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RESEARCH PAPER

Development of an enzyme-linked-immunosorbent-assay technique for accurate identification of poorly preserved silks unearthed in ancient tombs

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Abstract We report the preparation of a specific fibroin antibody and its use for the identification of unearthed ancient silk relics. Based on the 12-amino-acid repeat sequence "GAGA GSGAGAGS", which is found in fibroin of the silkworm *Bombyx mori*, a specific antibody against fibroin was prepared in rabbits through peptide synthesis and carrier-protein coupling. This antibody was highly specific for fibroin found in silk. Using this antibody we have successfully identified four silk samples from different time periods. Our results reveal, for the first time, a method capable of detecting silk from a few milligrams of archaeological fabric that has been buried for thousands of years, confirming that the ancient practice of wearing silk products while praying for rebirth dated back to at least 400 BCE. This method also complements current approaches in silk detection, especially for the characterization of poorly preserved silks, promoting the investigation of silk origins and of ancient clothing cultures.

Keywords Fibroin antibody · Archaeological fabric · ELISA · Identification · Silk

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Introduction

Silk is one of the main textiles of ancient times and has an important function in world civilization. Many ancient silk fabrics are now regarded as a heritage representing ancient clothing cultures and aesthetic features. Accurate assessment of these silks is necessary to obtain deeper insight into ancient civilizations and to design proper restoration and conservation methods [1]. A variety of approaches for the characterization of silks have been developed using microscopy (low-power binocular microscopy, high-power polarizing microscopy, and scanning electron microscopy) [2] and spectroscopy (near-infrared spectroscopy and Fourier-transform infrared spectroscopy) [3–5]. However, using these methods archaeological samples are characterized only by a simple comparison with modern specimen silks, and the composition cannot be ascertained without further analyses. Moreover, these methods are limited to characterizing well-preserved silks, and the difficulty of identifying ancient silks which have suffered from hydrolysis, corrosion, and oxidation has blocked further research on silk cultures. Amino-acid-ratio comparison could preliminarily characterize these poorly preserved silks, but without any precise protein identification [6, 7]. The earliest evidence to date for silk in China comes from an isolated find possibly as early as 2570 BCE from the Liangzhou Neolithic site of Qianshanyang. But silk fibers roughly contemporaneous with the earliest Chinese evidence for silk were also found in two Indus sites [2], which caused confusion regarding silk origins. Accurate identification of rotting silks of older age might reveal the origins of silks which are still a subject of debate, but no selective and sensitive method has been established.

In this study, we sought to develop a suitable procedure for the identification of aged or decayed silks unearthed from ancient tombs. Because of its advantages over traditional

analytical approaches, we emphasized the suitability of enzyme-linked immunosorbent assay (ELISA) for characterizing ancient silks. The idea of using this method in conservation science is not new. Since approximately 1990, several studies have used this technique to identify the species origin of archaeologically recovered bone, and of traces of blood either found on artifacts or even as a binder in paint [8, 9]. In recent years, this technique has been mainly used in the study of proteinaceous binders (including chicken-egg yolk, albumen, and animal glues) in ancient paintings [10, 11]. Some researchers have recently used the ELISA method as a supplemental technique to investigate the application history and scientific nature of blood-based materials in traditional Chinese mortar [12]. The binding materials in ancient Chinese textiles have also been investigated by using the ELISA method, and the result suggests that the use of ovalbumin and collagen I in textiles can enhance bond strength and cohesion between fibers [13]. Unlike microscopy, which can only be used to make speculative observations of the textiles, ELISA provides specific identification of denatured silk samples. Advances in sensitivity also led to the development of this method for studying silk relics.

Silk fibers are composed of a fibrous protein (fibroin) core and a surrounding glue protein (sericin) [14, 15]. Sericin is removed in the weaving process, leaving fibroin as the major constituent of silks [16]. Silk fibroin is not an enzyme, but a structural material like the keratin in hair or nails. As a large protein, fibroin consists of a heavy chain, light chain, and P25 protein [17]. The heavy chain is made of a low-complexity region bordered by short N and C-terminal segments. The bulk of the low-complexity region (4754 residues) is made of repeats of a GX dipeptide motif, where X is Ala in 65 %, Ser in 23 %, and Tyr in 10 % of the repeats compared with all residues in fibroin. The GX are distributed in 12 domains, designated GX domains, of 39 to 612 amino acids separated by 11 nearly identical copies of a boundary sequence. Most of the GX dipeptide units are present as part of the two hexapeptides GAGAGS (432 copies) and GAGAGY (120 copies), which together account for 72 % of the low-complexity region. X-ray diffraction patterns of silk fibers indicate that the bulk of the protein forms regular β-sheets similar to those of poly(Ala-Gly). Each GX domain could constitute a single β-sheet, and the boundary sequences break the β-strand, enabling the polypeptide chain to change direction [3, 18]. These extensive β-sheet crystalline regions in GX domains impart the characteristic strength of the fibers and a good chemical resistivity [5, 19].

