Identification of Proteinaceous Binders in Ancient Tripitaka by the Use of an Enzyme-linked Immunosorbent Assay

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Proteinaceous materials, such as ovabumin and collagen, were commonly used as binding media, and as adhesives and protective coatings. However, the identification of ancient proteinaceous binders is a great challenge for archaeologists, due to their limited sample size, complex combinations of various ingredients and reduced availability of the binder during the process of protein degradation. In this paper, an enzyme-linked immunosorbent assay (ELISA) provides to be a particularly promising method for the detection of proteinaceous binding materials in ancient relics. The present work focused on the specific identification of proteins in archaeological binders, which was brushed on the Tripitaka. Two samples, the adhesion area (S1) and the ink area (S2), were tested by ELISA. The results showed that both S1 and S2 reacted positively when treated with an anti-collagen-I antibody. It proved the existence of proteinaceous binders in Ancient Tripitaka, and the percentage of collagen in S1 and S2 was 61.44 and 15.4%, respectively. Compared with other conventional techniques, ELISA has advantages of high specificity, sensitivity, rapidity and low cost, making it especially suitable for the protein detection in the archaeological field.

Keywords | Enzyme-linked immunosorbent assay (ELISA), proteinaceous binder, Ancient Tripitaka, identification

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Introduction

The Ancient Tripitaka, derived from the Tang Dynasty, was originally defined as the name of a complete set of sutra collections.1 The existing copies cover a wide range of languages, including Chinese, Tibetan, Mongolian, Manchu, Tangut, Japanese, Pali, English and others. Among these, the Ancient Tripitaka, which was first compiled by the Tibetan Buddhists, was made with high-quality materials. There are limited numbers of Tripitaka that survived to today, which have undergone various degrees of damage. They have been widely investigated, mainly while focusing on their literary value, translation and reprint, whereas their scientific and technological values were usually overlooked. Until now, very few works have been reported concerning the materials of the Tripitaka, which made it very difficult to collect corresponding useful information, and then do research in the conservation of the Tripitaka.

Among naturally occurring organic substances of the Tripitaka and other verses, binders were widely used, playing an important role in maintaining the shape and enhancing the mechanical properties of the materials. In particular, proteinaceous binders, i.e. animal glue, egg (both yolk and albumen), and milk (or its byproduct casein), are mostly encountered as the main distinctive proteins in paintings. However, a detailed characterization of the binding materials in ancient heritage is still a challenging issue because of their intrinsic chemical complexity and their tendency to easily undergo degradation over long periods of time.

Immunological techniques have the potential to become powerful diagnostic tools in cultural heritage for highly specific and sensitive identification of proteins in microsamples of art and archeological materials. Among the immunological techniques used in conservation science, enzyme-linked immunosorbent assay (ELISA) has been the most frequently employed. ELISA has the advantages of being affordable, relatively simple, fast, with limited sample manipulation, and capable of multiple antigen recognition. These features make ELISA a promising technique for routine analysis of proteinaceous materials and biopolymers in the conservation of cultural heritage.

ELISA has been widely used in clinical and bioanalytical chemistry, in various branches of medicine, pharmaceutical and food industries, and in environmental monitoring. The identification of proteins by ELISA is feasible due to the high specificity of antibody-antigen binding, through which one can detect the presence of protein at very low quantities. However, although the potential of immunological techniques in
archaeology and conservation has been discussed and investigated sporadically over the last several decades, these techniques have found little practical application.13 Schweiter et al. used a material extracted from a well-preserved 100000 – 300000-year-old mammoth skull to produce antisera, indicating that ELISA is sufficiently sensitive to work with extremely aged proteins.26 Zou et al. demonstrated a novel concept for the development of phage-free peptide ELISA for the detection of OTA by using a biotinylated mimotope peptide as a competing antigen. It can be applied to sensitively detect other toxic small molecules during food safety monitoring.27 Le Bailly et al. used immunological techniques to detect protozoan infections in paleoparasitology and they came to the conclusion that ELISA tests for Entamoeba histolytica were positive in three of five samples.28 In addition, the ELISA technique was also occasionally used to identify the binding media in paints.29,30 Zhou et al. used ELISA to detect proteinaceous binders in ancient Chinese textiles. Both Silk String and Cinnabar Dyed, which were unearthed from No. 4 Chu tomb in Jingzhou Hubei province, gave a negative result for collagen-I.5 Palmieri et al. focused on selective identification, by use of the ELISA, of chicken-egg yolk and animal collagen in painting micro-samples.30 Cartechini et al. reported the immunodetection of Proteins in ancient paint media. It has been proved that casein, ovalbumin and the animal glue collagen were used as binders in ancient times.

