ANTHROPOLOGY

Ancient protein analysis in archaeology

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The analysis of ancient proteins from paleontological, archeological, and historic materials is revealing insights into past subsistence practices, patterns of health and disease, evolution and phylogeny, and past environments. This review tracks the development of this field, discusses some of the major methodological strategies used, and synthesizes recent developments in archeological applications of ancient protein analysis. Moreover, this review highlights some of the challenges faced by the field and potential future directions, arguing that the development of minimally invasive or nondestructive techniques, strategies for protein authentication, and the integration of ancient protein analysis with other biomolecular techniques are important research strategies as this field grows.

INTRODUCTION

Ancient protein analysis can be defined as the identification and study of proteins from archeological, historical, and paleontological remains and materials. Although work in the field stretches back to the 1950s, methodological advances in the field of mass spectrometry (MS) since the 2000s have revolutionized the scope and diversity of applications. In particular, techniques based on MS, generating protein sequence information, as well as insights into ancient proteomes (paleoproteomics) and metaproteomes are being applied to a diversity of paleontological and archeological materials in history and prehistory. While the analysis of proteins has somewhat lagged behind that of ancient DNA (aDNA), recent applications to archeological and paleontological samples beyond the limit of DNA preservation as well as more nuanced insights into cultural heritage and ancient lifeways are increasingly revealing the utility of ancient protein analysis.

Reviews of potential best practices (*1*), the application of ancient protein analysis to evolutionary studies (*2*, *3*), art and cultural heritage objects (*4*), and MS approaches in paleoproteomics (*5*) have recently been presented. In contrast, this review will highlight the diversity of applications of ancient protein analysis in the field of archaeology and, after outlining some of the identification strategies used for ancient protein identification, will focus on the application of this technique to distinct themes: subsistence practices and ecologies, osteobiographies and individual lifeways, material culture analysis, as well as studies of human evolution. Additionally, this review will highlight some of the challenges and potential future directions of this growing field, arguing that key areas for future research include investigations of protein survival and degradation, the development of minimally destructive or noninvasive techniques, and the integration of multiple biomolecular techniques in archeological science.

APPROACHES IN ANCIENT PROTEIN IDENTIFICATION

There is a diversity of methodologies used for the identification of ancient proteomes, metaproteomes, peptides, and amino acids. While there are multiple techniques for identifying the general presence of protein (or a proxy for protein), such as Fourier transform infrared (FTIR) [e.g., (*6*)], total organic content [e.g., (*7*)], and nitrogen content [e.g., (*8*)], this review will discuss some of the main analytical techniques used in protein identification, including the identification of individual amino acids (the building blocks of protein), individual target proteins by immunoassay analyses, techniques in proteomics (the analysis of a suite of proteins in a biological unit), and metaproteomics (the analysis of proteins from multiple taxa). While all of these techniques continue to be applied in studies of ancient proteins to varying degrees, the invention and adoption of MS markedly altered protein identification approaches in archaeology and paleontology.

Amino acid analysis

The survival of proteins in archeological and paleontological material has been explored since the discovery that amino acids can be detected in fossils, where Abelson (*9*, *10*) estimated total protein and amino acid content from Pleistocene mollusk shells and other fossils. The survival of amino acids continues to be explored to the present day, in particular, in investigations of amino acid racemization (AAR) dating, as well as bulk amino acid analysis to investigate the biological origin of particular artifacts and objects [e.g., (*11*–*13*)]. For example, proteins entrapped in shells have been extensively studied for their role in AAR [e.g., (*14*, *15*)] and their bulk composition has been analyzed as a taxonomic indicator (*16*, *17*). High-performance liquid chromatography (HPLC) is commonly used to identify ancient amino acids, but gas chromatography–MS (GC-MS) has also been used [e.g., (*18*)].

