Identification of Ancient Silk Using an Enzyme-linked Immunosorbent Assay and Immuno-fluorescence Microscopy

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The identification of ancient silk is of great importance in both archaeology and academia. In the present work, a specific antibody having the characteristics of low cost, easy operation and extensive applicability was developed directly through immunizing rabbits with complete antigen (silk fibroin, SF). Then, antibody-based immunoassays, i.e. enzyme-linked immunosorbent assay (ELISA) and immuno-fluorescence microscopy (IFM), were established and conducted in tandem to identify the corresponding protein in ancient silks. The anti-SF antibody exhibits high sensitivity and specificity for the identification of modern and ancient silks. The detection limit of the ELISA method is about 0.1 ng/mL, and no cross-reactions with other possible interference antigens have been noted. IFM makes it possible to localize target proteins in archaeological samples, and also ensure the reliability of the ELISA results. Based on these advantages, immunological techniques have the potential to become powerful analytical tools at archaeological sites and conservation science laboratories.

Keywords Ancient silk, identification, ELISA, IFM

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Introduction

Silk, which is a naturally occurring polymer, is painstakingly woven from natural protein fibers derived from reeling cocoons. As a symbol of ancient Chinese culture, silk has made an indelible contribution to world civilization. However, the origin of silk remains one of the most puzzling mysteries in both archaeology and academia. The identification of silk has become of great significance in cultural heritage research and conservation science.

Silk is easily affected by many factors, such as light, soil microbes and radiation, resulting in the degradation of macromolecular chains.1–2 During the past several decades, the identification of archaeological silks has been achieved using a variety of analytical methods, including optical and electron microscopies, capillary electrophoresis-mass spectrometry, FTIR spectroscopy, microbeam synchrotron radiation diffraction and nuclear magnetic resonance.3–6 However, external contamination, degradation of silk fibroin and different biological sources (i.e. silk fibroin derived from bombyx mori or tussah) all make identification particularly difficult, and sometimes even highly uncertain. Therefore, immunological techniques have been proposed as an alternative approach.

Immunological techniques show high specificity of the antigen-antibody interaction for analytical purposes.8,9 Samples are detected using a specific antibody, which is conjugated to a fluorescein or enzyme that can activate a fluorogenic or chromogenic substrate, thus producing an optical signal. In fact, immunological techniques have the potential to become powerful analytical tools at archaeological sites and conservation science laboratories.

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turns indicates the presence of the antigen in the sample.

Furthermore, to localize target proteins in embedded cross-sections, immuno-fluorescence microscopy (IFM) is undoubtedly the best choice. The use of IFM is based on the collection of images showing the distributions of fluorescent target molecules in samples. In comparison with ELISA, the IFM technique can visualize the immunoreaction in situ and simplify the pretreatments of sample. Similarly, to indirect ELISA, to obtain a stronger signal, an indirect method with higher sensitivity was usually applied. In this method, a non-labeled primary antibody first binds to the target protein, and then this antibody is detected by a fluorescently labeled secondary antibody, accordingly magnifying the response signals.

In our previous research, ELISA was firstly used to detect proteinaceous binders e.g., collagen-I and ovalbumin, in ancient silk textiles. Recently, we reported on the preparation of a tailored fibroin antibody through peptide synthesis and carrier-protein coupling as well as its use for the identification of poorly preserved silks. In the present work, a specific antibody that has the characteristics of low cost, easy operation and extensive applicability was developed directly through immunizing rabbits with complete antigen (silk fibroin). Then, antibody-based immunoassays, i.e. ELISA and IFM, were established to identify the corresponding protein in ancient silks. Archaeological silk samples derived from Song tomb (sample I) and Chu tomb (sample II) were analyzed. These two immunoassays are particularly useful when used in tandem, suggesting that immunological techniques have potential to become powerful analytical tools at archaeological sites and conservation science laboratories.