Briefly, our procedure included two main steps. The first part of this work describes the method and its development, including the preparation of the fibroin-protein antibody and determination of its sensitivity and selectivity, and the verification of the suitability of this analytical method for the identification of small silk samples. In the second part, we used the antibody to identify samples unearthed from ancient tombs of different time periods.

Materials and methods

Choice and preparation of assay

The amino-acid sequence of the fibroin heavy chain of *Bombyx mori* (Electronic Supplementary Material (ESM) Fig. S1) was retrieved from the NCBI public databases (*http://www.ncbi.nlm.nih.gov/pubmed/*). Some repetitive and conserved amino-acid sequences (Table 1) were generated using the software DNAman (Lynnon Biosoft, San Ramon, USA). The sequence "GAGAGSGAGAGS" was selected as the antigen for silk-antibody preparation because it had the highest frequency of occurrence in fibroin. The synthesis of the peptide "CGAGAGSGAGAGS" was performed using a peptide synthesizer (CS Bio, California, USA). The immunogen was prepared by coupling the synthetic peptide with keyhole-limpet hemocyanin (Sangon, Shanghai, China).

Immunization was performed as follows. The immunogen was mixed with complete Freund s adjuvant (volume ratio 1:1, Sigma, California, USA) and antibiotics (0.1 mg mL^{-1} streptomycin, 100 U mL^{-1} penicillin, Sigma, California, USA) using saline as a diluent. For the initial immunization, New Zealand white rabbits were subcutaneously injected multiple times into their thighs with 100 μL of the mixture above. At 2, 4, and 6 weeks after the initial immunization, boosters were administered. Starting from the third immunization, the rabbit serum titer was measured by indirect ELISA 10 days after each immunization. The rabbits were sacrificed for blood collection 6 weeks after the initial immunization. The blood coagulates were placed at 37 °C for 30 min and then at 4 °C overnight for complete clot contraction and precipitation. Antiserum was spun for 10 min at 3000*g* at 4 °C.

The antiserum was purified by affinity chromatography. Chromatographic media was coupled with the synthetic peptide and blocked with cysteine. The chromatography column (Sangon, Shanghai, China) was first equilibrated with 20 mL

Table 1 Repeating amino-acid fragments and their number of repeats in the heavy chain of silkworm fibroin

Fragment	Numbers of repeats in fibroin
GAGAGSGAGAGS	170
GAGAGSGAGAGYGA	48
GYGAGAGAGYGA	47
GAGYGAGAGAGY	31
GAGVGAGYGVGY	7
GAGAGAGAGAGA	5

50 mmol L^{-1} PBS (pH 7.4) at a flow of 60 mL h^{-1}. Antiserum (10 mL) was diluted with 10 mL 50 mmol L^{-1} PBS (pH 7.4) and was sampled at a flow of 60 mL h^{-1}. The silk antibody was eluted with 0.1 mol L^{-1} glycine–HCL (pH 3.0).

Direct ELISA was performed to determine the optimum dilution for horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (secondary antibody, Abcam, Cambridge, UK) solution. The silk antibody (primary antibody) for coating was diluted in coating buffer (0.015 mol L^{-1} Na_2CO_3, 0.035 mol L^{-1} $NaHCO_3$) to final mass concentrations of 8, 4, 2, 1, and 0.1 μg mL^{-1}. The secondary antibody for incubating was diluted in blocking solution (PBS, 1 % BSA (Sigma, California, USA)) to 1:800, 1:1600, 1:3200, 1:5000, and 1:10,000. Indirect ELISA was performed to determine the optimum dilution for the primary-antibody solution. Pure fibroin for coating was diluted in coating buffer to final mass concentrations of 8, 4, 2, 1, 0.5, 0.25, and 0.125 μg mL^{-1}. The primary antibody for incubating was diluted in blocking solution to 1:500, 1:1000, 1:2000, 1:5000, and 1:10,000. The secondary antibody for incubating was diluted in blocking solution to 1:5000 (see "Testing of model and artificially aged samples" section about ELISA procedures).

