Analysis of proteinaceous binding media used in Tang Dynasty polychrome pottery by MALDI-TOF-MS

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The proteinaceous binding media used in Tang Dynasty polychrome pottery was determined using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). The optimum conditions for the extraction and subsequent enzymatic hydrolysis of the protein sample were established, and applied to the pretreatment of model and historical samples. Complexation of EDTA in combination with dialysis was determined to be the most effective method for eliminating interferences in polychrome layers taken from historical samples. Model samples were aged by burial in loess so as to more closely replicate the ageing process of the historical samples. Using the aforementioned optimized conditions, the “peptide mass fingerprint” of the binding media of historical and model samples was determined by MALDI-TOF-MS. Comparisons between the “peptide mass fingerprint” obtained for the historical sample and those of the model samples proved that animal glue was used as the binding media for the Tang Dynasty polychrome pottery. It also shows that animal glue used as binding media enjoys a long history in China.

polychrome pottery, Tang Dynasty, proteinaceous binding media, animal glue, MALDI-TOF-MS

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The Tang Dynasty (AD618–AD907) was considered to be the golden age of Chinese history, and its capital established in Xi'an (formerly Chang'an), boasts a multitude of historic sites and ancient relics, particularly polychrome pottery [1,2]. The art of polychrome pottery is an example of the outstanding achievements in traditional Chinese culture [3,4]. Archaeological excavation and research have shown that the potteries were painted with rich polychrome (Figure 1) and that all polychrome layers were composed of natural pigments and binding media. Many natural pigments such as cinnabar [HgS], apatite [Ca₅(PO₄)₃OH] and malachite [Cu₂CO₃(OH)₂] have been found in polychrome layers [4,5].

However, very little information is available as to the identify of the binding media used in the polychrome layers of polychrome pottery, most probably because greater attention is given to the characterization of the pigments used. For the conservation and restoration of polychrome pottery, it is important to be able to identify and characterize the binding media within the polychrome layers. This knowledge not only provides fundamental information for art historians, but can also contribute to a better understanding of the deterioration processes accompanying aging, and ultimately assist in establishing suitable restoration and conservation methods.

The identification of proteinaceous binding media in the polychrome layers of polychrome pottery is problematic.

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Figure 1 Polychrome pottery of the Tang Dynasty.

Several analytical methods including Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy and various chromatographic techniques have been employed by other researchers in the identification of binding media [6–11]. These methods offer a number of advantages, but also come with deficiencies. FTIR and Raman spectrometry are unable to differentiate individual protein types in proteinaceous binding media. Chromatographic methods are not suitable for samples containing extremely low amounts of proteinaceous binding media, which are based on the characteristic ratio of certain amino acids, because of the losses of the amino acids or natural chemical reactions occurring during the hydrolysis step and the following derivatization in the sample pretreatment process [12,13].

Recently, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) has been successfully used in the identification of proteinaceous binding media [12–14]. MALDI-TOF-MS offers high sensitivity (in the range of pmol to fmole) [15], selectivity and simple pretreatment of samples, and its identification of different types of proteinaceous media is superior to that of other methods. In the present work, the proteinaceous binding media for the Tang Dynasty's polychrome pottery was identified by MALDI-TOF-MS. The conditions for sample pretreatment, proteinaceous extraction, enzymatic hydrolysis and peptide analysis were optimized. The extracted protein samples were cleaved with sequencing-grade trypsin to generate peptide fragments and the "peptide mass fingerprint" of the binding media from historical and model samples was determined by MALDI-TOF-MS. The results from this study identified animal glue as the binding media used in polychrome pottery from the Tang Dynasty.

1 Experimental

1.1 Reagents

Sequencing grade Trypsin was purchased from Promega (Madison, WI, USA). α -Cyano-4-hydroxycinnamic acid (CHCA), dithiothreitol (DTT) and acetonitrile were purchased from Merck (Darmstadt, Germany). Iodoacetamide (IAA) was purchased from Amresco (Ohio, USA). Trifluoroacetic acid (TFA) was purchased from Aladin (Shanghai, China). Urea and ammonium hydrogen carbonate were purchased from Xi'an Chemical Reagent Plant (Xi'an, China). Reversed phase ZipTip was obtained from Millipore (Billerica, USA). All chemicals were of analytical reagent grade. Deionized water was used throughout.