**Experimental**

**Archaeological samples**

Two precious archaeological silk samples were selected for immunological identification. The original condition of the samples is shown in Fig. 1. Sample I (Fig. 1A) was unearthed from Song tomb, Ningbo city, Zhejiang province. This tomb was excavated in 2011, and some precious silk fabrics of the Southern Song Dynasty (approximately 760 years old) were unearthed for the first time. Sample II (Fig. 1B) was unearthed from Chu tomb (approximately 2300 years old), Anji county, Zhejiang province. This tomb was excavated in 2006 and some bronze, pottery, lacquer wares and silks were unearthed. These findings are of great value for research on sericulture and textile industry in ancient China. However, the unearthed silk fabrics seem to be very fragile and concreted seriously, which makes them difficult to distinguish.

**Reagents and chemicals**

Bovine serum albumin (BSA), human serum albumin (HSA), chicken ovalbumin, collagen and casein were purchased from Sigma-Aldrich. Natural silks were obtained from Zhejiang Misai Silk Co., Ltd. Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (100 μg at 1 mg/mL) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (100 μg at 1 mg/mL) were prepared by Hangzhou Hu’an Bio-Technology Co., Ltd.

A phosphate buffered saline (PBS) solution at pH 7.4 was used for wash steps. A PBS solution at pH 9.6 was used as a diluent. 1% BSA in PBS (pH 7.4) was used to block the unbound sites of antigen. For preparing buffered glycerol, equal volumes of PBS at pH 9.0 and glycerol were mixed. All other reagents were of analytical grade and used as received. The water used in all experiments was purified by a TPM Ultrapure water system.

**Morphological characterization of archaeological samples**

The morphology of the archaeological samples was characterized by scanning electron microscopy (SEM, S4800, Japan). The samples were sputter-coated with gold for 30 s and observed by SEM at an operating voltage of 15 kV.

**Extraction of antigens**

Silk was firstly boiled in a 0.5% (w/w) Na2CO3 solution at a bath ratio of 1:100 for 30 min. Then, the insoluble fibroin was removed and the remaining solutions were dialyzed with a molecular weight cut-off of 3500 for 3 days. A sericin solution was thus obtained. Meanwhile, the insoluble silk fibroin was washed with water and dried at 60℃ overnight. Next, dried silk fibroin was dissolved in a 50% (w/w) CaCl2 solution at 98 ± 2℃ for 1.5 h at a bath ratio of 1:25. After filtering, solutions were dialyzed with a molecular weight cut-off of 3500 for 3 days to remove calcium and chloride ions. Finally, both silk fibroin and sericin solutions were freeze-dried and the pale-yellow silk fibroin and sericin powders were obtained.

![Digital images of ancient Chinese silks. (A) Silk fragment from Song tomb; (B) silk fragment from Chu tomb. The photos were taken by a Canon EOS700D digital camera in macro mode.](image)
To guarantee the same operating conditions and to avoid interference from other media, both cotton and hemp fibers were extracted using the similar procedures as silk fibroin.

The extraction of keratin can be summarized as follows. A mixture solution containing 2% (w/v) NaOH and 0.3% (w/v) \( \text{H}_2\text{O}_2 \) was prepared in advance. Then, shredded wool fabrics were completely immersed into the mixed solution at 50°C at a bath ratio of 1:50. Simultaneously, nitrogen was continuously replenished to the reactor. A clear liquid was obtained when the wool fabrics were completely dissolved by mechanical stirring at 200 r/min for 4 h. Next, solutions were dialyzed for 72 h with a molecular weight cut-off of 3500 to ensure that contaminating ions were completely removed. Finally, pure keratin solutions were freeze-dried and keratin powders were obtained.