By looking at overall proportions of amino acids in different biological tissues, it can be possible to identify their taxonomic origin, because the overall composition of amino acids can differ between taxa. Mollusk shell artifacts are abundant in the archeological record as objects of adornment or food processing objects and often survive over long archeological time scales. However, they usually lose diagnostic features when worked into different forms or become fragmented, preventing the identification of their taxonomic origin, a feature that enables an understanding of local resource use, or nonlocal trade and exchange. Applying bulk amino acid analysis to an archeological context, Demarchi *et al.* (*12*) examined the bulk composition of amino acids in archeological shell beads found in a funerary context from the Early Bronze Age site of Great Cornard, Suffolk. This revealed that the most likely taxonomic origin for the beads was *Nucella* or *Antalis*, both found locally, suggesting a local manufacturing, rather than long-distance trade of these precious personal ornaments. More recently, MS approaches have additionally been applied to the analysis of avian eggshells (*19*–*22*) and marine and freshwater mollusk shells (*23*) to reveal insight into the use,

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manufacturing practices, and trade of local or exotic shell raw material and ornaments.

In addition to serving as indicators of taxonomic origin, amino acids are also used for dating. AAR is a dating methodology whereby the relative proportion of D- and L-amino acid enantiomers is a measure of the time elapsed since an organism's death. In living tissues, the L-configuration of amino acids is typically present, but after death, L-amino acids are altered (racemized) to the nonbiological D-configuration. Therefore, the relative degree of these two forms is an indicator of postmortem time elapse (*24*). Complexities to AAR arise in the varying influence of environmental factors affecting rates of racemization, such as temperature, pH, and humidity, and at times, AAR has been subject to criticism and skepticism (*25*). However, AAR has seen particular success in highly mineralized substrates that act as closed systems (*26*). Such substrates for analyses include mollusk and avian shell (*11*, *26*, *27*) as well as tooth enamel (*13*, *28*), which are substrates where intracrystalline amino acids should remain endogenous and predictable. AAR has been extensively applied to understanding quaternary chronologies, for example, in identifying the presence of early human activity in Europe (*29*), the formation of terrestrial deposits in the British Isles (*30*), as well as the speed of local shell midden accumulations (*31*) and improved dating coverage in shell midden sites (*11*).

Immunoassays

Ancient proteins can be identified by immunoassay approaches. These assays are based on the identification of a reaction between a specific antibody and an antigen (a target protein) and are routine approaches used for detecting the presence or absence of particular target proteins of interest across the medical and food sciences (*32*, *33*). Multiple immunological approaches have been applied to archeological samples, including radioimmunoassays; gel-based separation immunoassays, such as crossover immunoelectrophoresis (CIEP); and enzyme-linked and immunofluorescence approaches [reviewed in (*4*, *34*)].

Immunoassay-based approaches have been extensively applied to understanding the use of artifacts for food acquisition, especially stone (lithic) tools and particularly in North American contexts. Examples include the reported identification of human, deer, and bovid protein residues from stone tools at the Boreal site of Cummins near Thunder Bay [7500 to 9000 before the present (B.P.)] (*35*), buffalo and elk proteins from the buffalo kill site, Head-Smashed-In Buffalo Jump in Alberta, Canada (*36*), and horse protein residues from Clovis points from Wally's Beach, southwestern Alberta, dating to 11,000 and 11,300 B.P. (*37*). A further controversial discovery was the reported identification of human myoglobin in ceramic vessels and a coprolite as evidence of cannibalism (*38*), an archeological interpretation that was met with criticisms (*39*) and response (*40*). Outside North American contexts, this approach has been used to understand food acquisition and processing at the Neolithic site of Çayönü Tepesi (*41*) in southeast Turkey, where evidence of *Bos primigenius*, sheep, goat, and even human blood were reported from stone objects using Labstix tests (dip-stick tests used for the presence of hemoglobin in urine) and patterns of hemoglobin crystallization. In a more recent example, analysis of flint artifacts in combination with use-wear analysis at the early Neolithic site of Almhov near Malmo, southern Sweden, has suggested evidence of fish consumption (*42*).

However, some of these approaches have been substantially criticized for their lack of reliability in archeological contexts. The main criticisms include the potential of false positives and negatives due to protein degradation, problems with the performance of kits or assays developed for the medical or food sciences, lack of blind testing and replication, and cross-reactions both within and beyond the family level. For example, Eisele *et al.* (*43*) performed tests on 54 modern stone tools with simulated archeological conditions, finding that evidence of protein residues were absent within 1 year of manufacturing. Additionally, tests on modern stone tools deliberately processed with different animals (*44*) found that a commercial laboratory performing CIEP analysis could make correct identifications in only 37% of samples and observed cross-reactions both within and beyond the family level. A lack of blind testing and replication has also been noted (*45*) [with a response (*46*)], and negative results in studies focusing on ancient (*47*) and modern experiment datasets (*48*) have also been presented. As such, research has tended to move away from studies of protein preservation on stone tools; moreover, many of these studies were performed before the invention and application of MS technologies.

