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DETERMINATION OF PROTEINACEOUS BINDERS FOR POLYCHROME RELICS OF XUMI MOUNTAIN GROTTOES BY USING ENZYME-LINKED IMMUNOSORBENT ASSAY AND IMMUNOFLUORESCENCE MICROSCOPY

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Abstract

Xumi Mountain Grottoes is a famous cave temple in China. There are a large number of polychrome mural paintings and clay sculptures in Xumi Mountain Grottoes. It has been an important issue to identify the composition of binders in polychrome relics in order to understand traditional painting technology used in ancient time. In this study, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy (IFM), which possess high sensitivity, high specificity and cost affordability, were utilized to determine proteinaceous binders in polychrome murals and clay sculptures in Xumi Mountain Grottoes. Animal glue and egg white, which were most likely to be used as binders in ancient Chinese relics, were identified by the existence of their corresponding main components, namely mammalian collagen and chicken ovalbumin, respectively. Positive signals of mammalian collagen and chicken ovalbumin were rapidly detected by ELISA and distribution of the detected protein was identified by IFM in the real sample of Xumi Mountain Grottoes. This is the first time that the proteinaceous binders for the polychrome layers of mural painting and clay sculpture in Xumi Mountain Grottoes are studied.

Keywords: *Xumi Mountain Grottoes; Animal glue; Egg white; Enzyme-linked immunosorbent assay; Immunofluorescence microscopy*

Introduction

Xumi Mountain Grottoes is located at Guyuan City, the Ningxia Hui Autonomous Region of China. It was listed into the national emphasis cultural relics in 1982 owing to its existing special grotto artworks. Xumi Mountain Grottoes was first constructed in 512 A.D. during the the Northern Wei Dynasty. At that time the governors believed in Buddhism and sponsored the construction of Xumi Mountain Grottoes for monks to cultivate and meditate and for the wide spread of Buddhism. Eventually a large number of grottoes were drilled and numerous mural paintings as well as clay sculptures were constructed in Xumi Mountain. During the following 1500 years, the grottoes were continuously repaired and reconstructed.

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Nowadays there are 132 grottoes, more than 70 of which have precious mural paintings, clay sculptures as well as stone carvings [1].

Ancient Chinese polychrome relics including mural painting, clay sculptures and other forms are attractive artworks owing to their delicate polychrome layers. These relics usually have multilayered structure which mainly consists of the ground layer, the white preparation layer and the painting layer [2]. In order to smooth the painting layer and thus make it more exquisite, elastic and adhesive, natural organic binders were added during the constructing process. The employed organic binders were usually made of egg white and/or egg yolk, milk, animal glue, wax, drying oil, rubber, etc. Among these binders, egg white and animal glue are the most commonly used binders in ancient China due to their satisfactory effect and easy accessibility. Wei et al. [3] discovered animal glue in Western Han's (206 B.C. – 8 A.D.) polychrome terracotta army in Qingzhou County, Shandong Province. Hu et al. [4] found egg white and Yan et al. [5] found animal glue in Qin Shi Huang's Terracotta Warriors in Xi'an City, Shanxi Province. Determination of organic binders utilized in ancient artworks can help people understand the techniques created by ancient craftsmen and find the suitable measures to protect these artworks remained today. Moreover, it may also play an important role on dating and determining the regional provenance of a specific mural since choosing of binders for ancient artworks varies with different ages and regions.

Many analytical methods have been used to identify proteinaceous binders in cultural relic, for example Fourier transform infrared spectroscopy (FTIR) [6], Raman spectroscopy [7], chromatographic techniques such as thin-layer chromatography (TLC) [8], gas chromatograph (GC) [9] and high performance liquid chromatography (HPLC) [10], proteomic strategies such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) [5, 8, 11-13] and nano LC-MS/MS with electrospray ionization (ESI) [14] and so on. However, these techniques are limited by the requirement of large sample size, high expenses, the natural degradation of materials and pre-separation of target proteins since it is difficult to identify proteins when two kinds of proteins mixed together. Nowadays, immunological methods, which use highly specific antigen-antibody interaction to detect the presence of proteinaceous binders, have gained more attention. There are a lot of immunological methods, among which enzyme-linked immunosorbent assay (ELISA), immunofluorescence microscopy (IFM) and chemiluminescent immunochemical imaging technique [15, 16] were used frequently in determination of binders in polychrome samples.