**Preparation of anti-SF primary antibody**

Three New Zealand white rabbits (14 – 16 weeks old) were injected subcutaneously at multiple points with silk fibroin immunogen (500 μg in PBS, mixed with an equal volume of Freund’s complete adjuvant to form an emulsion). For subsequent boosters, Freund’s incomplete adjuvant was substituted for the Freund’s complete adjuvant as an emulsifier every 2 weeks. Ten days after the third and the fourth booster immunization, the titer of the antiserum was measured by drawing venous blood. Sera were collected 1 week after the last injection. Anti-fibroin antibodies were further purified by a Protein A montage spin column. The column was pretreated by washing 3 times with 50 mM PBS (pH 7.4). Then, 15 mL antiserum was added and the non-conjugated molecules were removed by washing with 50 mM PBS (pH 7.4). Next, the antibodies were eluted with a 0.2 M glycine–HCl solution (pH 3.0). Immediately afterwards, 0.1 M NaPO₄ was added to adjust to neutral pH so as to avoid any irreversible denaturation of antibodies. The supernatant containing the antibodies was collected and stored (3.15 mg/mL) at –20°C before use.

**ELISA procedures**

An appropriate amount of sample was first dissolved in PBS solutions (pH 9.6). Then, 100 μL of the sample solution was added to each well and incubated at 4°C overnight to ensure that proteins were completely bound to the plastic surface. After the solution was removed, the wells were washed 3 times with PBS. Next, a 200-μL of blocking solution was added to each well, and the plate was incubated at 37°C for 1 h to occupy the unbound sites. The solution was removed, followed by 3 washings with PBS. Then, 100 μL of the indicated anti-SF primary antibody was added to the wells, followed by incubation at 37°C for 1 h. After removing the solution, the wells were washed 3 times with PBS. Next, 100 μL of AP-labeled goat anti-rabbit secondary antibody was added, followed by incubation at 37°C for 1 h. After washing with PBS for 3 times, 100 μL of substrate solution (TMB color system) was added to each well in a dark environment at room temperature for 10 min. Finally, 100 μL of 1 mol/L H₂SO₄ was added to terminate the color reaction, and the sample absorbance at \( \lambda = 450 \text{ nm} \) was measured using a microplate reader (Model 550, Bio Rad).

To ensure the specificity of the anti-SF primary antibody, a series of controls were set up. PBS was used as a blank control, and only experiments in which it presented negative results were considered to be reliable. For negative control, an anti-SF primary antibody was replaced by a PBS solution for the assay to ensure that the secondary antibody did not react nonspecifically with the sample. Silk fibroin and extracts of modern silk were employed as positive controls. BSA and other possible interference antigens (ovalbumin, collagen, casein, sericin, keratin, extracts of cotton fiber and extracts of hemp fiber) were used to verify the specificity of the anti-SF primary antibody.

**IFM procedures**

The IFM analytical procedure was optimized by comparing different conditions, including antibody dilution, incubation time and temperature. The adapted IFM protocol can be summarized as follows.

An archaeological sample was firstly placed in a 35-mm petri dish, and then a suitable amount of PBS was added to the surface of the sample, completely wetting the sample. Next, excess water was aspirated off and 100 μL of anti-SF primary antibody (dilution ratio of 1:20) was added to each petri dish, followed by incubation at 4°C for 24 h. Then, the sample was washed 3 times with PBS for 5 min. Next, 100 μL of FITC-labeled secondary antibody (dilution ratio of 1:400) was added to the sample surface and incubated at 37°C for 1 h. After washing 3 times with PBS, the sample was protected from light so as to avoid photobleaching. Then 100 μL of buffered glycerol was added to the sample surface. Finally, the sample was observed using confocal laser scanning microscopy (CLSM, LSM510, Zeiss, Germany). The wavelengths of the excitation source and the emission filter used in immune-fluorescence microscopy were 488 and 525 nm, respectively.

Similarly to indirect ELISA, a series of controls were also set up for the IFM techniques. For negative control, the anti-SF primary antibody was replaced by the PBS, while, the other procedures remained the same. Silk fibroin, derived from degummed silk, was prepared as a positive control. An archaeological sample wetted only with PBS was used as a blank control.

**Results and Discussion**

**Morphology of the archaeological samples**

To better understand the sample surface, SEM was employed to observe the microstructure of two archaeological samples, derived from Song tomb (Fig. 2A) and Chu tomb (Fig. 2B), respectively. As shown in Fig. 2A, most of the silk fibers were completely broken, and no parallel fibers were observed. Instead, piles of fibers were crushed and stacked together, which make them impossible to distinguish. In Fig. 2B, silk fibers were well woven, but some fibers were severely rotted. Besides, some attachments were firmly deposited on the fabric surface and the identification of silk could not be performed with ease.

