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Sidestepping the challenge of casein quantification in ancient paintings by dot-blot immunoassay

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ABSTRACT

A straightforward procedure based on dot-blot immunoassay is proposed as an effective diagnostic tool suitable for detecting and quantifying milk casein in cultural heritage samples. A polyclonal primary antibody, denaturing conditions and the standard addition method were used to overcome barriers common to the traditional analysis of protein-based artistic materials, providing the possibility of achieving specific and detailed results in an easy and cost effective way. The optimized procedure detected, and successfully quantified, casein in both freshly dried and artificially aged model samples prepared with milk casein and various pigments (azurite, calcite, cinnabar, minium and red ochre). Moreover, the experiments revealed that pigments as well as artificial ageing did not differently influence the dot-blot response. Thermogravimetric analysis confirmed the obtained results. The detection and quantification of casein applied to canvas 70-years ago for conservation purposes provided final proof of the feasibility of the methodology. Despite the ageing, the complex matrix and the micro-size of the sample, it was possible to detect and quantify casein by dot-blot immunoassay. The specific and unambiguous result makes the proposed protocol a suitable procedure to recognize aged proteins with a degraded amino acid pattern.

1. Introduction

The determination of the chemical composition of paint samples is a useful tool in helping conservators to prevent deterioration and plan conservation treatments. Moreover, an accurate knowledge of the painting materials is extremely interesting from the art history point of view, and also helps specialists assess the authenticity of artistic work [1].

In recent decades, researchers have put a lot of effort into the development of analytical methods suitable for the detection and characterization of the chemical components found in the complex structure of paintings [2,3]. Protein recognition, especially, is a source of great interest as artists have been using proteinaceous substances as raw material since ancient times [4]. However, the detection and identification of proteinaceous materials is challenging [5]. Indeed, the uniqueness of a sample and its dimensions, the simultaneous occurrence of organic and inorganic materials, the chemical modifications undergone by these materials over time (ageing), and the presence of non-original restoration materials all give rise to analytical problems that make the reliable identification of the proteinaceus material a complex task [1,5,6]. The most employed techniques for the analysis of the protein components of paintings have been, to date, chromatographic and spectroscopic techniques, due to their great versatility in obtaining analytical information from both inorganic and organic materials [1,3,5]. However, despite their marked contribution to organic binder studies in art work, these techniques require complex sample pre-treatment, expensive equipment and a level of knowledge and competence not common in conservation laboratories [7–9]. Moreover, most of these analytical methods give little quantitative and structural information, and proteinaceous materials are detected without the identification of their biological origin [10].

On the other hand, immunochemical techniques represent a promising alternative tool to efficiently and selectively detect proteinaceous materials as they are sensitive to nanogram quantities of protein, and are based on the highly specific antigen-antibody reaction [9].

The present work proposes a procedure based on the dot-blot immunoassay as a simple and inexpensive method to identify proteinaceous binders in samples from works of art. The dot-blot immunoassay offers the advantage of analysing, with minimal sample

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pre-treatment, complex samples containing a protein mixture, facing the outstanding problem of structural alterations in aged proteinaceous material, and providing unambiguous significant and detailed results of easy interpretation [11]. Recently, the dot-blot immunoassay was successfully employed for the detection and quantification of egg white in samples from works of art [12]. To the best of our knowledge, no dot-blot immunoassay protocol has been reported for the detection of other proteinaceous materials in works of art.

Among proteinaceous materials, milk and casein were a valid alternative to egg tempera for artists in the past. Though less popular than egg tempera, milk and casein have been used as paint binders [13] especially for mural painting and polychrome objects as well as a conservation material [8,14–17]. In this work, a dot-blot immunoassay protocol for casein detection has been set up. The protocol was optimized on pigmented model samples, analysed both freshly dried and artificially aged, and then applied to the characterization of a naturally aged sample collected from a canvas used to detach a mural painting decorating the Monumental Cemetery walls in Pisa (Italy). Certainly the availability of an easy technique to detect and quantify casein in art work offers a significant advance in discerning casein as a major or minor organic component in artwork, helping conservators to better define deterioration processes undergone by art materials, and to choose the best and most suitable conservation treatment.

2. Materials and methods

2.1. Reagents

Primary affinity-purified rabbit polyclonal anti-bovine casein antibody (1 mg/mL) (RCAS-10A) was purchased from the Immunology Consultants Laboratory (Portland, OR, USA). According to the manufacturer, the antibody, raised using highly purified bovine casein from milk as immunogen, does not react with the serum proteins α-lactalbumin, whey, β-lactoglobulin, or lactoferrin. Secondary goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (A3687), fish gelatine (G7765), casein from bovine milk (C7078), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (SigmaFast BCIP/NBT, B5655), chicken egg albumin (A5378), bovine milk α-lactalbumin (L6010), bovine milk β-lactoglobulin (L0130), bovine serum albumin (A2153) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calf skin collagen (234112) was purchased from Calbiochem (Merk KGaA, Darmstadt, Germany). Skimmed fresh and powdered bovine milk, as well fresh goat milk, were bought from a local market. Milk casein for conservation was obtained from the restoration product supplier Bresciani (Milano, Italy).