ELISA and IFM were used as complementary techniques for identification of animal glue and egg white in this work. ELISA is fast, sensitive and cost-effective but as dissolution of the sample is needed before analysis and physical separation of layers is practically difficult, the detection of proteins in individual layer is very hard [17]. IFM could successively redress the limitations of ELISA by spatially resolving the target protein in the cross-section of samples. IFM also has limitation of unspecific emission which comes from light scattering phenomena at the surface of the cross-section and interferes with the observation of specific fluorescence [18, 19]. Though this phenomenon can be solved by using confocal fluorescence microscopy to obtain images instead of conventional fluorescence one [20], this confocal fluorescence microscopy is not general in conservation laboratory. As both technologies have limits and strengths, they were used sequentially in this experiment.

In ELISA, protein extracted by phosphate buffer saline (PBS) is captured by the surface of a well plate and then recognized by enzyme labeled antibody [21]. To improve the detection specificity and sensibility, indirect ELISA is adopted in this experiment. It means that enzyme, which is used to catalyze reaction of chromogenic substrate to get an optical signal, is not linked to the primary antibody; it is, instead, conjugated with the secondary antibody, several of which reacts specifically with one primary antibody and thus magnifies the signal [22]. In IFM, protein is not extracted and the reaction happens on the surface of the cross-section of polychrome relics. To prevent unspecific adsorption of antibody to the porous surface, which

would lead to unspecific fluorescence, a blocking solution was used to fill in the pores and block the non-specific binding sites in the protein [19]. As same as ELISA, this procedure is performed in an indirect way, which means that a primary antibody binds to corresponding target protein and is then recognized by several fluorescent labeled secondary antibodies [19]. The image is captured with a fluorescence microscope and thus the position of target protein can be indicated by IFM accurately.

Immunological methods being used in archaeological science are not novel [23, 24]; while using these methods to detect boiled collagen remained in culture relics is rare [16]. In this work, different antibodies were examined to choose the appropriate antibody to identify prolonged boiled collagen. In ELISA, the concentrations of antibodies were optimized and the sensitivity and specificity were determined. The method of IFM was tested on positive and negative mimetic samples to validate its reliability. These two methods were then applied to real samples of Xumi Mountain Grottoes to determinate the existence and to acquire accurate information about the position of animal glue and egg white in the samples.

Experimental

Reagents

The used primary antibodies were:

- Rabbit polyclonal antibody to collagen type I (cod. AB749P, Millipore, Temecula, USA).
- Rabbit polyclonal antibody raised against native collage I (cod. PAB13485, Abnova, Taipei, Taiwan).
- Mouse monoclonal antibody to collagen type I (cod. Ab23446, abcam, New Territories, Hong Kong).
- Mouse monoclonal antibody to ovalbumin (cod. A6075, Sigma-Aldrich, St. Louis, USA).

The secondary antibody goat anti-mouse and anti-rabbit IgG conjugated enzyme alkaline phosphatase (AP-MAb) were used for colorimetric detection by enzymatic reaction in presence of para-nitro-phenyl-phosphate (p-NPP) as substrate for the enzyme. Both the secondary antibodies and p-NPP were provided by Sigma-Aldrich (St. Louis, USA).

The secondary antibody used in IFM were goat anti-mouse and anti-rabbit IgG (H+L) conjugated Alexa Fluor 488 and 594, respectively. These two antibodies were used for fluorescence detection with microscope in corresponding exciting light and were received from Invitrogen-MP (Carlsbad, USA). Phosphate-buffered saline solution (PBS, 150mM NaCl, 5.2mM Na_2HPO_4, 1.7mM KH_2PO_4, pH = 7.4, 0.2% Tween 20) was used to dilute antibodies and for washing steps. Bovine serum albumin (BSA) from Sigma-Aldrich (St. Louis, USA) with a concentration of 0.1% in PBS was used as a blocking solution in ELISA. The blocking solution used in IFM was bought from Beyotime (Beijing, China).

Bovine skin, porcine skin, dog skin, goat skin and fish maw were purchased at the local market, and each of them were then boiled for 3h to make different types of animal glues respectively. Fresh hen's egg and bovine milk were purchased at local supermarket, and gelatin used as dried animal glue was obtained from Jiangsu Jiang Si Xutang. Cinnabar (HgS), azurite ($Cu_3(CO_3)_2(OH)_2$) and malachite ($Cu_2(OH)_2CO_3$) were purchased from Rock Color Sky Elegance (Beijing, China) and were used as pigments in the preparation of mimetic samples.

Instruments

ELISA tests were performed in 96 wells polycarbonate microliter plates (Sigma-Aldrich, St. Louis, USA). Absorbance of sample at λ = 405 nm, expressed as optical density (OD_{405nm}), was measured by using R-Biopharm Well Reader (R-Biopharm, Beijing, China).