Fresh hen eggs were obtained from the countryside in the northern region of Shaanxi Province, China. Animal glues (ox skin, hide and bone glue) were purchased from a local market, and were made using traditional Chinese methods. Pigments (cinnabar, azurite, malachite and apatite) were purchased from Beijing Jinbizai Art Pigment Plant (Beijing, China).

1.2 Model samples

The model samples were prepared by mixing different pigments with either animal glue or egg. The model samples were composed of the pigments cinnabar, azurite, malachite and apatite (94% w/w for animal glue, or 84% w/w for egg), animal glue (6% w/w) or egg (8% v/v egg white and 8% v/v egg yolk) as the binding media. The mixture of pigment and binding media was spread as a thin film across several microscope glass slides and evaporated at room temperature.

The model samples were artificially aged by either burial in the loess or storage in a box at ambient temperature and humidity.

1.3 Historical samples

Fragments of polychrome layers taken from polychrome pottery of the Tang Dynasty were procured from the Engineering Research Center of Historical Cultural Heritage Conservation, Ministry of Education, Shaanxi Normal University. Microsamples were collected by gently scraping fragments from the polychrome layers using a scalpel. Every effort was made to prevent contamination in the sampling process.

1.4 Pretreatment and analysis

Proteins were extracted from the model samples (10 mg) or historical samples (50 mg) with 0.5 mL 0.1% TFA. The extraction solution was subjected to ultrasonic baths (3×15 min). The supernatant was obtained after centrifugation (15 min, 4000 r/min). The extracted proteins were hydrolyzed using a protocol adapted from the literature [14,16] with a little modification here. In brief, the protein solution was denatured in 0.5 mL of 100 mmol/L Tris-HCl (pH 7.25) by urea at a final concentration of 8 mol/L. Then, it was reduced with 1.9 mg of DTT and incubated for 3 h at 60°C, chilled, and 45 mg IAA added for alkylation in the dark at room temperature. Finally, 5 µL of 10 µg/mL trypsin was added to the solution (50 µL) and the mixture incubated for 10 h at 37°C.

All peptide mass fingerprint spectra were obtained using an AutoflexIII Smartbeam MALDI-TOF mass spectrometer (Bruker, Germany) equipped with a standard nitrogen laser (337 nm) in linear mode. A 1.0 µL aliquot of the peptide mixture was mixed 1:1 with the matrix (CHCA) solution. The resulting mixture was spotted on the stainless steel MALDI target and air-dried.

At least 300 laser shots were collected for each spectrum. All searches using peptide mass fingerprint spectra were carried out using the MSFIT program of Protein Prospector (<http://prospector.ucsf.edu/>).

2 Results and discussion

2.1 Protein extraction and enzymatic hydrolysis

It is important for the identification of proteinaceous binding media to select the appropriate method of protein extraction and condition of enzyme hydrolysis. The extraction of protein using concentrated HCl, NaOH or NH₃ have been proposed [12,14,17], but these methods often lead to selective degradation of amino acids, which affects the identification of protein binding media. In the current study, TFA was selected to extract the protein from both the historical

and model samples [16]. Different concentrations of TFA (0.05%, 0.1%, 0.5%, 1.0%, 1.5%) were tested for the extraction of protein. The 0.5 mL of 0.1% TFA was selected for the extraction of protein from the historical and model samples in the experiment.

To optimize the conditions for enzymatic hydrolysis of the protein sample, the concentration of trypsin and time of incubation was investigated. The extracted protein was incubated in the presence of differing concentrations of trypsin (2.5, 5.0, 7.5, 10 µL of 10 µg/mL trypsin) for a nominated period of time (8, 10, 12, 14, 16, 18, 24 h) at 37°C. The results demonstrated that the optimum conditions for enzymatic hydrolysis of the extracted protein were performed with 5.0 µL of 10 µg/mL trypsin at 37°C for 10 h.