2.2. Samples

2.2.1. Pigmented model samples

The optimized protocol was tested on pigmented model samples prepared according to Gambino et al. [12]. Briefly, two sets of glass slides were set up with a painted layer of a water mixture composed of milk casein for conservation and azurite ($Cu_3(CO_3)_2(OH)_2$), calcite ($CaCO_3$), cinnabar (HgS), minium (Pb_3O_4) and red ochre (Fe_2O_3), according to traditional painting techniques [18]. One set of painted glass slides was used as freshly dried samples, the other set was artificially aged in an ageing chamber Solarbox 1500e RH (Erichsen Instrumentation, Hemer, Germany). Samples were exposed for 720 h at 25 °C and 40% relative humidity with a Soda-lime glass UV filter to simulate indoor exposure, according to the normative UNI 10925: 2001 [19].

2.2.2. Sample from the Monumental Cemetery of Pisa

The naturally aged sample was a fragment of canvas used in 1945 to detach a mural painting, and glue it onto an asbestos cement support during a conservation treatment carried out soon after the Second World War [15]. The mural painting belongs to a cycle decorating the Monumental Cemetery walls in Pisa (Italy). Previous studies carried out on samples collected from these paintings, including the paint surface itself, the layer between the canvas and the support structure and the asbestos support, always showed the presence of animal glue and casein [15,16,20].

2.3. Protein extraction

One mg of each sample (freshly dried, artificially aged and naturally aged) was scraped from the surface and ground to a fine powder with pestle and mortar. The powder was suspended in 1 mL of 6 M urea prepared in 100 mM Tris-HCl pH 8.0 (6 M urea-Tris). The suspension was vortexed for 15 min, centrifuged for 15 min at 11000*g* at room temperature and submitted to dot-blot immunoassay. Supernatants from freshly dried and artificially aged model samples were 20-fold diluted with 6 M urea-Tris prior the dot-blot immunoassay, while supernatant from the naturally aged sample was analysed directly.

Standard proteins used in the optimization of the experimental conditions, and in the antibody specificity assay, were suspended in 6 M urea-Tris and submitted to the same extractive procedures.

2.4. Dot-blot immunoassay

2.4.1. Experimental procedure

The dot-blot immunoassay was performed according to Gambino et al. [12] with some modifications. Briefly, a MiniFold 1 Systems dot-blotting apparatus (Whatman) was assembled according to manufacturer instructions and samples were spotted onto nitrocellulose membrane (N9763–5EA, Sigma-Aldrich, St. Louis, MO, USA), let stand for 20 min and then adsorbed by gentle vacuum application. After washing steps (four times) with Tris-buffered saline (TBS; 0.1 M Tris-HCl pH 7.4, 5 M NaCl), the dot-blotting apparatus was dismantled and the membrane was: i) blocked with 2% fish gelatine in TBS for 12 h at 4 °C; ii) incubated with the primary anti-bovine casein antibody diluted 1:8000 in TBS and 1% fish gelatine for 3 h at room temperature; iii) washed four times with 0.05% Tween 20 in TBS (TBS-T) at room temperature (4 min each washing); iv) incubated with the secondary antibody diluted 1:3000 in TBS and 1% fish gelatine for 2 h at room temperature; v) washed three times with TBS-T and once with TBS at room temperature (4 min each washing). The membrane was finally incubated with the SigmaFast BCIP/NBT chromogenic substrate dissolved in 12 mL of deionised water (1 tablet, the resulting solution contained 0.12 mg/mL BCIP, 0.25 mg/mL NBT, 83 mM Tris buffer and 4.16 mM $MgCl_2$, pH 9.25–9.75) for 4 min and stopped by dilution with 50 mL of water.

After washing three times with water, the membrane was dried on filter paper and the image was digitized using Expression 1680PRO scanner (Seiko Epson Corporation, Amsterdam, Netherlands), removing all scanner automatisms and using the scanner cover as white reference for the white point correction.

Densitometric analyses were performed using ImageMaster 1D Elite software (Nonlinear Dynamics Ltd./Amersham Pharmacia Biotech, Cologno Monzese, Italy). Density values were obtained subtracting the ratio between volume (sum of intensities of every pixel within the defined area) and the defined area (pixel number) with the background density.

2.4.2. Interval of detection

The interval of detection was established for each single assay. Three analytical replicates of 15 two-fold serial dilutions (0.2–3500 ng) of standard bovine casein in 6 M urea-Tris were spotted onto the same membrane of the sample to be analysed. Background density was obtained by spotting 3 replicates of 6 M tris-urea only. Density values were plotted against standard casein quantity, and a calibration dose-response four-parameter logistic curve was obtained via GraphPad Prism (GraphPad Software, San Diego, CA, USA) providing, along with others, the bottom density and the top density value parameters. The