Optical microscope (OM) images were collected at 400× magnifications using three-dimensional microscopic system (VHX-700FC, Keyence Corporation, Osaka, Japan). The fluorescence images of 400× magnifications were obtained by Olympus CX41 fluorescence microscope (Tokyo, Japan) with a liquid-nitrogen-cooled ultra-sensitive CCD camera

(QImaging, Surrey, Canada). The microscope was equipped with a tungsten lamp (Olympus, Tokyo, Japan) and light filter set specifically for the FITC fluorophore (excitation filter BP = 450–480nm, beam splitter FT = 500nm, emission filter LP = 515nm for fluorophore emitting green light; and excitation filter BP = 510–550nm, beam splitter FT = 570nm, emission filter LP = 590nm for fluorophore emitting orange-red light).

Preparation of mimetic samples

Mimetic samples were prepared according to polychrome relics in Xumi Mountain Grottoes. First, the ground layer was made as follows. Soil and sand were made through a 100mesh sieve, and hemp rope was cut into 5mm and beaten in a mortar to make it fluffy. Then soil, sand and hemp rope were mixed in a weight ratio of 64:36:3, and water was added and this slurry was put into a 5×5×0.5cm mould. Second, after drying and demoulding, the mixture of $Ca(OH)_2$ and water was smeared on the surface to imitate the white preparation layer in mural paintings. Finally, the painting layer was made with pure pigment or the mixture of pigment and proteinaceous binder in a weight ratio of 2:1. The proteinaceous binders used in this step were bovine skin glue, porcine skin glue, dog skin glue, sheep skin glue, fish maw glue, chicken egg white and bovine milk. Samples with multiple painting layers were also made to simulate repainted relics. All the samples were analyzed after three months of natural aging.

Real Samples from Xumi Mountain Grottoes

In the present work, three fragments from clay sculptures (Fig.1, sample XM1-3) and four from mural painting (Fig.1, sample XM4-7) from Xumi Mountain Grottoes were collected and examined. The description of each sample is listed in Table 1.

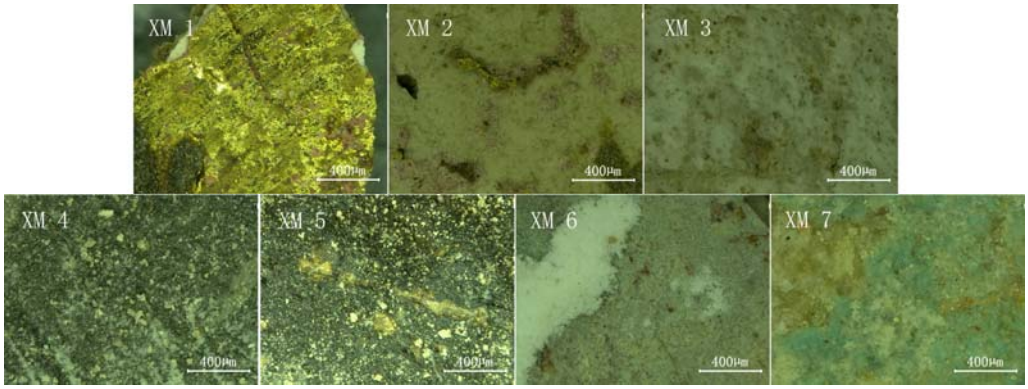


Fig. 1 The surface images of sample XM 1-7 collected from the Xumi Mountain Grottoes

Table 1 Descriptions and sources of sample XM 1-7

Sample	Description of sample	Dynasty
XM 1	Gilded sample from clay sculpture of cave 48 shrine3[a]	Ming
XM 2	White sample from clay sculpture of cave 48 shrine 2	Ming
XM 3	White preparing layer from clay sculpture of cave 48 shrine 4	Ming
XM 4	Black paint from mural painting of cave 44	Ming
XM 5	Red paint from mural painting of cave 69	Ming
XM 6	Red paint from mural painting of cave 69	Tang
XM 7	Green paint from mural painting of cave 62	Tang

[a]shrine means a small cabinet which is used to consecrate the sculpture of Buddha

Sample XM1, which was gilded, was taken from a finger of the third sculpture in cave 48. Other six samples were taken from the colored relics with different pigments. A representative number of samples of different caves which were built in different dynasties

were selected in order to identify the binders and to figure out painting techniques used in ancient time. The image of surface condition of each sample is shown in Figure 1.