Samples and optimization of extraction

Artificial-aging fiber samples included silk (approximately 2 mg), linen (approximately 3 mg), cotton (approximately 3 mg), and wool (approximately 4 mg), and were provided by the Materials and Textiles Institute of Zhejiang Sci-Tech University (Hangzhou, China). They were separately subjected to dry thermal ageing (125 °C) for 20 days, high-temperature and humidity ageing in a closed chamber (100 °C, 100 % RH) for 20 days, and sunlight-equivalent ageing for 20 days (for more details about aging conditions, see Ref. [3]).

Four different archaeological samples (approximately 2 mg each) were obtained from the Chinese Textiles Identification and Conservation Center of China Silk Museum. They dated back to 400 BCE, 0 CE, 400 CE, and 1000 CE, with each separated by a time interval of approximately 400 years from the next. The color diversity *s* of the samples might be caused by the use of different dyes in the silk-dyeing process, and by the color change of aging silks.

The artificial-aging silk, linen, cotton, and wool and the four archaeological samples were each crushed in liquid nitrogen using a pestle and mortar. Extracting solution (20 μL) (the molar ratio of calcium chloride–water–ethanol was 1:8:2) was added to 2 mg of each crushed sample. The mixture was incubated in a 56 °C water bath for 1 h, followed by centrifugation for 5 min at 12,000*g*. Supernatant (10 μL) was transferred into a clean centrifuge tube and 1 mL coating buffer was added. The mixture was allowed to stand for 10 min, followed by centrifugation for 5 min at 12,000*g* to obtain the supernatant for coating.

Testing of model and artificially aged samples

The artificially aged samples were separately extracted. Artificially-aged-sample extracts (100 μL) were added to coated wells (Greiner Bio-One GmbH, Frickenhausen, Germany) separately and incubated overnight at 4 °C. All wells were washed, and unless otherwise mentioned all washings throughout the procedure were performed three times (2 min each wash) using PBST (PBS, 150 mmol L^{-1} NaCl, 5.2 mmol L^{-1} Na_2HPO_4, 1.7 mmol L^{-1} KH_2PO_4, pH 7.4). Approximately 100 μL blocking solution was then added and incubated for 1 h at 37 °C, followed by washing. The primary antibody was diluted 1:5000 with blocking solution and 100 μL was added and incubated for 1 h at 37 °C, followed by washing. The secondary antibody was diluted 1:5000 with blocking solution and 100 μL was then added and incubated for 1 h at 37 °C, followed by washing. Finally, 100 μL of the colorless substrate solution (3,3 ,5,5 -tetramethylbenzidine) was added and the color was allowed to develop in the dark for 10 min. Stopping solution (50 μL) (2 mol L^{-1} H_2SO_4) was added to stop the reaction, and the amount of enzymatic activity was assessed by optical-density (OD) readings at 450 nm using a microplate reader (BioTek, Vermont, USA). Each analysis was repeated at least three times for all samples, and the OD (450 nm) readings were reported with the corresponding standard deviations (SD).

Testing of naturally aged samples and testing of archeological samples

The archeological aged samples were extracted separately. Archeological-sample extracts (100 μL) were coated to separate wells and incubated with the primary antibody and secondary antibody at a dilution of 1:5000, one after the other (see "Testing of model and artificially aged samples" for ELISA procedures).

Results and discussion

Preparation of silk antibody

Antibody preparation is crucial for the successful use of ELISA in archaeology. Self-prepared antibodies should meet the specific needs of this field, providing highly sensitive recognition of aged or denatured protein in inorganic matrices for the purpose of distinguishing their biological sources (possibly within the same biological family). As a primary component of silk in relics, fibroin might have been denatured and would otherwise be unidentifiable using a general fibroin

antibody as a result of possible epitope loss [20, 21]. For this reason, we decided to use repetitive and conserved amino-acid sequences of the fibroin of silkworm, *Bombyx mori*, to investigate its primary structure, which has been the traditional focus of most research [22, 23]. Sequence analysis of fibroin s primary structure was performed, and some of the sequences with their number of repeats are listed in Table 1. On the basis of the finding that the sequence "GAGAGSGAGAGS" is the most repeated sequence in the primary structure of *B.mori* fibroin, the utility of this peptide as a target antigen for silk-antibody preparation was established. Starting from peptide synthesis, we attempted the immunization of rabbits to obtain a selective and sensitive silk antibody.

