Journal of Archaeological Science 63 (2015) 65–71

Contents lists available at ScienceDirect

Journal of Archaeological Science

journal homepage: http://www.elsevier.com/locate/jas

Molecular evidence of use of hide glue in 4th millennium BC Europe

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ARTICLE INFO

Article history:
Received 3 February 2015
Received in revised form
6 August 2015
Accepted 16 August 2015
Available online 18 August 2015

Keywords:
Ancient proteins
Collagen
Animal glue
Domestic animals
Neolithic Europe

ABSTRACT

A well-preserved bow, dated by dendrochronology to 3176–3153 BC, was found at the waterlogged Neolithic site "Parkhaus Opéra" in Zurich (Switzerland). The surface of the bow, made of yew (*Taxus baccata*), was decorated with bark strips from a different, broad-leaved, tree species. In order to investigate whether the bark decoration was fixed to the bow with hide or fish glue, mass spectrometry (MS)-based ancient protein sequencing was attempted to detect possible traces of collagen residues. The sequences retrieved, in particular collagen type 3 (*COL3A1*), indicate that most probably skin, and possibly other slaughtering by-products, were used as the initial materials to produce hide glue. Amongst the candidate animal species that the glue could have originated from, cattle and domestic ovicaprids were confidently identified. This is, to the best of our knowledge, the oldest evidence of the use of animal-based glue in Europe. It demonstrates that in the late 4th millennium BC human communities, aside from benefitting from more commonplace primary and secondary products, also exploited domestic animals to extract a high value-added biochemical.

1. Introduction

Protein residues are probably the most abundant class of ancient biomolecules preserved in bones and soft tissues from archaeological and palaeontological contexts (Di Lullo et al., 2002; Lynnerup, 2007; Prockop and Kivirikko, 1995). Moreover, due to their chemical and mechanical properties, proteinaceous materials have also been used to satisfy primary human needs, including sheltering (Crone, 2007), transportation, clothing and food production (Hollemeyer et al., 2008; Kirby et al., 2013; Pinhasi et al., 2010). Production of adhesives, although less intuitive and more technologically refined, represented another way to use abundant animal proteins. In particular, hide glue, can be relatively easily extracted from animal skin, bone and other slaughtering by-products immersed in hot water. During this process, known as gelatinization, high temperature hydrolyses, denatures and solubilises collagen (Kuckova et al., 2009, 167). Collagen is a heterotrimer composed of three amino acid chains in a triple-helix structure. The transformation of collagen into gelatin is interpreted as the disintegration of the helical structures into random coils (Chen et al., 2014).

The use of adhesives is one of the most ancient human technological solutions for producing elaborate tools with superior mechanical properties, overcoming the technical limitations associated with products derived from a single component (Koller et al., 2001). Therefore, by allowing the production of objects made out of different parts and materials, the introduction and use of natural adhesives represents one of the most important breakthroughs in object manufacturing. Although easy to produce, even in rudimentary conditions, hide glue presents remarkably interesting properties, thus making it the adhesive of choice for many applications, until the relatively recent introduction of glues produced by organic synthesis (e.g. Brauns, 1858).

It is well documented that birch bark tar was used as early as the Middle Palaeolithic (Koller et al., 2001; Grünberg, 2002; Mazza et al., 2006; Wadley, 2010). Other adhesives, such as conifer resins and their heated derivatives, have been identified as adhesives in later periods (Regert, 2004). Conifer resin has been documented on a limited number of archaeological finds from the 7th Millennium BC in Canada (Helwig et al., 2008), and on a 5000 year-old copper age arrow point (Strnad, 1990). Together with beeswax, other substances, such as pistacia resins and plant oils, have also been found on archaeological objects from Bronze and Iron Age in Europe (Regert and Rolando, 2002). Similarly, the most ancient

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http://dx.doi.org/10.1016/j.jas.2015.08.012
0305-4403/

archaeological traces of a more elusive type of glue, based on collagen, originate from a site in Israel, radiocarbon-dated between 8310 and 8110 years before present (Nissenbaum, 1997). In Egypt, where glue was identified as an ingredient of plaster from the third millennium BC, an ingot of pure hide glue was also found at Deir-el-Bahari dating to the late second millennium BC (Lucas, 1934). To the best of our knowledge, however, no occurrence of animal glue has been documented in Neolithic Europe.

Analytical methods for detection of proteinaceous residues in archaeological and artistic material initially relied on gas or liquid chromatographic methods (GC or HPLC) coupled to mass spectrometry (MS) (Schneider and Kenndler, 2001; Chiavari et al., 1998). These approaches only allow amino acid identification and tentative reconstruction of the proteins they possibly originated from. Immunological methods, namely Enzyme-Linked Immuno Sorbent Assay (ELISA), and ancient DNA sequencing were also occasionally reported for the identification of collagen and hide glue in artworks (Cartechini et al., 2010; Albertini et al., 2011). However, none of these approaches allow for protein sequencing. This limit was overcome with the recent introduction of mass spectrometry-based methods for ancient protein identification (Ostrom et al., 2000) and sequencing (Nielsen-Marsh et al., 2002). The use of liquid chromatography, coupled to tandem mass spectrometry (LC-MS/MS), allowed species identification of biological samples, such as bone and skin, from archaeological contexts (Buckley et al., 2009; Brandt et al., 2014). The same approach was also applied to successfully identify protein binders in historical paintings (Tokarski et al., 2006), as well as food residues in pottery (Solazzo et al., 2008). Finally, a proteomics strategy based on LC-MS/MS was also used to identify the origin species of glues used in gilt from the 18th century (Dallongeville et al., 2011).