ELISA procedure

A painted micro sample (ca. 5mg) was scraped from the painting layer and added into a 1.5mL Eppendorf tube with 300μL PBS. The mixture was sonicated for 0.5h and incubated at room temperature overnight. Then 90μL of the supernatant solution was placed into an ELISA well plate and incubated for 1 h at 37°C. Then the blocking solution, primary antibody and secondary antibody were added sequentially by incubating 100μL for 1 h at 37°C. The coloring enzymatic reaction is performed by adding 100μL of colorless p-NPP and incubating at room temperature (25°C) for 15min. Finally, 50μL of NaOH (2N) was added to stop the reaction, and the ELISA plate was put into the spectrophotometer to measure the optical density at 405 nm. The well was rinsed with 200μL of PBS for five times between each step to remove unbounded compounds. All the ELISA tests were repeated at least three times on each sample, and the average values of absorbance at OD405 nm were reported with the corresponding standard deviations (SD) in following Table 2, 3 and 4 as well as Figures 2 and 3.

Table 2. Antibody panel titration results of ELISA for gelatin

Sample (dilution, v/v)	Primary antibody dilution (v/v)	OD_{405nm}±SD		
		Secondary antibody dilution (v/v)		
		1:250	1:500	1:1,000
Gelatin (10^{-4})	1:250	1.250±0.4	1.371±0.06	0.807±0.3
	1:500	0.926±0.2	**0.843±0.2**	0.856±0.1
	1:1,000	0.778±0.03	0.655±0.02	0.435±0.001
Gelatin (10^{-5})	1:250	1.258±0.4	0.990±0.3	0.763±0.3
	1:500	0.614±0.1	**0.603±0.1**	0.466±0.03
	1:1,000	0.589±0.2	0.531±0.1	0.401±0.1
PBS	1:250	1.142±0.1	0.571±0.2	0.473±0.2
	1:500	0.488±0.03	**0.399±0.02**	0.412±0.04
	1:1,000	0.390±0.02	0.379±0.006	0.319±0.002

Table 3. Antibody panel titration results of ELISA for chicken egg white

Sample (dilution, v/v)	Primary antibody dilution (v/v)	OD_{405nm}±SD		
		Secondary antibody dilution (v/v)		
		1:250	1:500	1:1,000
Chicken egg white (10^{-1})	1:250	2.396±0.43	2.215±0.39	1.780±0.39
	1:500	2.123±0.42	**2.157±0.33**	1.729±0.48
	1:1,000	1.636±0.38	1.777±0.34	1.340±0.31
	1:2,000	0.703±0.01	0.799±0.00	0.308±0.00
Chicken egg white (10^{-2})	1:250	1.309±0.29	1.290±0.26	0.956±0.22
	1:500	1.303±0.33	**1.189±0.34**	0.890±0.30
	1:1,000	0.822±0.12	0.598±0.27	0.522±0.24
	1:2,000	0.304±0.005	0.301±0.004	0.272±0.001
PBS	1:250	0.482±0.21	0.471±0.12	0.453±0.13
	1:500	0.488±0.12	**0.327±0.015**	0.310±0.02
	1:1,000	0.357±0.002	0.253±0.00	0.327±0.01
	1:2,000	0.231±0.000	0.234±0.00	0.224±0.00

Table 4. The result of real samples from Xumi Mountain Grottoes using ELISA and IFM

Sample	Results of ELISA		Results of IFM	
	Animal glue	Egg white	Animal glue	Egg white
XM 1	**0.828±0.101**	0.201±0.001	++	-
XM 2	0.308±0.033	0. 236±0.062	-	-
XM 3	0.269±0.031	**0. 875±0.094**	-	++
XM 4	**0.872±0.102**	0.304±0.013	++	-
XM 5	0.314±0.053	0.248±0.005	-	-
XM 6	**0.719±0.019**	**0.619±0.001**	++	++
XM 7	**0.741±0.057**	0.246±0.001	++	-

[a](-) not fluorescent; (+) fluorescent; (++) strongly fluorescent.