**ELISA**

Optimal antibody dilutions (v/v) corresponding to the best specificity and sensitivity of the ELISA method were obtained by panel titrations. Silk fibroin, PBS and BSA were used as positive control, blank control, and negative control, respectively. High sensitivity and low background should give a good compromise in the antibody optimizations. In this study, the values of “\( \text{OD}_{450} \text{nm} \)” and “\( \text{OD}_{450} \text{nm} / \text{OD}_{600} \text{nm} \)” were employed as criteria to evaluate the appropriate antibody concentration. First, to keep a high sensitivity, antibody concentrations with a high \( \text{OD}_{450} \text{nm} \) value of positive control (silk fibroin) should be chosen for silk detection. However, a low background should never be ignored. The larger is the \( \text{OD}_{450} \text{nm} / \text{OD}_{600} \text{nm} \) value, the greater is the difference between the \( \text{OD}_{450} \text{nm} \) values of silk fibroin and PBS. The background interference was relatively low, accordingly, as shown in Table 1. The antibody dilutions of 1:3000 for the anti-SF primary antibody and 1:5000 for the
secondary antibody gave the best compromise between the sensitivity and the low background. At these antibody concentrations, silk fibroin shows a mean OD450nm value of 1.625, while PBS and BSA show mean OD450nm values of 0.105 and 0.087, respectively; that is, the “ODsilk fibroin/OPBS” value is 1.625, while PBS and BSA show mean OD450nm values of 0.087 and 0.058, respectively; that is, the “ODsilk fibroin/ODnegative control” and “ODBSA/ODnegative control” were analyzed. It showed that all of the ODsilk fibroin/ODnegative control are substantially above 2.0, while all of the ODbulk/ODnegative control and ODBSA/ODnegative control are substantially below 2.0. False-positive result has greater impact than a false-negative result in the ancient silk detection. To avoid such a result, we defined “S = ODsamples/ODnegative control” as the standard to precisely analyze the optical density results of all of the samples. This means that S > 2.1 was considered to be positive, while S ≤ 2.1 was considered to be negative for the immunological test. The ELISA protocol was then employed to test the detection limit for the silk fibroin. The results are presented in Fig. 3. Silk fibroin was diluted to 10–2, 10–1, 10, 102, 103, 104, 105, and 106 (ng/mL) by PBS (pH 9.6) respectively. It clearly demonstrates that the detection limit of silk fibroin was about 0.1 ng/mL, indicating that silk fibroin cannot be detected using the ELISA method when its concentration is below 0.1 ng/mL.

Next, further investigation was applied to evaluate the specificity of the anti-SF primary antibody. As shown in Fig. 4, a series of possible interference antigens, including ovalbumin, collagen, human serum albumin (HSA), casein, sericin, cotton fiber extraction, hemp fiber extraction and keratin (10 μg/mL), were tested. All of the above samples showed clearly negative results, while modern silk extractions and silk fibroin showed positive results. These results indicate that the silk fibroin antibody is an acceptable antibody for detecting archaeological silk due to its high specificity.

Since the ELISA protocol had been optimized for the identification of silk fibroin, it was employed to detect archaeological samples. Both sample I and sample II were
treated by using a CaCl₂ solution as the extraction solvent. As shown in Fig. 5, the archaeological samples, modern silk extractions and silk fibroin (10 μg/mL) all showed positive results, while PBS and BSA behaved negative, which correctly identified the presence of silk.