In our previous research, several traditional methods were applied to identify the composition of Ancient Tripitaka binders, such as elemental analysis, attenuated total-reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and amino acid analysis (AAA). However, no reliable result was obtained due to sample contamination and difficulty in distinguishing protein mixtures. The study was aimed at identifying the species of proteinaceous binders of Ancient Tripitaka using the ELISA test combined with other analytical methods. The protocol has been optimized with respect to the protein solubilization conditions, the antibody’s concentrations, the incubation time and the temperature. The results reported herein provide a deeper understanding of the interactions between antibody and immunoreactive antigen in archaeological proteinaceous samples.

### Experimental

#### Archaeological samples

Information concerning two archaeological samples is summarized in Table 1. Samples are friendly supported by China National Silk Museum. Both of them are subject to different degrees of damage and aging. One of the samples is from the adhesion area (coded as S1, Fig. 1A), which was used to bond the Ancient Tripitaka between two layers of paper. The other is from the ink area (coded as S2, Fig. 1B), that is an Ancient Tripitaka ink brushing paper, on which mud silver writing verses exist.

#### Reagents and chemicals

Anti-collagen-I antibody (100 μl at 1 mg/ml) was purchased from Abcam. Goat anti-Rabbit IgG (H+L) HRP conjugated (100 μl at 2 mg/ml) was purchased from Hua’an Biological. Gelatin from bovine skin, gelatin from cold water fish skin, urea, sodium dodecyl sulfate (SDS), disodium ethylene-diaminetetraacetate dihydrate (EDTA), and albumin from bovine serum (BSA) were purchased from Sigma-Aldrich. All other reagents were of analytical grade and used as received. The water used in all experiments was purified by a Milli-Q water system.

Elution buffer (EB) was prepared in advance; 180 g of urea was added to a solution containing 5 ml 1 mol/l Tris-HCl, 1 ml 0.5 mol/l EDTA, and 25 ml 20% SDS and diluted to 500 ml.

### Table 1 Origin, primary antibodies, corresponding secondary antibodies and dilutions of the samples tested by ELISA

| Sample No. | Sample Description | Sample Origin | Primary Antibody (Dilution) | Secondary Antibody (Dilution) |
|------------|-------------------|---------------|----------------------------|-----------------------------|
| S1         | Adhesion area used to bond the Tripitaka between two layers of paper | Ancient Tripitaka (14th – 17th century A.D., Ming Dynasty) | AbCam rabbit polyclonal anti-collagen-I antibody (1:200) | Goat anti-Rabbit IgG (H+L) HRP conjugated (1:3000) |
| S2         | Tripitaka ink brushing paper, marked as ink area | Ancient Tripitaka (14th – 17th century A.D., Ming Dynasty) | AbCam rabbit polyclonal anti-collagen-I antibody (1:200) | Goat anti-Rabbit IgG (H+L) HRP conjugated (1:3000) |

![Fig. 1 Digital images of the Ancient Tripitaka dated from Ming Dynasty. (A) Adhesion area (S1); (B) ink area (S2). These pictures were taken using a Canon EOS700D digital camera in the macro mode. The side length of the monochrome square was 1 cm.](image-url)
The solution was adjusted to pH 7.4 and sterilized before use. Phosphate-buffered saline solution (PBS) was prepared as follows: 0.2 g KCl, 0.2 g KH₂PO₄, 8.0 g NaCl and 2.16 g NaH₂PO₄·7H₂O were added to water and diluted to 1000 ml. The solution was adjusted to pH 7.4 and sterilized before use.