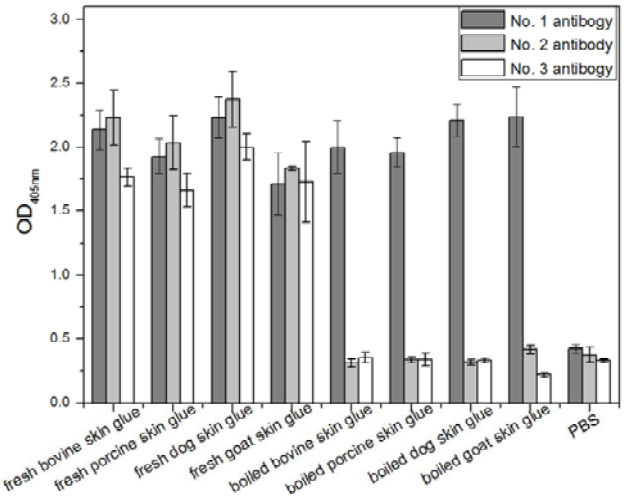
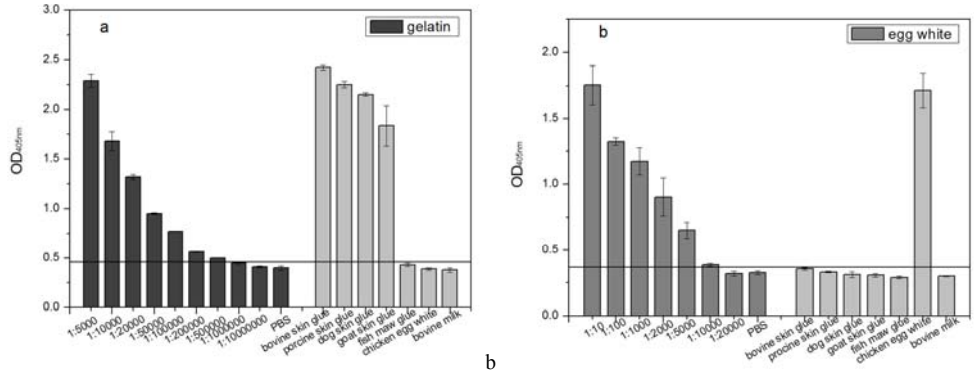


Fig. 2. The performance of three anti-collagen antibodies to detect fresh and boiled mammalian skin glue. Results for No. 1 (dark gray), No. 2 (light gray) and No. 3 (white) antibody and the corresponding SD are reported for the tested animal glues showed on the X axis



a b

Fig. 3. Optical densities obtained from ELISA for different dilution ratios of proteinaceous binders to evaluate the sensitivity of the method for (a) gelatin and (b) egg white at the optimized primary and secondary antibody dilution of 1:500 and 1:500. OD_{405nm} responses for different antigens (light grey) are also shown in each graph to detect the specificity of the developed method. The error bars in graphs represent standard deviations

IFM procedure

A very small amount of painted materials (each the size of several mm^2) was carefully separated and embedded in acrylic resin. Heat or ultraviolet was not used during the solidifying of resin since they will denature the proteinaceous binders in the samples. The resin block was

cut into thin slices with the thickness of about 0.1-0.5mm and then these slices were glued on glass slides with a drop of acrylic resin and polished with a series of increasingly finer grades of micromesh (600, 1000, 2000 and 5000 mesh) to make the surface smooth and the cross-sections of samples exposed.

The IFM procedure was performed on the cross-sections of the samples. Firstly, a drop of the blocking solution was used to avoid antibodies to react nonspecifically with the sample. The primary antibody was added and incubated overnight at 4°C. Then the fluorescein-labeled secondary antibody was added and incubated in the dark at room temperature for 2h. The samples were observed under the microscope with corresponding excitation wavelengths of fluoresceins. Between each step the cross-sections were rinsed with 5mL PBS for three times. All the IFM observations were performed at least three times on each sample and the sample slices were not used repeatedly.

Results and Discussions

Selection of antibody to detect collagen

Animal glue used as proteinaceous binder in ancient Chinese artworks was prepared through prolonged boiling of connective tissues (bones, skins, tendons and other cartilaginous parts) from animals with the temperature of approximately 100°C. As collagen contained in animal glue was totally denatured after being boiled, the primary antibody used to detect animal glue preserved in ancient artwork must be capable of recognizing and binding denatured collagen. Three antibodies numbered 1-3 in *reagents* in Experimental section were examined to detect fresh and boiled mammalian animal glue and PBS was used as blank control. The antibody numbered 3 has been used in some previous articles [4, 16]. Figure 2 showed that all the three antibodies can detect fresh mammalian animal glues which were extracted from animal skin with distilled water without heating but only No.1 antibody can detect boiled ones. The principle of these three antibodies to react with fresh and boiled collagen was not studied and it could only be supposed that high temperature destroyed antigenic determinant of other two antibodies but reserved the antigenic determinant of the first antibody. It can also be derived from figure 2 that similar results were obtained among different mammalian animal glues, which meant that antibodies could not distinguish between different species of animal glues. As the similarity between structures of collagen in different mammal animal is generally larger than 95%, antibody could not recognize this subtle difference. Moreover, No. 1 and 2 antibodies were polyclonal antibodies which increased the probability of cross-reaction with collagen from different species. Based on the facts mentioned above, No. 1 antibody was chosen and standard condition of using this antibody to detect boiled collagen was shown in the following section.