The effect of extraction methods on the detection of archaeological sample was further investigated. In this study, PBS (pH 9.6) and CaCl₂ (50 wt%) were used as extracted solutions, while a sample from the Song tomb (sample I) was chosen as a representative sample. As shown in Fig. 6A, when an archaeological sample was extracted by PBS, clearly positive results were obtained at a sample concentration of 10 μg/mL or less; however, when an archaeological sample was extracted by a CaCl₂ solution (Fig. 6B), a clearly positive result was still obtained at a sample concentration of 10 ng/mL. A comparison of these two extraction methods showed that samples extracted by a CaCl₂ solution displayed a much lower detection limit than those extracted by PBS. The minimum detection limit of archaeological samples extracted by PBS and CaCl₂ solutions was approximately 10 μg/mL and 10 ng/mL, respectively. These results indicate that CaCl₂ solutions produced a higher protein extraction efficiency than PBS solutions, although PBS solutions allowed a simpler pretreatment for operation. Consequently, the simplicity and efficiency should offer a good compromise in the practical detection of archaeological samples.

IFM

Archaeological silks undergoing thousands of years of erosion in underground environments have been mostly degraded, and are partially soluble, which makes IFM procedures become particularly difficult. In order to solve this problem, a washing solution (PBS) was refrigerated prior to archaeological silks being investigated by IFM. At the same time, optimized experimental conditions for immuno-fluorescence detection were established.
An archaeological sample treated only with PBS (blank control) was firstly monitored for auto-fluorescence. Figures 7A and 7B show fluorescent and bright field images of blank control. They clearly show no green light emission. This result ensures that immuno-fluorescence experiments can be carried out smoothly under the emission of FITC fluorophore. Figures 7C and 7D show fluorescent and bright field images of the negative control. They further demonstrate that the secondary antibody does not react nonspecifically with the sample in the absence of anti-SF primary antibody. Finally, the archaeological sample treated with both anti-SF primary antibody and FITC-conjugated secondary antibody was examined in further detail. As shown in Figs. 7E and 7F, the sample emits strong green fluorescence. The fluorescence is ascribable to a FITC-conjugated secondary antibody, which signals the presence of an anti-SF primary antibody, and in turn confirms the presence of silk fibroin in the archaeological sample from the Song tomb.

Figure 8 shows fluorescent and bright field images of another archaeological fabric from the Chu tomb. Similarly to the sample from Song tomb, both the blank control (Figs. 8A and 8B) and negative control (Figs. 8C and 8D) show no fluorescence, indicating that the auto-fluorescence is negligible and a low fluorescence background is obtained. However, an archaeological sample treated with both anti-SF primary antibody and FITC-conjugated secondary antibody (Figs. 8E and 8F) clearly show greenish fluorescence emission. All of the above results were consistent with the ELISA results, demonstrating the high specificity of the immunoreactions for silk recognition in archaeological samples.

Conclusions

In the present work, a specific antibody was developed directly through immunizing rabbits with silk fibroin. Then, antibody-based immunoassays, ELISA and IFM, were established and conducted in tandem to identify the corresponding protein in ancient silks. Specifically, ELISA is particularly suitable for rapid, sensitive and specific analysis, while IFM makes it possible to localize target proteins in embedded cross-sections, and to also ensure the reliability of the ELISA results. Based on these advantages, immunological techniques have the potential to become a particularly useful tool at archaeological sites and conservation science laboratories. However, before these techniques can be widely applied to archaeological sites, there is still much work to do. The next priority is the micro-trace detection of silk, which is crucial with respect to promoting the practical applications of immunodetection at the archaeological sites.

