analytical chemistry

TECHNICAL NOTE

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Dating Silk By Capillary Electrophoresis Mass Spectrometry

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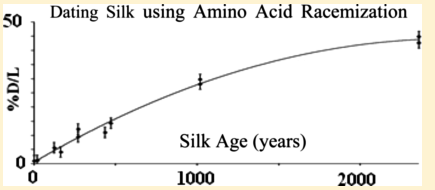
ABSTRACT: A new capillary electrophoresis mass spectrometry (CE-MS) technique is introduced for age estimation of silk textiles based on amino acid racemization rates. With an L to D conversion half-life of ~2500 years for silk (*B. mori*) aspartic acid, the technique is capable of dating silk textiles ranging in age from several decades to a few-thousand-years-old. Analysis required only ~100 μg or less of silk fiber. Except for a 2 h acid hydrolysis at 110 °C, no other sample preparation is required. The CE-MS analysis takes ~20 min, consumes only nanoliters of the amino acid mixture, and provides both amino acid composition profiles and D/L ratios for ~11 amino acids.



Silk fiber is one of the oldest proteinaceous fibers used to produce clothing and textiles. Silk production can be traced back millennia to China, where sericulture and even draw-loom weaving were well developed before it became a more widespread luxury commodity, spreading to other parts of Asia and the Middle East and later to Europe.[1] In addition to its use in the making of sumptuous garments and hangings, silk has been used in works of art such as tapestries, carpets, banners, Far Eastern paintings, etc.[2] Silks are naturally occurring polymers extruded when the larvae pupate and are composed of a filament core protein (fibroin) and a glue-like coating consisting of sericin proteins.[3] The raw silk fiber most commonly used in fabric manufacture is reeled from the cocoons of the silk worm *Bombyx mori* (*B. mori*) and is usually degummed by the removal of the sericin during processing. Because of the existence of a large number of silk artifacts in museums and private collections, the identification of the nature of the degradative state of such historic masterpieces is often critical to their preservation. In most cases, however, there is insufficient uniform material available from museums' silk samples for accurate stress−strain measurements or for ^{14}C dating since these techniques usually require a few milligrams of specimen, which for silk is a relatively large amount of fabric and is almost impossible to obtain from precious objects.[4−8] Moreover, ^{14}C dating of more recent samples (<400 years) is often hindered by the very poor time resolution of radiocarbon dating over this period.[9,10] Due to this, dating silk has largely been a speculative endeavor that has mostly relied on historical evidence, as well as the physical and chemical characteristics of the silk textiles.

For several decades, archeologists and forensic scientists have used amino acid racemization (AAR-D/L ratio) to determine the relative age of biological materials such as bone, shells, and teeth. AAR is a post-translational modification, which stems from the intrinsic instability of certain AA residues that leads to racemization. On earth, proteins are synthesized from L-amino acids (L-AAs); however, after incorporation into proteins, bound L-AAs start to racemize to their D-form under a reversible first order kinetic reaction until equilibrium is reached.[9−20] Each amino acid has a different intrinsic AAR rate, which is a function of the physical, chemical, and biological (protein sequence and its secondary, tertiary, and quaternary structures) states of the specimen and acts as an independent biological clock. For bound amino acids, the proteinaceous specimens are digested by hydrochloric acid to free amino acids, D- and L-amino acids are separated by a chiral GC or a chiral HPLC column, and the ratio of the D/L is measured to estimate the age of the specimen.[9−18] Among AAR rates, only the aspartic acid racemization rate is fast enough to be used for more recent specimens (<2500 years), and in this time period, the AAR rates for other amino acids are generally too slow to provide consistent results from sample to sample.[15]

In this article, we introduce a new sensitive and fast technique for AAR measurement using capillary electrophoresis mass spectrometry. As a proof of concept, this technique was applied to dating silk textiles fabricated from the cocoons of the silk worm *Bombyx mori* by measuring the D/L ratio of the aspartic acid for several well dated textiles ranging in age from the present to ~2500 years ago. Silk was chosen because AAR is a chemical reaction, and as such, it is affected by chemical and environmental factors such as temperature, UV radiation, humidity, chemical form, matrix, pH, treatment history, impurities, metal contents, etc.[10] All of these factors affect the rate of racemization, rendering the age identification more complex. However, unlike the AAR dating of proteinaceous specimens in natural environment, AAR measurement of silk is expected to be less complicated since these textiles are usually stored under extraordinary care in religious or royal treasuries and later in museums, where many of these factors are maintained fairly constant.[10−13,19,20] Moreover, proteinaceous fabrics made from silk have simple protein compositions with a high degree of purity, rendering the D/L measurement more reliable.