Assay of standard condition of ELISA

To achieve the maximal sensitivity and minimal background of the method, antibody panel titrations of solutions of the proteinaceous binders were used to obtain the optimum dilution ratio of antibody for mammalian collagen and chicken ovalbumin. Different dilution ratios of gelatin and egg white were used as positive controls and PBS was chosen to be the blank control. Gelatin was used as dried animal glue. The results are shown in Tables 2 and 3. By comparing the measured optical densities of positive and negative controls, the dilution ratios of 1:500 for the primary antibody and 1:500 for the secondary antibody were chosen both in animal glue and chicken egg white determination experiments. At these antibody concentrations, the best signal-to-noise ratio was obtained. If increasing the concentration of

antibody, the mean OD_{405nm} of PBS blank control will rise, while if decreasing the concentration of antibody, the mean OD_{405nm} of positive controls will reduce.

From the results of the replicated analysis of PBS blanks, the detection limit (obtained by the blank mean OD_{405nm} plus three times the corresponding standard deviation) of animal glue and egg white was fixed at OD_{405nm}= 0.46 and 0.37, respectively. This indicates that samples with the average OD_{405nm} greater than the detection limit were considered positive in the immunological test, on the contrary, were considered negative. Calibration curves were depicted in figure 3.

In order to examine the sensitivity of this method, different dilution ratios of gelatin and egg white with concentration gradient were tested at the optimized primary and secondary antibody dilutions of 1:500 and 1:500, respectively. It could be seen from the calibration curves that the detection limit was reached at maximum binder dilutions of 1:500000 for gelatin (Fig. 3a) and 1:10000 for egg white (Fig. 3b). The response of the immunoassay for the two binders was very different because gelatin was dried binder and thus had a high content of collagen while the weight ratio of ovalbumin to total egg white was just around 12% [22]. It could be calculated that the minimum dilution of binder detectable by using of the developed method was cca. 0.2 g/L for gelatin and cca. 10 g/L for egg white.

Primary antibody specificity was examined by checking cross-reactivity with bovine skin glue, porcine skin glue, dog skin glue, goat skin glue, fish maw glue, egg white and milk. The results were shown in figure 3. It can be derived that the primary antibody (rabbit polyclonal antibody to collagen type I), which was used to identify mammalian collagen, was specific to mammal animal glue but not to fish maw glue, egg white and milk (Fig. 3a); meanwhile the primary antibody (mouse monoclonal antibody to ovalbumin), which was used to identify chicken ovalbumin, was only specific to egg white (Fig. 3b).

Assay of mimetic sample using IFM

The IFM procedure, applied on mimetic samples with and without corresponding binders which have undergone prolonged aging and were mixed with inorganic pigment, was performed to identify the feasibility of this method. During the experiment, we found that the autofluorescence is unavoidable [6]. Therefore, in order to avoid such interference, both the images before and after the fluorescence-labeled treatment at the same location was shown in figures 4 and 5. If the fluorescence appeared only after the IFM staining, it is regarded as specific emission and the target antigen exists in the place of fluorescence. As Fluor 488 was labeled in the determination of mammalian collagen, bright green fluorescence excited by blue light could be obtained; and as Fluor 594 was labeled in the determination of chicken ovalbumin, specific red fluorescence excited by green light could be observed.

The sample in figure 4a has three painting layers, i.e. malachite mixed with porcine skin glue in the first painting layer, azurite mixed with egg white in the second painting layer and cinnabar mixed with bovine skin glue in the third painting layer. It can be seen that bright green fluorescence was emitted from the first and third painting layer but not the second painting layer. This indicated that IFM could not only detect protein specificity and sensitively but also determine the location of the target protein accurately. Fig. 4c demonstrates the positive result of egg white detection. The negative results in Fig. 4b and 4d show that no specific fluorescence images were acquired for blank samples. The results indicated that IFM could detect protein accurately. As the specificity of primary antibody has been examined in ELISA, IFM staining tests with irrelevant antigens were not carried out.

Analysis of samples from Xumi Mountain Grottoes

The ELISA and IFM protocols for the identification of animal glue and egg white have been applied to investigate the proteinaceous binders in the real samples from Xumi Mountain Grottoes and the results are presented in figure 5 and summarized in Table 4. The results of both ELISA and IFM found that sample XM 1(Fig. 5a), XM 4 (Fig. 5c) and XM 7 (Fig. 5f) show positive results to animal glue; sample XM 3 (Fig. 5b) shows positive result to egg white; sample XM 6 (Fig. 5d and 5e) shows positive result to both animal glue and egg white. Sample XM 1 consists of four layers, *i.e.* the thin gilded layer, the red painting layer, the white preparation layer and the ground layer, and bright green fluorescence is gathered in the ground layer. It can be derived from this phenomenon that animal glue is mixed with clay to enhance the bonding effect of the finger of the sculpture. Expect sample XM1, fluorescence is gathered in the surface painting layer. Therefore, it is highly possible that both animal glue and egg white are used as binders in the polychrome layers of mural painting and clay sculpture on Xumi Mountain Grottoes.