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References

1. M. Y. Li, Y. Zhao, T. Tong, X. H. Hou, B. S. Fang, S. Q. Wu, X. Y. Shen, and H. Tong, Polym. Degrad. Stab., 2013, 98, 727.
2. X. Zhang, I. V. Berghe, and P. Wyeth, J. Cult. Herit., 2011, 12, 408.
3. A. Hermes, R. Davies, S. Greiff, H. Kutzke, S. Lahlil, P. Wyeth, and C. Riekel, Biomacromolecules, 2006, 7, 777.
4. M. Moini, K. Klauenberg, and M. Ballard, Anal. Chem., 2011, 83, 7577.
5. X. Zhang and P. Wyeth, Sci. China: Chem., 2010, 53, 626.
6. Z. Zhu, D. Gong, L. Liu, and Y. Wang, Anal. Bioanal. Chem., 2014, 406, 2709.
7. M. Wilding, “Optical microscopy for textile fibre identification. In identification of textile fibers”, 2009, Woodhead Publishing, Cambridge.
8. J. R. Crowther and J. M. Walker, “The ELISA guidebook”, 2009, Vol. 149, Springer.
9. A. Voller, D. E. Bidwell, and A. Bartlett, “The enzyme linked immunosorbent assay (ELISA). A guide with abstracts of microplate applications”, 1979, Dynatech Europe, Borough House, Rue du Pré.
10. J. Arslanoglu, S. Zaleski, and J. Loike, Anal. Bioanal. Chem., 2011, 399, 2997.
11. L. Cartechini, M. Vagnini, M. Palmieri, L. Pitzurra, T. Mello, J. Mazurek, and G. Chiari, Acc. Chem. Res., 2010, 43, 867.
12. A. H. Heussner, I. Winter, S. Altaner, L. Kamp, F. Rubio, and D. R. Dietrich, Chem.-Biol. Interact., 2014, 222C, 10.
13. M. Johnson and E. Packard, Stud. Conserv., 1971, 16, 145.
14. M. E. Malainey, “Blood and Protein Residue Analysis. A consumer’s Guide to Archaeological Science”, 2011, New York.
15. B. Ramírez-Barat and S. de la Viña, Stud. Conserv., 2001, 46, 282.
16. W. Shi, J. He, H. Jiang, X. Hou, J. Yang, and J. Shen, J. Agric. Food Chem., 2006, 54, 6143.
17. R. Wierzbicka, L. Eyer, R. Landberg, A. Kamal-Eldin, and M. Franek, J. Immunol. Methods, 2014, 413, 12.
18. L. Chen, Z. Wang, M. Ferreri, J. Su, and B. Han, J. Agric. Food Chem., 2009, 57, 4674.
19. H. J. Lee, T. Watanabe, S. J. Gee, and B. D. Hammock, Bull. Environ. Contam. Toxicol., 2003, 71, 0014.
20. A. Heginbotham, V. Millay, and M. Quick, J. Am. Ins. Conserv., 2006, 45, 89.
21. L. Kockaert, P. Gausset, and M. Dubi-Raucquoy, Stud. Conserv., 1989, 34, 183.
22. M. Palmieri, M. Vagnini, L. Pitzurra, P. Rocchi, B. G. Brunetti, A. Sgamellotti, and L. Cartechini, Anal. Bioanal. Chem., 2011, 399, 3011.
23. J. Pavelka, L. Kovačíková, and L. Šmejda, Comptes Rendus Palevol, 2011, 10, 61.
24. M. Potenza, G. Sabatino, F. Giambi, L. Rosi, A. M. Papini, and L. Dei, Anal. Bioanal. Chem., 2013, 405, 691.
25. M. Vagnini, L. Pitzurra, L. Cartechini, C. Milliani, B. G. Brunetti, and A. Sgamellotti, Anal. Bioanal. Chem., 2008, 392, 57.
26. M. Gambino, F. Cappitelli, C. Cattò, A. Carpen, P. Principi, L. Ghезzi, I. Bonaduce, E. Galano, F. Pucci, and L. Birolo, J. Biosci., 2013, 38, 297.
27. M. Palmieri, M. Vagnini, L. Pitzurra, B. G. Brunetti, and L. Cartechini, Anal. Bioanal. Chem., 2013, 405, 6365.
28. T. Holzhauser and S. Vieths, J. Agric. Food Chem., 1999, 47, 603.
29. J. Arslanoglu, J. Schultz, J. Loike, J. Loike, and K. Peterson, J. Biosci., 2010, 35, 3.
30. Y. Zhou, B. Wang, M. Sui, F. Zhao, and Z. Hu, Stud. Conserv., doi: 10.1179/2047058414Y.0000000150.
31. Q. Zheng, X. Wu, H. Zheng, and Y. Zhou, Anal. Bioanal. Chem., 2015, 470, 3861.