Characterization of archaeological samples

The morphology of the archaeological samples was characterized by field-emission scanning electron microscopy (FESEM). The SEM was equipped with detectors to image secondary and backscattered electrons and an analytical Oxford ISIS energy dispersive spectrometer (EDS) system. The samples were sputter coated with gold for 10 s and examined by SEM at an accelerating voltage of 3 kV. For EDS analysis, certain zones of the sample were selected and detected at an accelerating voltage of 10 kV.

ELISA procedure

The indirect ELISA technique used was a modified procedure based on the literature. In an indirect ELISA test, the primary antibody was targeted by enzyme-conjugated secondary antibody instead of being conjugated with enzymes directly. The use of a secondary antibody increased the specificity of ELISA, and reduced the unspecific background due to the linkage of antibodies on the plastic surface of the well plate. In order to investigate the specificity and sensitivity of the primary antibody used in this experiment, several negative and positive controls were employed for the ELISA test. The negative controls included phosphate buffered saline (PBS), an elution buffer (EB) and BSA solution, while gelatins from the bovine and the fish skin served as positive controls.

Samples (2.5 mg) were first immersed in a 1-ml EB solution, and incubated at room temperature for 4 days, followed by adding 100 mmol/l NaHCO₃ to a 15-fold dilution. Then, 80 μl of the sample diluents were added to the wells of a 96-well ELISA plate (U-Bottom, 12w × 8). The plate was covered with an adhesive plastic film and incubated at 4°C overnight. As the antigen to be detected attached to the plastic of the well, the sample was removed and the plate was washed with 300 μl PBS (0.01 M, pH 7.4) for 3 times. Then 300 μl of a blocking buffer (1% BSA in PBS) was added and the plate was covered again and incubated at 37°C for 1 h in and to block any non-specific binding sites in the coated wells. After washing with 300 μl PBS for 3 times, 80 μl of the antigen-binding primary antibody (used at an assay dilution of 1/200, the final concentration: 0.2 – 2 μg/ml) was added and incubated at 37°C for 2 h. The plate was then washed with 300 μl PBS (0.01 M, pH 7.4) for 3 times and the secondary antibody Goat anti-Rabbit IgG (H+L) HRP conjugated (used at an assay dilution of 1/3000) was added to the wells and incubated at 37°C for 2 h. After removing the secondary antibody and washing with PBS for 3 times, 100 μl of the 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 in and to terminate the color reaction, and the sample absorbance at λ = 450 nm was measured using a microplate reader (Model 550, Bio Rad).

Statistical analysis

Five parallel wells were used for each sample and control, and run simultaneously. All of the values are expressed as mean ± standard deviation.

Results and Discussion

Morphology of the archaeological samples

To understand the distribution of the binder in the Ancient Tripitaka, SEM was employed to visualize the microstructure of...
archaeological samples. As shown in Fig. 2A, there are large amounts of irregularly shaped sediment adhered onto the surfaces of paper fibers, which contains some spherical objects. Besides, many gaps and holes are also presented in S1 from the adhesion area of the Ancient Tripitaka. The spherical objects, gaps and holes are indicated by red, blue and yellow arrows, respectively. Figure 2B shows an image of S1 with higher magnification; pieces of flaky crystals and a rough layer are found around the fiber surface. Based on the above results, the existence of the proteinaceous binders is speculated. In Fig. 2C, it scan a paper surface from ink area of the Ancient Tripitaka (S2) can be seen, but with many cracks and uneven objects. This illustrates the possibility that the ancients wrote verse on the paper surface. Figure 2D shows an image of S2 with higher magnification, from which it can be seen that the intact structures of brushing substances have degraded into flake structures. These results demonstrate that both the adhesion area and ink area have undergone varying degrees of degradation along with long period of physical, chemical, and biological erosion.