In the past two decades, we have introduced a variety of analytical techniques for the analysis of amino acids.[21−24] While to date, most AAR measurements have been achieved using GC or HPLC utilizing chiral columns,[6−20,25,26] (see ref 26 for

Received: July 6, 2011
Accepted: August 30, 2011

Table 1. Amino Acid Compositions of Fibroin

AA symbols	heavy chain #	heavy chain %	light chain #	light chain %	P25 chain #	P25 chain %	overall %
A (alanine)	1593	30.3	37	14.1	16	7.3	28.7
R (arginine)	14	0.3	10	3.8	13	5.9	0.6
N (asparagine)	20	0.4	18	6.9	13	5.9	0. 9
D (aspartic acid)	25	0.5	17	6.5	14	6.4	1.0
C (cysteine)	5	0.1	3	1.1	9	4.1	0.3
E (glutamic acid)	30	0.6	5	1.9	7	3.2	0.7
Q (glutamine)	10	0.2	15	5.7	8	3.6	0.6
G (glycine)	2415	45.9	22	8.4	9	4.1	42.6
H (histidine)	5	0.1	5	1.9	8	3.6	0.3
I (isoleucine)	13	0.2	21	8.0	14	6.4	0.8
L (leucine)	7	0.1	20	7.6	22	10.0	0.9
K (lysine)	12	0.2	5	1.9	7	3.2	0.4
M (methionine)	4	0.1	2	0.8	2	0.9	0.1
F (phenylalanine)	29	0.6	8	3.1	15	6.8	0.9
P (proline)	14	0.3	9	3.4	12	5.5	0.6
S (serine)	635	12.1	25	9.5	14	6.4	11.7
T (threonine)	47	0.9	8	3.1	12	5.5	1.2
W (tryptophan)	11	0.2	2	0.8	3	1.4	0.3
Y (tyrosine)	277	5.3	11	4.2	10	4.5	5.2
V (valine)	97	1.8	19	7.3	12	5.5	2.2

comparison between different chiral separation techniques) application of capillary electrophoresis mass spectrometry (CE-MS) to AAR is on the rise.[27−30] This is because CE-MS offers several unique advantages for the AAR analysis, including high sensitivity and high enantiomeric resolution. CE-MS is an ideal technique for attomole detection and separation of most D- and L-amino acids in one run in ∼20 min, and it is simply achieved by adding (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TCA, MW 440) to the CE background electrolyte as a complexation reagent.[23] Under this condition, D- and L-amino acids were separated and detected as protonated amino acid/18-C-6-TCA complexes. For example, aspartic acid was detected at m/z 574 (440 + 133 + 1).[23] The technique uses underivatized amino acids in conjunction with an underivatized capillary, significantly reducing both cost and analysis time. Another major advantage of the use of CE-MS for AAR rate measurement that makes it especially attractive in conservation is its low sample consumption. CE-MS analysis usually requires less than 100 μg of silk specimen, and it only consumes a few nanoliters or less of sample volume for each run.