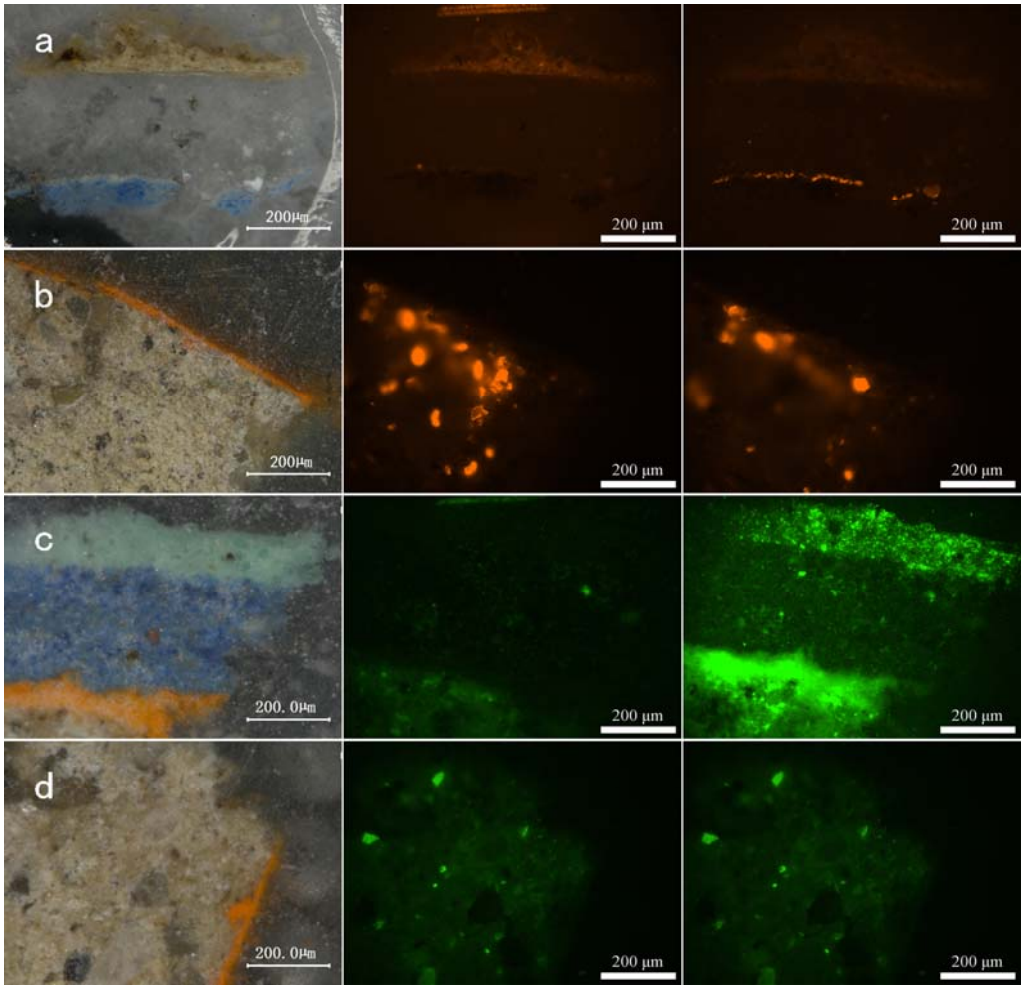


Fig. 4. IFM staining for mammalian collagen and chicken ovalbumin in mimetic samples, from left to right: OM 400X image, before and after IFM 400X image; (a) positive results for malachite mixed with pigskin glue in the first painting layer and cinnabar mixed with cowhide glue in the third painting layer but negative result for azurite mixed with chicken egg white in the second painting layer; (b) negative result for pure cinnabar; (c) positive result for azurite mixed with chicken egg white; (d) negative result for pure cinnabar