Assay performance

After immunization of rabbits and purification of antiserum, polyclonal antibody against fibroin was obtained. On the basis that fabrics unearthed from archaeological sites were once used as funeral objects and represented the burial clothing culture [24], all fibers that might have been used to manufacture clothes were investigated to confirm the specificity of the new antibody. For this reason, four artificially aged fiber samples (silk, cotton, hemp, and wool, Fig. 1) were used for antibody-specific validation.

To determine the optimum secondary-antibody dilution, serial dilutions of primary antibody were coated to the wells followed by incubation with the secondary antibody in gradient dilutions. For each secondary-antibody dilution, the obtained OD values were plotted against primary-antibody concentrations (Fig. 2). No difference in OD response was observed for secondary-antibody dilutions of up to 1:5000, but a significant reduction arose at the dilution of 1:10,000. This indicated that much primary antibody was not bound with secondary antibody when the secondary antibody was at a dilution of 1:10,000. Therefore, the threshold value for secondary-antibody dilution was 1:5000; below this concentration, the two antibodies could not bind well with each other and the sensitivity of immunoassays was affected. A parallel experiment was performed to determine the optimum primary-antibody dilution. For each primary-antibody dilution, the obtained OD values were plotted against fibroin concentrations (Fig. 3). The dilution of 1:5000 was also the threshold dilution for primary antibody, because a significant reduction in OD response was again observed when the primary antibody was at a dilution of 1:10,000, revealing that the coated fibroin could just be fully bound with primary antibody which was at a dilution of 1:5000. In sample testing, the optimum primary and secondary-antibody dilutions were both set at 1:5000; at these antibody concentrations, sensitivity of immunoassays was suitable for silk detection.

To achieve quantitative determination of the relationship between OD response and fibroin concentration, the OD value for standard fibroin solution was plotted against the denary logarithm of fibroin concentration (Fig. 4). The best linear fit was obtained and the equation was:

$$\mathrm{OD}_{450\ \mathrm{nm}} = 1.82 \lg C - 2.42 \left(r^2 = 0.961\right) \quad (1)$$

where C is the concentration of fibroin in ng mL^{-1}. The detection limit of the assay, estimated as the concentration of the fibroin giving an OD value at 450 nm lower than 0.34 (the average of the OD value at 450 nm of the phosphate buffered saline (PBS) plus three times its standard deviation), was 32.8 ng mL^{-1} [25].

The first difficulty for sample testing was to find the extraction solution adapted to protein extraction from the samples, considering that silks were mixed with dyes and other inorganic compounds. Some solutions, for example lithium bromide aqueous, usually need to be removed by dialysis after