EXPERIMENTAL SECTION

Methods and Instrumentation. A porous tip was used for interfacing CE (ProteomeLab PA 800, Beckman-Coulter; Fullerton, CA) to MS (LCQ Duo, Finnigan; San Jose, CA).[31] Underivatized 150 μm o.d. fused-silica capillaries with nominal inner diameters of 15 and 20 μm and lengths varying from 95 to 115 cm were utilized. The MS was scanned in the m/z range 525−700, for monitoring complexed AAs, under positive ionization mode. By selecting this narrow scan range, the scan times were minimized and the number of data points acquired across the narrow electrophoretic peaks were maximized, which provided more accurate peak definition/quantitation. The temperature of the heated capillary used in these experiments was set to 200 °C in order to minimize AA/18-C-6-TCA complex dissociation. The CE was operated in the forward polarity mode (25−30 kV were applied to the CE inlet electrode) with 3−5 psi of forward pressure, and 1.1−1.25 kV was applied to the CE outlet/ESI electrode. A 30−60 mM solution of 18-C-6-TCA in water was used as the background electrolyte and complexation reagent in order to increase the sensitivity of the amino acid detection. All samples were injected using the pressure injection mode. The injection volume was determined experimentally by measuring the time it took to fill the capillaries at a specific pressure and using this information to calculate the injection volume based on the injection time and pressure. Under the experimental conditions used here, D and L isomers for 10 or 11 amino acids were separated in one run. However, only Asp D/L values are reported here because the other amino acid D/L values are generally too low. D/L values were calculated from the peak−area ratios obtained from at least three electropherograms of each sample.

Sample Preparation. All chemicals, other than HPLC grade water and 49% HF (Fischer Scientific; Pittsburgh, PA), were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Solutions of (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid in the range of 15−60 mM were used as the BGE during the AA analyses. Samples were prepared by digesting 0.05 to 1 mg of silk in 6 N HCl at 110 °C for 2, 4, 8, or 24 h. The digested samples were then dried to complete dryness and brought to a final concentration of ∼5 μg/μL using 0.1 N HCl. For example, when 0.05 mg of sample was used for HCl digestion, the dried sample was brought up to 10 μL. Therefore, since the minimum volume of sample needed for injection using the Beckman CE was 5 μL, the minimum amount of sample needed for this type of analysis is ∼25 μg. The UV irradiation of the silk samples was performed at Oka studio (Oka Bokkodo Co., Ltd., Kyoto, Japan). The UV exposure time was from ∼2 weeks to ∼11 weeks, which is consistent with other studies in which the effects of UV radiation on silk tensile strength have been investigated.[32,33]

dx.doi.org/10.1021/ac201746u |Anal. Chem. XXXX, XXX, 000–000

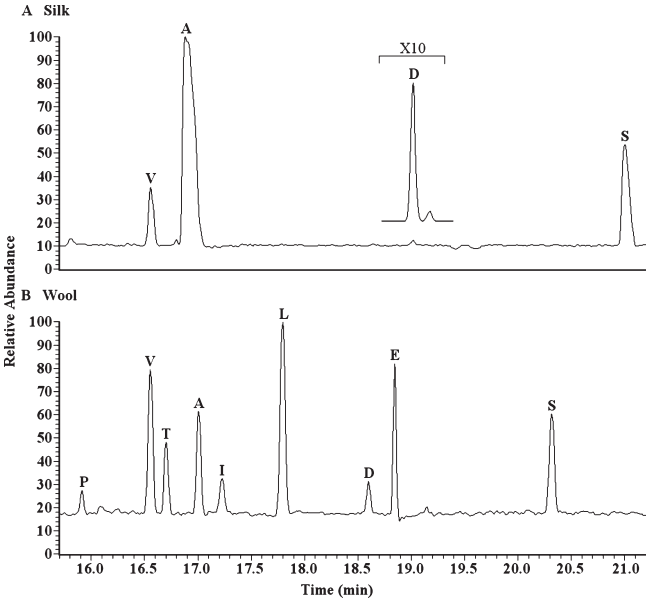


Figure 1. CE-MS electropherograms of silk (top) vs wool (bottom) acid hydrolysis. Inset in the top panel shows the 10× magnification of the ion electropherogram of aspartic acid.

RESULTS AND DISCUSSION

CE-MS Analyses of Silk and Wool. Silk Fibroin consists mainly of three proteins, a heavy chain with 5263 amino acid residues, a light chain with 262 residues, and the P25 glycoprotein, with 220 residues.[3] The amino acid compositions of the individual proteins, as well as the combined proteins, are listed in Table 1. As shown in Table 1, only 4 chiral amino acids, alanine (A, 28.7%), serine (S, 11.7%), tyrosine (Y, 5.2%), and valine (V, 2.2%) are more than 2%. The amino acid composition of silk in Table 1 is consistent with the peak intensity of amino acids in CE-MS electropherogram obtained from HCl digestion of degummed silk (Figure 1), with the exception of tyrosine, since its ionization efficiency is considerably less than these three amino acids.[22,23] For comparison, the electropherogram of the HCl digest of wool in the same mass range is also provided (Figure 1, bottom panel). As shown, silk and wool can be easily distinguished by their CE-MS electropherograms. The inset of Figure 1, top panel, compares the intensity of aspartic acid with the other major amino acids of silk.