2.2 Elimination of interferences

The identification of binding media in the polychrome layers of Tang Dynasty's polychrome pottery is made all the more difficult because of the complexity and extremely low quantity of binding media present, interference from natural pigments and other existing species, and the effects of denaturation. To eliminate interference from natural pigments and other species residing in polychrome layers, we tested the following separation methods: (1) dialysis, (2) reversed phase ZipTip, (3) dialysis with reversed phase ZipTip, and (4) complexation of metal ions with EDTA followed by dialysis against deionized water. The results are presented in Table 1. The choice of the superior separation method for the removal of interference was based on comparisons between the number of peaks related to peptides and the signal to noise ratio (SNR) observed in the MALDI-TOF-MS spectra recorded in the *m/z* ranges of peptide. The data indicate that the peptide mass fingerprint spectra have a higher background (low SNR) and that the number of peaks related to peptides is less for dialysis, separation with reversed phase ZipTip, and combined dialysis with reversed phase ZipTip. These results may be due to the influence of metal ions, such as Cu²⁺, Hg²⁺, Ca²⁺ and Mg²⁺, within the pigment of the polychrome layer and adhering soil. The metal ions can interact with the medium predominantly through the formation of strong complexes and give rise to interferences during the determination procedures [17,18]. Evidently, the best result, as evidenced by the greater number of peaks related to peptides, higher SNR and lower background, was achieved when complexation of EDTA was combined with dialysis (Table 1). Therefore, the separation method of combined complexation of EDTA with dialysis was selected for all further experimental work in the current study.

2.3 Effect of the ageing processes

Considering that the polychrome pottery of the Tang Dynasty has been buried in the loess for 1200 years, the ageing process could strongly influence the identification and characterization

Table 1 Peptide mass peaks and SNR in the MALDI-TOF-MS spectra for the animal glue model samples using different separation methods

Method	<i>m/z</i>	SNR
Dialysis	767.6, 1096.0, 1112.4, 1270.1, 1586.4, 1671.7, 1958.8, 1974.0, 2018.2, 2054.6, 2329.6, 2700.9, 2715.9, 2858.2, 3310.1, 3426.1	Low
Reverse phase ZipTip	798.1, 1129.5, 1799.3, 1974.0, 2055.7, 2685.5, 2697.9, 2808.7	Low
Dialysis with reverse phase ZipTip	780.8, 1060.1, 1196.4, 1270.5, 1286.7, 1474.8, 1611.7, 1703.8, 1973.2, 2052.1, 2699.4, 3114.2	High
Complexation of EDTA with dialysis	785.8, 837.0, 853.0, 887.1, 1089.7, 1096.4, 1106.5, 1112.5, 1268.7, 1329.7, 1429.0, 1437.1, 1461.0, 1564.1, 1588.9, 1650.4, 1801.5, 1834.6, 1924.7, 1977.8, 2059.0, 2183.2, 2217.9, 2350.1, 2473.0, 2483.6, 2707.2, 2783.2, 2856.0, 2969.6, 3103.3	Higher

of proteinaceous binding media in the polychrome layers. For this reason, the ageing process was investigated in model samples. One part of the prepared model samples (50%) was aged by storage in a box at ambient temperature and humidity, and the other part (50%) was aged by burying them in the loess. It was discovered that the peptide mass fingerprint spectra differed markedly between the two ageing processes. The number of peaks present in the peptide mass fingerprint spectra for the model sample aged in the loess was less than that for the sample stored in a box. The loss of peaks for the peptide mass fingerprint for the sample buried in the loess is most probably due to environmental factors such as changes in humidity, the activity of micro-organisms and other aggressive processes that have affected the integrity of the proteins structure. Not surprisingly, the

peptide mass fingerprint spectra of the model samples aged by burial are in close agreement with those observed for the historical sample. It should be emphasized that some of the identified peptides are characteristic and can certainly be used by animal glue or egg markers, such as 1476 and 2706 Da for animal glue, 2285 and 2903 Da for egg. As a result, the model samples were aged by burial 1 m deep in the loess for 6 months prior to analysis.

2.4 Comparison between the animal glue and egg model sample

The peptide mass fingerprints for the animal glue and egg model samples were determined and are presented in Figure 2. As illustrated, there is a distinct difference in the peptide

Figure 2 MALDI-TOF-MS spectra of the egg model samples (a) and the animal glue model samples (b).

mass fingerprint when animal and egg are used as the binding media in the model samples. However, the peptide mass fingerprints detected for the different types of animal glue (ox skin glue, hide glue and bone glue) would be expected to share some commonalities because they contain similar amino acid sequences. These results demonstrate that MALDI-TOF-MS can distinguish among classes of binding media, i.e. between egg and animal glue, and that it is an appropriate technique for the identification of proteinaceous binding media in the polychrome layers of the Tang Dynasty's polychrome pottery.