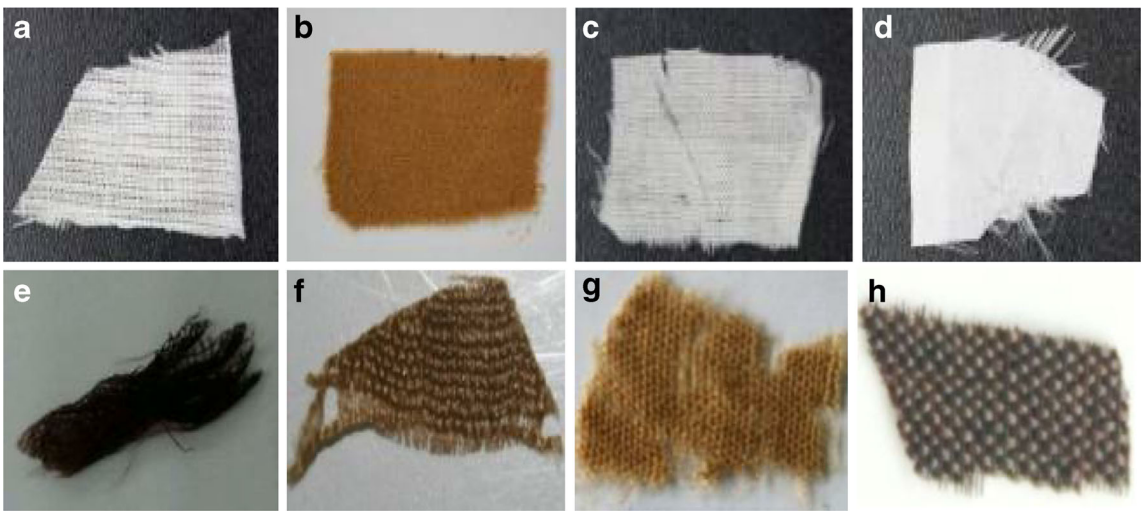

Fig. 1 Samples chosen for analysis. (**a**–**d**) Artificial-aging fibers of (**a**) linen, (**b**) wool, (**c**) cotton, and (**d**) silk. (**e**–**h**) Ancient silks, which date back to (**e**) 400 BCE, (**f**) 0 CE, (**g**) 400 CE, and (**h**)1000 CE

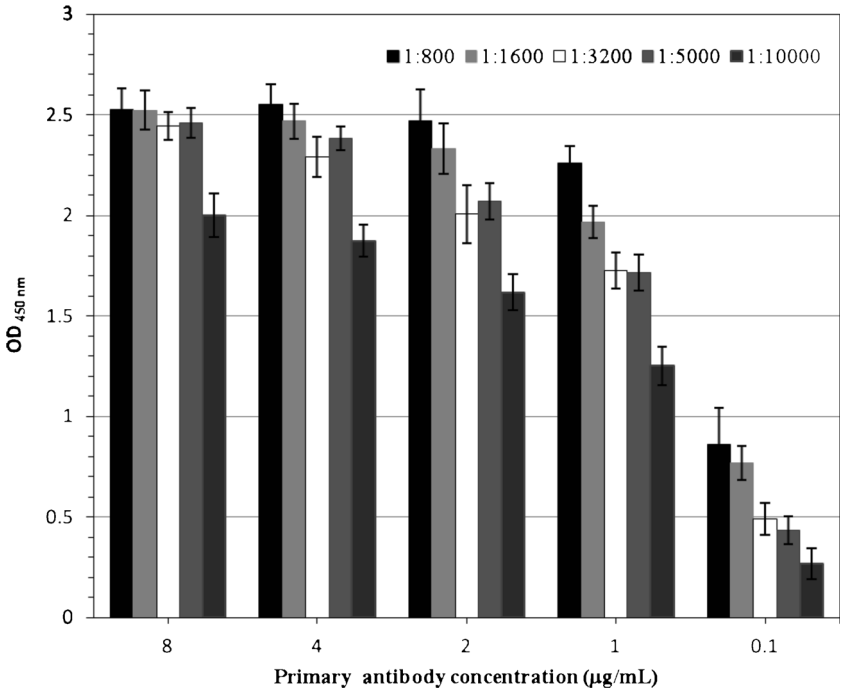

Fig. 2 ELISA curves obtained for primary antibody at different secondary-antibody dilutions. Standard solutions of the primary antibody were prepared by progressive dilution of 8 mg mL^{-1} primary antibody with carbonate–bicarbonate buffer at pH 9.6. The secondary antibody was diluted in blocking buffer to 1:800, 1:1600, 1:3200, 1:5000, and 1:10,000

fibroin extraction, which may cause the loss of fibroin and is incompatible with the small amounts of archaeological samples available. A solution with 1 mol L^{-1} calcium chloride, 8 mol L^{-1} water, and 2 mol L^{-1} ethanol has already been tested on modern samples, and proved to be efficient for extracting fibroin from silks. Furthermore, calcium ions in this extraction solution could be precipitated from the extract simply by adding excessive amounts of coating buffer consisting of sodium carbonate and sodium bicarbonate. The sediment formed included calcium carbonate and calcium bicarbonate, which could be separated from the remaining fibroin solution by centrifugation. This purification method had two advantages: one was that the loss of fibroin could be reduced simply by substituting dialysis with centrifugation; the other was that negative ingredients, for example ethanol, in the extract could be greatly diluted by adding excessive amounts of coating buffer.