EDS analysis of the archaeological samples

Despite that one can observe the morphology of the Ancient Tripitaka from the SEM images, EDS can more intuitively identify any distinction from the aspect of sample components. As shown in Fig. 3, the content of every element of the Ancient Tripitaka was detected. In disregard to Au, which probably resulted from the sample treatment of gold plating and other potential interferences from the paper’s filler, Figs. 3A and 3B both show sharp and intense peaks of K, Ca, Al and Si. The presence of Ca is very likely derived from the lime treatment in the paper-making process, Al and Si should be derived from the paper filler (kaolin). As early as in the Northern Wei Dynasty, kaolin had been used as a paper coating, in order to improve the brightness, smoothness and ink receptivity of the paper. In the process of ancient papermaking, the fiber materials were subjected to retting, cooking, exposure, cleaning and cutting processes, in order to remove any lignin and pectin, and to improve the binding force between the fibers. Particularly, lime or plant ash, whose main ingredients are CaCO3 and K2CO3, respectively, was usually added in the cooking processes.

Although the nitrogen element is one of the major components of collagen, no obvious peak of nitrogen is detected in the EDS spectra. It is speculated that the nitrogen element content is below the detection limit of the EDS measurement. Accordingly, a highly sensitive and specific method, enzyme-linked immunosorbent assay (ELISA), was implemented for further analysis in order to identify the composition of the binders used in the Ancient Tripitaka.

The optimized dilution of primary antibody

Optimal antibody dilutions, corresponding to the best specificity and sensitivity of the ELISA method, were obtained by panel titrations. Gelatin and PBS were used as positive and negative controls, respectively. As the two archaeological samples are taken from the Tibetan tripitaka heritage, which are very precious, only a small amount of samples was allowed for conducting research. Also, the total mass of S1 and S2 are 8 and 5 mg, respectively. For the ELISA test, 2.5 mg samples were immersed in a 1-ml EB solution, and the concentrations of both S1 and S2 in the EB solution were set at 2.5 mg/ml. Therefore, gelatin samples with the same concentration were used to determine the optimal antibody dilutions.

High sensitivity and a low negative value should allow a good...
compromise in the antibody optimization. In this study, the optical density of the samples at 450 nm was abbreviated as OD\textsubscript{gelstat}. The values of “OD\textsubscript{gel}” and “OD\textsubscript{gel}/OD\textsubscript{PBS}” were employed as criteria to evaluate the appropriate primary antibody concentration. First, to keep a high sensitivity, primary antibody concentrations with a high OD\textsubscript{gelstat} value of positive control (gelatin) should be chosen for the detection of proteinaceous binders. However, a low negative value should never be ignored. The larger is the OD\textsubscript{gel}/OD\textsubscript{PBS} value, the greater is the difference between the OD\textsubscript{gelstat} values of gelatin and PBS. The negative value was relatively low, accordingly. As shown in Fig. 4 and Table 2, gelatins from bovine skin and cold water fish skin were marked as Gel 1 and Gel 2, respectively. This showed that the OD\textsubscript{gelstat} values of Gel 1, Gel 2 and PBS decreased with an increase of the dilution ratios of the anti-collagen-I primary antibody. In particular, when the dilution ratio of primary antibody was 1:200, it not only showed the highest sensitivity, but also had the lowest negative value. As a result, the antibody dilution of 1:200 for the anti-collagen-I primary antibody gave the best compromise between the sensitivity and the low negative value. At this antibody concentration, the “OD\textsubscript{gel}/OD\textsubscript{PBS}” value is 5.91, while the “OD\textsubscript{gel}/ OD\textsubscript{PBS}” value is 4.43. According to the protocol of anti-collagen-I antibody, the dilution ratio of 1:200 is the minimum dilution multiple permitted in the ELISA test. In addition, the higher is the concentration of the antibody, the greater is the probability of obtaining false positive results and the higher is the cost. This was not conducive to archaeological research. Therefore, a dilution ratio of 1:200 for anti-collagen-I primary antibody was considered to be optimum in view of the compromise between high sensitivity and a low negative value.