Effect of Digestion Time on Racemization. To investigate the effect of digestion time on aspartic acid racemization, modern silk specimens were digested at 110 °C for 2, 4, 8, and 24 h (Figure 2). As shown, increasing HCl digestion time almost monotonically increases D/L ratio until at 24 h where the curve starts to plateau. At 2 h digestion time, conversion of L enantiomer to D due to heating is only ∼1%, while at 24 h, this contribution is almost 4%. Proteomics analysis of silk samples after 2, 4, 8, and 24 h HCl digestion indicated that, even at 2 h digestion time, no detectable peptide peaks were observed, indicating most proteins were digested to amino acids. The results clearly indicate that digestion of silk samples for 2 h is adequate for efficient D/L analysis of silk samples, while minimizing L to D conversion due to sample preparation.

Racemization of Silk Aspartic Acid and Asparagine by Time. To obtain an AAR rate for silk (*B. mori*) specimens, several well dated silk samples from several museums were analyzed by

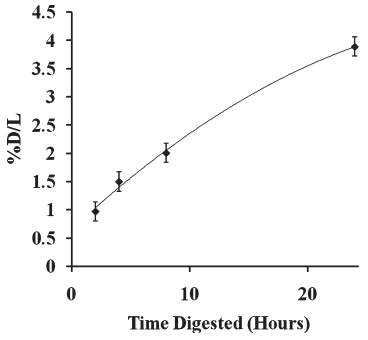


Figure 2. Average D/L ratios for fresh silk samples, HCl digested for 2, 4, 8, and 24 h.

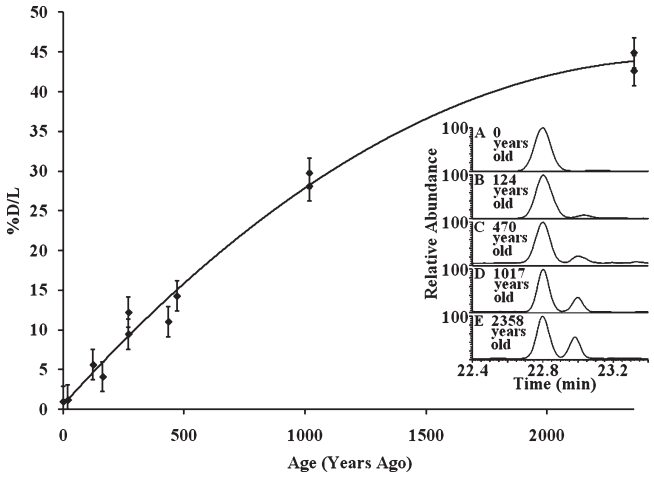


Figure 3. Average D/L ratios for silk samples. Silk samples include fresh silk (2010), untreated silk from the Oka Studio (∼1990), a silk flag from 1883−8, a silk flag from the Mexican War (1845−6), pink (top) and blue (bottom) silk from a man's suit coat (1740), silk from the 1540s, yellow (top) and blue (bottom) silk from Egypt (993), and brown (top) and red (bottom) silk from China (475−221 BC). The inset shows the electropherogram of each sample's aspartic acid D and L peaks (547 *m*/*z*).

CE-MS. The silk samples included fresh silk (2010); untreated silk from the Oka Studio, Freer-Sackler Museum, Smithsonian Institution (SI), Washington, DC (∼1990); Sheridan flag, National Museum of American History, SI (1883−8); Mexican War flag, National Museum of American History, SI (1845−6); a man's suit coat, pink (top) and blue (bottom), Museum of the City of New York (1740); a silk textile from Istanbul, Textile Museum, Washington, DC (1551−1599); a silk tapestry from the Fontainebleau Series, Kunsthistorisches Museum, Vienna, Austria (1540s); Tiraz silk yarns from Egypt, yellow (top) and blue (bottom), Textile Museum, Washington, DC (993); and silks from the Warring States Period, China, brown (top) and red (bottom), Metropolitan Museum of Art, New York City (475−221 B.C.). The results are shown in Figure 3, with an inset showing the relative increase of the D vs L enantiomer of aspartic acid (*m*/*z* 574). From Figure 3, it is clear that the data points fit very well with a theoretical curve obtained from reversible first order chemical kinetics (solid curve). Investigation of the electropherograms of the silk sample hydrolysates revealed no peak for asparagine in any of the samples, even when the molar ratio of aspartic acid to asparagine was ∼1.1. This is