The color variance of the wells coated with artificially aged fiber samples was obtained (ESM Fig.S2a). By comparing the color variance of sample wells with the control well, a preliminary judgment could be made of whether the sample contained fibroin, although this needed to be verified by measuring their OD values. Color variance was only observed for the well coated with artificial-aging silk, indicating that the specific binding between the antibody and fibroin existed in this well. Comparing the well OD (450 nm) (Fig. 5) values revealed that the responses of the immunoassay to the four artificially aged fiber samples were very different. For silk,

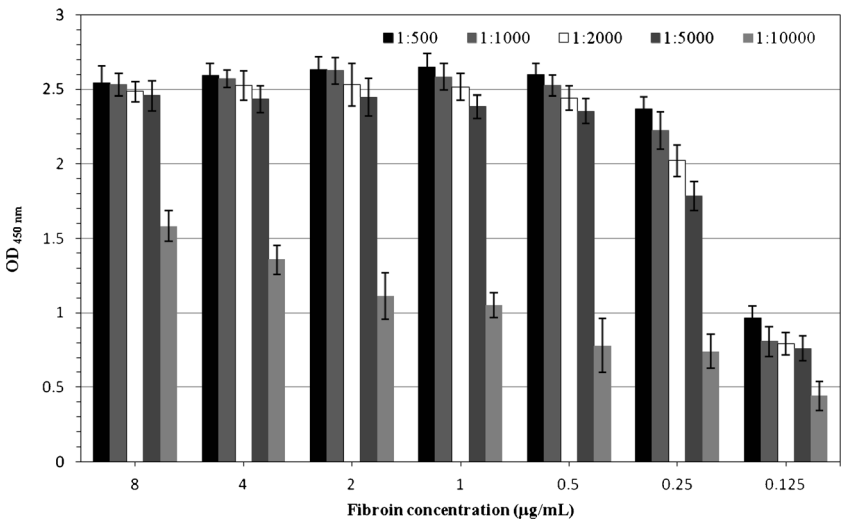

Fig. 3 ELISA curves obtained for fibroin at different primary-antibody dilutions and a secondary-antibody dilution of 1:5000. Standard solutions of the reference proteins were prepared by progressive dilution of 8 μg dried fibroin in 1 mL carbonate–bicarbonate buffer at pH 9.6. The primary antibody was diluted in blocking buffer to 1:500, 1:1000, 1:2000, 1:5000, and 1:10,000

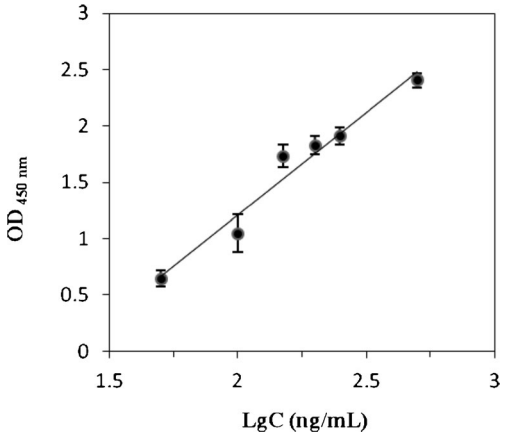

Fig. 4 ELISA calibration curves obtained for fibroin at the optimised primary and secondary-antibody dilutions, each of 1:5000, expressed as the relationship between OD at 450 nm and the denary logarithm of fibroin concentrations (*C*). Standard solutions of the reference proteins were prepared by progressive dilution of 1 mg dried fibroin in 1 mL carbonate–bicarbonate buffer at pH 9.6

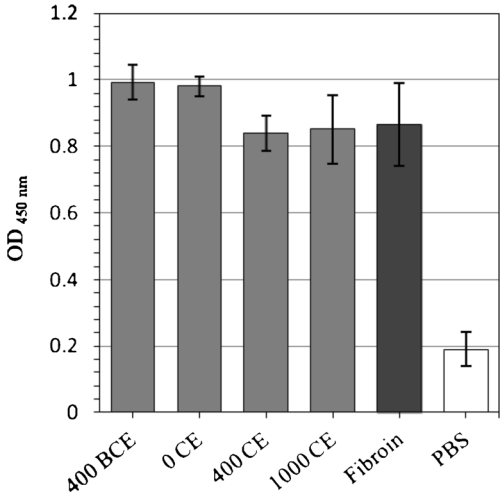

Fig. 6 ELISA optical densities obtained for ancient silk samples treated with polyclonal antibody to fibroin. Results for silks of different ages and the corresponding SD are reported for the tested fibroin, which is shown on the *x*-axis