2.5 Analysis of proteinaceous binding media

The proteinaceous binding media used in the polychrome pottery of the Tang Dynasty was analyzed using MALDI-TOF-MS. The MALDI-TOF-MS spectra of the historical sample are presented in Figure 3. Many characteristic peptides can be observed from the "peptide mass fingerprint" and these peaks can respectively be attributed to different proteins (Table 2). The "peptide mass fingerprint" of the proteinaceous binding media in polychrome layers of the

polychrome pottery from the Tang Dynasty was compared with that of the model samples of egg and animal glue (Figure 2 and Table 3). The results show that the historical sample and the model sample with animal glue as the binding media shared very similar peptide mass fingerprints. The match in peptide masses between the historical sample and the model sample composed of animal glue as binding media exceeded 80% (Table 3). Any losses in peptide matches could be attributed to protein denaturation and deterioration of the historical sample during its 1200-year lifetime. The results for the historical sample are in agreement with data obtained for animal glue as binding media, confirming the existence of animal glue in the polychrome layers for the Tang Dynasty's polychrome pottery. This result is consistent with the research reported for polychrome terracotta army, wall paintings and sculptures in different periods of Chinese history [19–22].

3 Conclusions

The proteinaceous binding media used in polychrome pottery

Figure 3 MALDI-TOF-MS spectra of the historical sample of polychrome pottery.

Table 2 The assignments of protein fragments obtained from the historical sample of polychrome pottery

<i>m/z</i>	Sequence	Position	Protein
878.3	GYPLDGAK	291–299	Collagen α-1(II)
1180.8	GVAGKPGPRGQR	638–649	Collagen α-1(XI)
1477.5	GFPGTPGLPGVKGHR	276–290	Collagen α-1(II)
	SGETGASGPPGFVGEK	829–844	Collagen α-2(I)
1640.7	GEPSGVDGAPGKDGP	588–605	Collagen α-1(III)
	GERGFPLPGPSGEPGK	990–1066	Collagen α-1(II)
	GERGFPLPGPSGEPGK	967–983	Collagen α-1(I)
	GFPGADGVAGPKGPAGER	493–510	Collagen α-1(I)
2152.3	GDAGAPGAPGSQAGPLQGMPGER	709–732	Collagen α-1(I)
2213.4	GVPGIPGTRGPIGPPGIPGPGSK	443–466	Collagen α-1(X)
2904.9	GPTGPIGPPGAPGQPGDKGESGAPGVP	606–638	Collagen α-1(III)
	GIAGPR		
3267.8	GPPGPMGLTGPRGPVGGPGSSGAKG	296–331	Collagen α-1(XI)
	EMGDPGPQGPR		
3355.4	VFCNMETGETCVYPTQPSVAQKNWYISK	1288–1315	Collagen α-1(I)

Table 3 Comparison of the most relevant m/z of historical and model samples^{a)}

Sample	m/z
S ₁	990.5, 1047.6, 1248.2, 1347.2, 1437.0, 1557.4, 1583.2, 1689.7, 1775.5, 1861.4, 1962.1, 1974.3, 2084.2, 2238.2, 2239.3, 2285.7, 2562.2, 2645.4, 2903.4, 3390.1, 3860.8
S ₂	861.2, 877.2*, 893.1*, 1066.1, 1180.6*, 1308.7*, 1476.6*, 1640.1*, 1853.3*, 1995.5*, 2151.6*, 2212.5*, 2285.1*, 2385.2*, 2511.6*, 2511.8*, 2706.7*, 2781.3, 2808.8, 2904.1*, 3122.7, 3224.9*, 3266.2, 3313.8*, 3352.7, 3637.9, 4974.4
S ₃	878.3*, 893.7*, 1180.8*, 1309.1*, 1477.5*, 1640.7*, 1854.0*, 1996.0*, 2152.3*, 2213.4*, 2286.2*, 2386.4*, 2512.0*, 2512.2*, 2707.5*, 2904.9*, 3225.9*, 3267.8, 3315.1*, 3355.4, 4976.1

a) S₁, S₂ are egg and animal glue model samples, respectively (in Figure 2). S₃ is the historical samples of the polychrome pottery (in Figure 3). *The data indicate the match between historical and model sample.

obtained from the Tang Dynasty was identified using MALDI-TOF-MS. In the current study, complexation of EDTA with dialysis was successful in eliminating interferences from the polychrome layers. Model samples were aged by burial in the loess so as to replicate the natural aging process encountered by the historical samples. The proteinaceous binding media used in the polychrome layers of the polychrome pottery of the Tang Dynasty was identified as animal glue. The results support the evidence that animal glue been used as binding media in polychrome pottery, wall paintings and paintings in China for a great number of years.

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