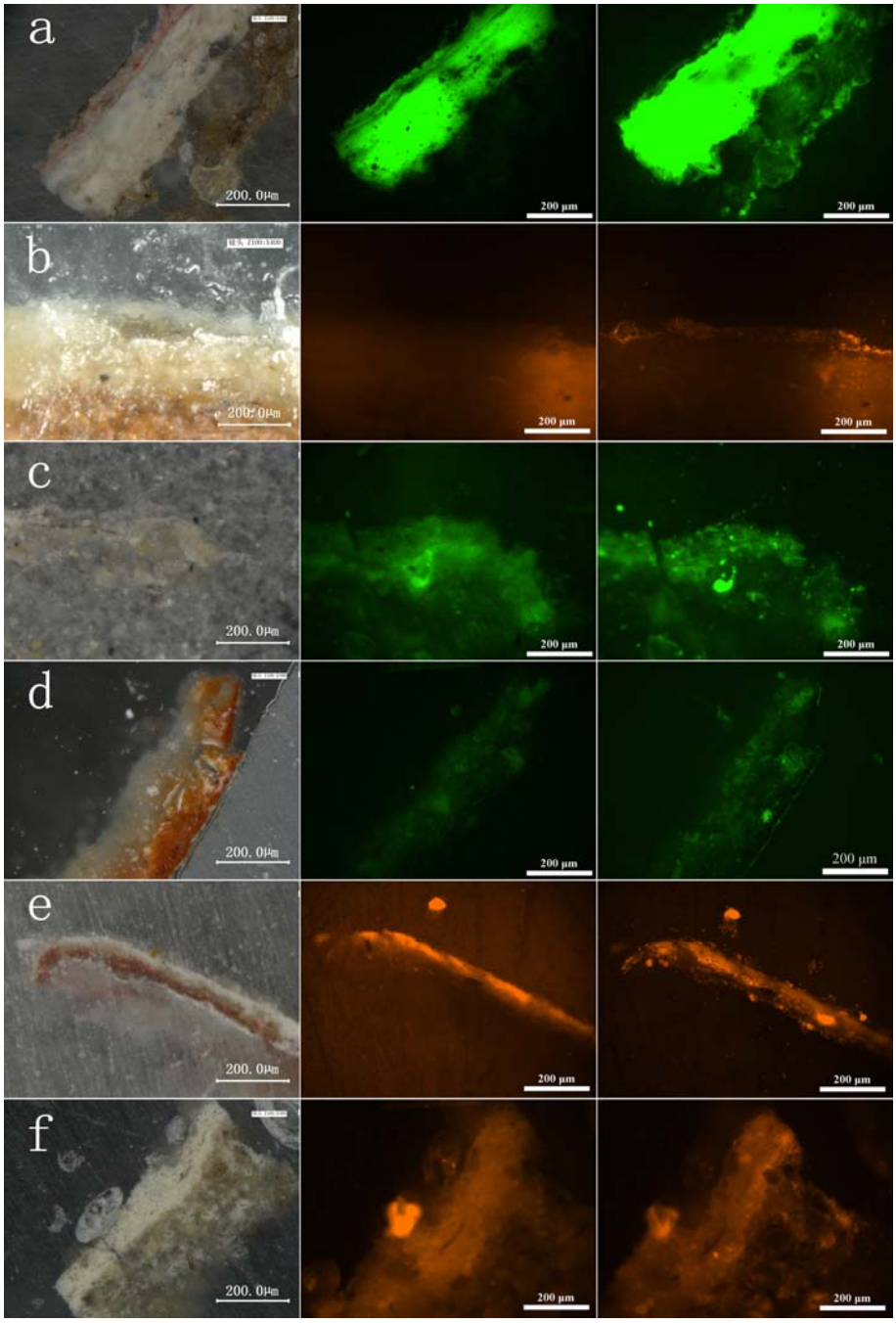


Fig. 5. IFM staining for real samples from Xumi Mountain Grottoes, from left to right: OM 400× image, before and after IFM 400× image; (a) the sample XM 1 presents positive result to animal glue; (b) the sample XM 3 presents positive result to egg white; (c) the sample XM 4 presents positive result to animal glue; (d) the sample XM 6 presents positive to animal glue; (e) the sample XM 6 present positive to egg white; (f) the sample XM 7 presents positive result to egg white

Conclusions

In the current study, we have successfully found the antibody which was positive to totally denatured animal glue. The animal glue and chicken egg white in the polychrome layers of mural painting and clay sculpture were identified in Xumi Mountain Grottoes by the specific antibody. To the best of our knowledge, this is the first study that verified the existence of the proteinaceous binding media and indicated its exact location in polychrome samples from Xumi Mountain Grottoes. These results validated that ELISA and IFM which use highly specific antigen-antibody interaction to detect the presence of the antigen are appropriate methods for the identification of the proteinaceous binders. By using these two methods in tandem, proteinaceous binders can be detected and located even when these proteinaceous binders have been denatured during the aging process. Moreover, these two methods possess the advantages of high sensitivity, good specificity, cost-effectiveness and need of only micro-scale samples. Coupled with other discoveries [3-5], it can be indicated that animal glue and chicken egg white were chosen as binders of polychrome layers extensively in artworks of ancient China.

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