the OD (450 nm) was 0.8. This was higher than the OD of 0.2 obtained for the other three fibers. The unique positive response of silk clearly indicated the excellent specificity of the new antibody. It has been established that modifications of fibroin during aging can be quite complex, because many factors including light, oxygen, humidity, heat, and other organic components affect this process. Prior study also revealed that cleavage of the peptide chain arises as silk suffers from oxidation conditions, resulting in a decrease in fibroin molecular weight and fibroin crystallite disorientation [3]. However, the results in Fig. 5 reveal that the response of aged silk was, on average, comparable with that of fibroin extracted from fresh silk. This indicated that the sequence "GAGAGSGAGAGS" in GX domains remained intact and β-sheet crystalline regions may not be seriously damaged even if the silk suffers from severe aging conditions.

Overall, these results reveal that the proposed methods of preparation and analysis were successful in generating a silk antibody that is both sensitive and selective. What is more, damage caused by light, oxygen, humidity, and heat had no effect on silk identification using this method.

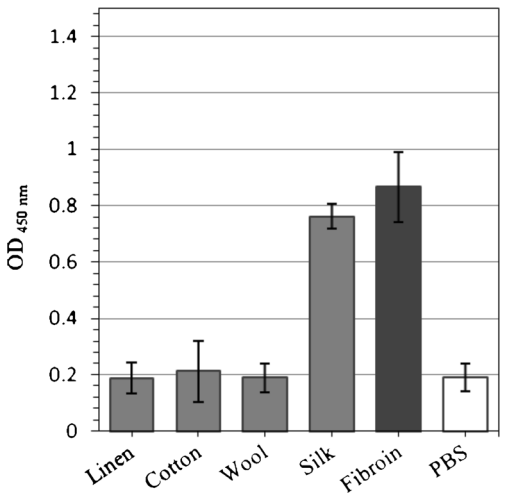

Fig. 5 ELISA optical densities obtained for artificial-aging fibers treated with polyclonal antibody to fibroin. Results for different fibers and the corresponding SD are reported for the tested fibroin, which is shown on the *x*-axis

Application of the new method to archaeological samples

Once tested and optimized, the developed procedures were used to identify a few milligrams of real silk samples unearthed from Chinese ancient tombs (Fig. 1). In China, inspired by the phenomenon of silkworm pupation, it was believed that people buried in silk products would be reborn as silkworms [26]. As time passed, buried silk products were damaged by years of decay. These spoiled silk artifacts have recently been unearthed from different ancient Chinese burial sites, some of which may date back to the dynasties of the Warring States Period (475 BCE to 221 BCE), Western Han (202 BCE to 9 CE), Eastern Jin (317 to 420 CE), and the Northern Song (960 to 1127 CE). All denatured samples were recognized as part of the traditional burial attire. They were identified by extracting protein from trace amounts of sample, which was then tested using ELISA. Traces of fibroin were detected in four tested samples, for which dramatic color variance of the corresponding wells was observed (ESM Fig. S2b). Tests were repeated and the results are reported in Fig. 6. Comparison of the average OD value of the PBS control (0.2) with that of the four samples (nearly 1.0) was sufficient to verify the presence of fibroin in these samples. No significant difference was found between the OD values of the four real samples, revealing that the responses of the antibody

to silks from different eras were similar. This indicated that decay in tombs was too slow to greatly change the silks; at the least, the crystalline regions containing the sequence "GAGA GSGAGAGS" were well preserved. The existence of β-sheet crystalline regions in fibroin results in much more stable physicochemical properties of silk than of standard protein. We proposed that the intimate relationship between silk dyes and fibroin might also contribute to the preservation of silks, because these dyes might cover the silk surface and protect the fibroin from poor conditions including high humidity and microorganisms.

Strikingly, the investigation of four archaeological samples revealed the longstanding nature of the ancient Chinese aspiration to be resurrected by being buried wearing silk garments. The presence of silk in a sample from 400 BCE suggested that this Chinese tradition existed as far back as the 4th century BCE.

