan, China, and Thailand. Notably, the Koishimaru race was the most expensive compared to the other Japanese races. It could be possible to identify cocoon races by MALDI biotyping analysis within 10 min at minimal cost. This technique could be applied to the study of old historical silk textures for old cultural property restoration or the examination of criminal evidence to identify the races, which were impossible to analyze by DNA analysis.

**ABBREVIATIONS**

CAA, α-cyano-4-hydroxycinnamic acid; FA, formic acid; PMF, peptide mass fingerprint; TFA, trifluoroacetic acid

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**CONFLICT OF INTEREST**

No potential conflicts of interest were disclosed.

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APPENDIX

Appendix Fig. 1A.

Appendix Fig. 1B.

Appendix Fig. 1C.

Appendix Fig. 1D.