The specificity of primary antibody

Next, further investigation was applied to evaluate the specificity of the anti-collagen-I antibody. An investigation of the ELISA procedure for recognition of collagen began with antibody panel titration of standard solutions of the proteinaceous binders, using PBS as a control. The specificity of the primary antibody was tested by checking the cross-reactivity with a series of possible interference antigens, including BSA, silk fibroin, and keratin. The detection limit was set as the mean OD\textsubscript{gelstat} value of PBS plus three times the corresponding standard deviation. As shown in Fig. 5, all of the interference antigens, i.e. BSA, silk fibroin and keratin, clearly showed negative results, while both Gel 1 and Gel 2 showed positive results. The above results proved the effectiveness and specificity of the antibody. It is further indicated that the anti-collagen-I antibody is an acceptable antibody for detecting the presence of collagen in binding materials, due to its high specificity.

Immunodetection of proteins in archaeological samples

Since the ELISA protocol had been optimized for the identification of proteinaceous binders, it was employed to detect archaeological samples. To ensure the high reliability of the ELISA test, PBS and EB were set as negative controls, while gelatin from the bovine (Gel 1) served as positive control. As shown in Fig. 6, the archaeological samples (S1, S2) and Gel 1 all showed a positive results, while PBS and EB proved to be negative, which correctly identified the presence of collagen. It proved the existence of proteinaceous binders in the Ancient Tripitaka; what is more, the composition of the proteinaceous substance was collagen. In order to estimate the ratio of collagen to the total protein, a quantitative ELISA test using a standard curve for collagen was performed. First, gelatin from bovine (Gel 1) was diluted to 1, 2, 4, 6, 8, 10 mg/ml with the EB solution, followed by incubating at room temperature for 4 days. The following procedures were the same as the ELISA described above. The values of OD\textsubscript{gelstat} were measured at the optimized dilution of the primary antibody. As shown in Fig. 7, the relationship between OD\textsubscript{gelstat} and the gelatin concentrations was described in the ELISA calibration curves obtained for gelatin from the bovine (Gel 1). According to the OD\textsubscript{gelstat} values of S1 and S2, the concentration of collagen in S1 and S2 was calculated to be 1.536 and 0.385 mg/ml, respectively. Therefore, in combination with the concentration of the samples (2.5 mg/ml), the percentage of collagen in S1 and S2 was 61.44 and 15.4%, respectively. In our ongoing work, immunofluorescence microscopy and other chemical analytical tools are being employed to investigate the distribution of collagen in samples, thus, allowing for confirming of the ELISA results, and thus decreasing the risk of false positive results.
y = 0.131x + 0.120
R² = 0.9999

Fig. 7 ELISA calibration curves obtained for gelatin from the bovine (Gel 1), expressed as the relationship between OD₄₅₀ nm and the gelatin concentrations.

Conclusions

This study focused on the selective identification of proteinaceous binders in the Ancient Tripitaka. Morphological observation showed that there is large amount of irregularly shaped sediment adhering onto the surface of the Ancient Tripitaka. For further accurate analysis, ELISA was used to detect proteinaceous binders in the Ancient Tripitaka. The optimal antibody dilutions corresponding to the best specificity and sensitivity of the ELISA test were obtained by panel titrations. It is further indicated that the anti-collagen-I antibody is an acceptable antibody for detecting the presence of collagen in binding materials due to its high specificity. The results showed that both S1 and S2 reacted positive where treated with anti-collagen-I antibody. This proved the existence of proteinaceous binders in the Ancient Tripitaka; what is more, the composition percentage of collagen in S1 and S2 was 61.44 and 15.4%, respectively. ELISA is particularly suitable for rapid, sensitive and specific analysis, which is a successful application of immunological techniques in the analysis of trace proteins in archaeological samples. With such advantages, the ELISA technique may become a promising tool and play an important role in archaeological research.

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