In 2009, during the archaeological excavation of the Neolithic settlement at the site “Parkhaus-Opéra” in Zurich (Switzerland), Fig. 1a and b, the discovery of an object made of yew (*Taxus baccata*), suggested the possible use of hide glue, Fig. 1c. The finding, dated by dendrochronology to 3176–3153 BC (Bleicher and Burger, in press.), was identified as the symbolic representation of a hunting bow. The preservation of wood was excellent due to the anaerobic conditions in the waterlogged deposit encasing the bow (Madigan et al., 1997; Bleicher and Schubert, 2015).

The bark on its surface was shaped in silvery-reddish strips showing large lenticels, a feature absent in yew. Although a more detailed anatomical characterisation was not possible, these traits were sufficient to confidently assign the bark to a broadleaved species and tentatively identify the origin of the bark to a young cherry tree. The straight edges and regular distribution of the strips along all the length of the bow, Fig. 1c-inset, strongly indicated that they were intentionally glued as a decoration on the surface of the bow.

In the Swiss Neolithic site of Cham Eslen, birch tar was utilised to glue birch bark to an axe handle (Gross-Klee and Hochuli, 2002). For the new find from Zurich, though, the use of birch tar can be ruled out since no dark residue was detected under the bark. Although in the Near East the use of collagen-based adhesives is recorded at much earlier times (Nissenbaum, 1997), so far no Neolithic European evidence of animal-based glue has been found. The earliest trace of adoption of hide glue in Europe is possibly a mention by Aristoteles (Anheuser, 2001). If confirmed by molecular evidence, the case we describe would represent the most ancient occurrence of hide glue in Europe, and provide the first positive evidence for its use in this area predating the written sources by several millennia.

We set out to test whether hide glue or, taking into account that the prehistoric settlement was situated on the lakeshore, fish glue was used to affix the bark to the body of the bow. To achieve this, we attempted LC-MS/MS proteomics analysis of organic residues extracted from wood flakes collected under the bark.

2. Materials and methods

Immediately after unearthing the object, small wood flakes from two areas underneath two different bark strips, lifted from the body of the bow, were carefully removed using a razor blade. Since the sampled area had been covered by the bark for over 5000 years, chances of contamination before or during sample collection can be considered extremely limited. A negative control prepared and analysed following exactly the same procedure adopted for the archaeological samples, except for the initial addition of ancient material, was processed together with the samples. Each of the two samples, approximately 15 mg, was then transferred in a protein LoBind 1.5 mL tube (Eppendorf, Germany). Each sample was processed independently as follows. Each sample was re-suspended in 500 μL of 100 mM ammonium bicarbonate solution at pH 8.00 and was briefly washed by mechanical shaking at room temperature to remove soil residues and other debris, prior to pelleting by centrifugation at 14,000 g for 5 min. The supernatant was discarded and this step was repeated two times in total. The pellet was re-suspended in 1 mL 1.2 M HCl and incubated at 4 °C for 24 h to gently hydrolyse collagen. This approach was chosen with the aim of maximising recovery of collagen altered by prolonged exposure to protein crosslinkers present in soil, such as clay, humic and fulvic acids, as well as wood lignin. After centrifugation at 20,000 g for 20 min, the supernatant was removed and stored, while the pellet was re-suspended in 200 μL 50 mM ammonium bicarbonate pH 8.00 and incubated at 70 °C for 24 h, to solubilise collagen. The pH was checked using pH indicator strips and adjusted to 8.00 with diluted ammonium hydroxide. The sample was then pelleted by centrifugation at 14,000 g for 10 min and the supernatant was collected and reduced by incubation for 1 h at 60 °C with 5 mM (final concentration) of dithiothreitol (VWR-BDH, England), dissolved immediately before use. The reduced cysteines were then alkylated by incubating in the dark, at room temperature, for 45 min with 15 mM (final concentration) chloroacetamide (Sigma–Aldrich, Denmark), dissolved immediately before use. The pH was then checked using pH-indicator strips, adjusted to 8.00 with diluted ammonium hydroxide and digestion was started by adding 4 μL of 0.5 μg/μL sequencing grade trypsin solution (Promega, Nacka Sweden) and incubating at 37 °C overnight. The following morning, 2 μL of fresh trypsin were added and digestion was extended for 6 additional hours. Digestion was quenched with 10% trifluoroacetic acid to a final concentration of 0.2–0.8%, as necessary to reach pH < 2.00. After centrifugation at 14,000 g for 10 min, to precipitate any eventual insoluble residue, the tryptic peptides in the supernatant were immobilised on C18 stage tips as previously described (Cappellini et al., 2012).

Peptide mixtures were analysed by online nanoflow reversed-phase C18 liquid chromatography tandem mass spectrometry (LC-MS/MS), as described previously (Cappellini et al., 2012). Briefly, the LC-MS system consisted of an EASY-nLCTM system (Proxeon Biosystems, Odense, Denmark) connected to the LTQ-Orbitrap Velos (Thermo Electron, Bremen, Germany) through a nano-electrospray ion source. An aliquot of 5 uL of each peptide sample was auto-sampled onto and directly separated in a 15 cm analytical column (75 μm inner diameter) with a 90 min gradient from 5% to 30% acetonitrile in 0.5% acetic acid at a flow rate of 250 nL/min. The effluent from the HPLC was directly electrosprayed into the mass spectrometer by applying 2.2 kV through a platinum-based liquid-junction.

The LTQ-Orbitrap Velos instrument was operated in data-dependent mode to automatically switch between full scan MS