Conclusion

Studies using microscopy as the main method to empirically test for complete silk in ancient relics have been sufficient in the past. However, identifying denatured silk using this method is difficult, if not impossible. Here we were able to extract the protein from ancient fabric and to identify the fibroin component of silk using a novel antibody. To accomplish this, we focused on the repetitive and conserved amino-acid sequences appearing in the heavy chain of *B.mori* silk fibroin. In particular, the repetitious nature of these sequences makes them suitable markers for the identification of silk. Starting from the preparation of an antibody that recognizes the amino-acid sequence of "GAGAGSGAGAGS", an ELISA-based method was developed to successfully identify fibroin in four archaeological samples. Thus, for the first time, we are able to confidently conclude that these pieces unearthed from ancient Chinese tombs were made of silk. Furthermore, our conclusion suggests that silk products were used as grave goods at least 2500 years ago.

This study is an example of the valuable outcomes that can be attained from interdisciplinary approaches based on analytical and bioanalytical methods. As far as we are aware, this is the first study to use a bioanalytical method for the characterization of archaeological silk. It is probable that this method will be used in the future to provide more information regarding the cultural uses of silk. Furthermore, accurate identification of earlier but seriously denatured silks by this method will promote the investigation of the origins of silk, which are in either the Chinese Civilization or the Indus Civilization.

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References

1. Cartechini L, Vagnini M, Palmieri M, Pitzurra L, Mello T, Mazurek J, Chiari G (2010) Acc Chem Res 43:867–876
2. Good IL, Kenoyer JM, Meadow RH (2009) Archaeometry 51:457–466
3. Garside P, Lahlil S, Wyeth P (2005) Appl Spectrosc 59:1242–1247
4. Kim J, Wyeth P (2009) Preservation 6:60–67
5. Kim J, Zhang X, Wyeth P (2008) E-ps 5:41–46
6. Becker MA, Magoshi Y, Sakai T, Tuross NC (1997) Stud Conserv 42:27–37
7. Caroline S, Jolon MD, Santanu DCT, Stefan C, Paul W (2012) Photochem Photobiol 88:1217–1226
8. Cattaneo C, Gelsthorpe K, Phillips P, Sokol RJ, Smillie D (1991) Antiquity 65:878–881
9. Scott DA, Newman M, Schilling M, Derrick MR, Khanjian HP (1996) Archaeometry 38:103–112
10. Potenza M, Sabatino G, Giambi F, Rosi L, Papini AM, Dei L (2013) Anal Bioanal Chem 405:691–701
11. Yan F, Ge QY, Li Q, Yu M, Zhu XD, Pan J (2014) Sci Conserv Archaeol 26:71–75
12. Zhang K, Zhang BJ, Fang SQ (2013) Sci Conserv Archaeol 25:94–102
13. Zhou Y, Wang B, Sui M, Zhao F, Hu Z (2014) Studies in Conservation
14. Dash R, Mukherjee S, Kundu SC (2006) Int J Biol Macromol 38: 255–258
15. Dash R, Ghosh SK, Kaplan DL, Kundu SC (2007) Comp Biochem Physiol Part B: Biochem Mol Biol 147:129–134
16. Creighton M (2001) Japan Stud 21:5–29
17. Mhuka V, Dube S, Nindi MM (2013) Int J Biol Macromol 52:305–311
18. Zhou C, Confalonieri F, Medina N, Zivanovic Y, Esnault C, Yang T, Jacquet M, Janin J, Duguet M, Perasso R (2000) Nucleic Acids Res 28:2413–2419
19. Zhou CZ, Confalonieri F, Jacquet M, Perasso R, Li ZG, Janin J (2001) Proteins 44:119–122
20. Zhang X, Yuan S (2010) Chin J Chem 28:656–662
21. Chujo R, Shimaoka A, Nagaoka K, Kurata A, Inoue M (1996) Polymer 37:3693–3696
22. Jin HJ, Kaplan DL (2003) Nature 424:1057–1061
23. Degano I, Biesaga M, Colombini MP, Trojanowicz M (2011) J Chromatogr A 1218:5837–5847
24. Hermes AC, Davies RJ, Greiff S, Kutzke H, Lahlil S, Wyeth P, Riekel C (2006) Biomacromolecules 7:777–783
25. Palmieri M, Vagnini M, Pitzurra L, Rocchi P, Brunetti BG, Sgamellotti A, Cartechini L (2011) Anal Bioanal Chem 399: 3011–3023
26. Zhao F (1996) Southeast Cult 1:67–74