dx.doi.org/10.1021/ac201746u |*Anal. Chem.* XXXX, XXX, 000–000

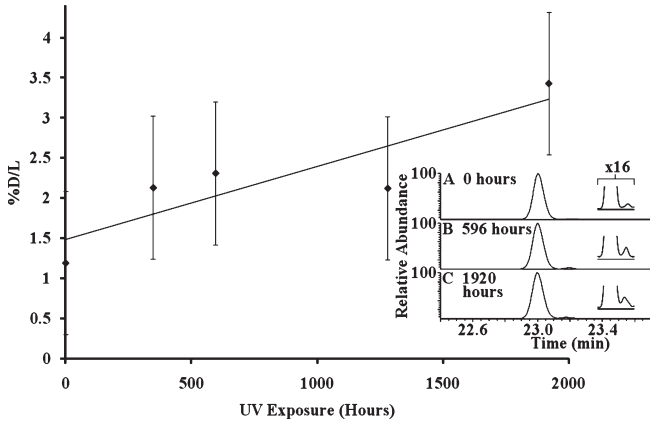


Figure 4. Average D/L ratios for silk samples treated with UV radiation for 0, 348, 596, 1280, and 1920 h. The inset shows the electropherogram for the samples treated with UV radiation for 0, 596, and 1920 h, focusing on the aspartic acid D and L peaks (547 m/z), along with 16× magnification of the D peak.

consistent with previous reports, in which the lack of an asparagine peak was attributed to the conversion of asparagine to aspartic acid during the hydrolysis.[34] In addition, since the data points of Figure 3 fit well with a simple reversible first order reaction, we predict that during the first ∼2500 years, the rapid initial rate of racemization of asparagine is the main contributor to the D/L ratio observed.[14]

Effect of UV Radiation on D/L Ratio. To investigate the effect of UV exposure on L to D conversion of silk proteins, silk samples which had been exposed to UV light for various lengths of time (ranging from 0 to 1920 h) were analyzed for their D/L ratio (Figure 4). It was found that exposure to UV light increased the D/L ratio by about 2% (∼100 years) over an exposure time of 1920 h.

CONCLUSION

D/L analysis of silk samples from museum collections using CE-MS is a viable technique for the dating of historical silk-based textiles. The amino acid racemization measurement of silk is typically less complicated than the measurement of proteinaceous specimens removed from the natural environment, since silk textiles are usually stored under extraordinary care in religious or royal treasuries and in museums, where many of the environmental factors that affect racemization rates are maintained fairly constant. The calibration curve for silk specimens spanned in time from the present to ∼2500 years ago and followed the expected reversible first order reaction.

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ACKNOWLEDGMENT

We wish to thank the Smithsonian's Museum Conservation Institute Director and Deputy Director, Drs. Robert J. Koestler and Paula T. DePriest, for their support of this project. We also wish to thank several curators and conservators who have provided expertise as well as silk samples: Dr. Sumru Kordy and Ms. Esther Methe of the Textile Museum; Dr. James C. Y. Watt and Ms. Joyce Denney of the Metropolitan Museum of Art; Dr. Blythe McCarthy of the Smithsonian's Freer and Sackler Galleries; Ms. Laura Mina of the Smithsonian Museum Conservation Institute; Ms. Phyllis Magidson of the Museum of the City of New York; Ms. Jennifer Jones and Ms. Suzanne Thomassen-Krauss of the National Museum of American History; and Dr. Katja Schmitz-von Ledebur from the Kunsthistoriches Museum, Vienna, Austria. We also wish to thank intern Casey Kohnhorst for her assistance in proteomics analysis of silk acid hydrolysates.

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