Peptide mass fingerprinting

Approaches to identifying ancient proteins fundamentally changed with the invention and adoption of MS-based approaches. In contrast to immunological approaches, which are based on detecting the presence or absence of particular target proteins, MS involves the ionization of molecules, whose mass and charge are precisely detected. In contrast to techniques based on immunoassays, this means that it is possible to study ancient proteomes (paleoproteomics) rather than just individual target proteins of interest. In one of the first studies to apply MS-based techniques to archeological material, Ostrom *et al.* (*49*) applied matrix-assisted laser desorption/ionization MS (MALDI-MS), alongside other approaches, to investigate the survival of bone proteins. The authors generated purified extracts and used MALDI-MS to identify peptides (protein fragments) from the bone protein osteocalcin in 53,000-year-old bison bone.

Peptide mass fingerprinting using MALDI–time-of-flight (TOF) MS is the basis of zooarchaeology by MS (ZooMS) (*50*). Peptide mass fingerprinting involves the analysis of extracted peptides using MALDI-TOF MS, whereby a "fingerprint" consisting of masses of individual, diagnostic peptides is generated. These masses are then matched against reference fingerprints, which are generated from samples with known taxonomies. ZooMS uses peptide mass fingerprinting to identify the taxonomic origin of archeological and historic material. The degree of taxonomic specificity is variable and depends on the evolutionary distance between taxa. It is typically possible to distinguish between family or even genera-level taxa, but differentiating between species can be challenging due to insufficient sequence variability between protein sequences of different taxa. The approach was built upon work that examined the taxonomic origin of pelts, furs, feathers, and other animal products to aid in the identification of animal product trade and import/export (*51*). For archeological questions, this analysis not only is typically applied to fragmented or morphologically nondiagnostic bone fragments but also has been applied to a range of other archeological material culture, including parchments (*52*–*54*), ivory (*55*), eggshell (*19*, *22*), combs (*56*), and leather objects (*57*).

Tandem MS (LC-MS/MS)

In contrast to the peptide fingerprinting approach described above, which relies on fingerprints of mass spectra, liquid chromatography–tandem

mass spectrometry (LC-MS/MS)–based approaches result in the identification of peptide or protein sequences. This identification is achieved through two or more fragmentation steps, whereby the mass of a peptide (parent ion) is detected in one mass analyzer, before further fragmentation into smaller peptides or amino acids ahead of detection of these smaller masses in a second mass analyzer. The peptide sequence of the parent ion can then be identified through the reconstruction of unique mass fragments. Like peptide mass fingerprinting, LC-MS/MS approaches have been applied to a diversity of substrates and archeological inquiries, including human and animal skeletal tissues, artifacts and artifact residues, and well-preserved organic remains, which are highlighted in sections below.

Top-down LC-MS/MS

Typically, LC-MS/MS approaches in paleoproteomics are "bottom-up" protein identifications. In this approach, proteins are fragmented into peptides during the extraction process, and these peptides are identified using LC-MS/MS, enabling protein identification of complex mixtures of extracted peptides. In contrast, top-down–based proteomics involves protein identification while the protein is still in its unbroken (native) state. Some advantages of such a strategy are that the data produced can inform on protein fragmentation and posttranslational modifications (PTMs) (alterations to amino acid side chains). However, this emerging approach has so far seen limited application to ancient protein archeological contexts but offers great potential for understanding protein survival and degradation (*4*).

Quantification

Bottom-up approaches used by LC-MS/MS analyses in ancient proteomics have typically not been quantitative, meaning it is challenging to make reasonable comparisons of protein abundance between samples. However, recent studies have adopted stable isotope labeling and label-free approaches to assess differences in protein expression in ancient samples. Isobaric tags for relative and absolute quantitation approaches (*58*) have been applied in the examination of animal age of parchments, by looking at the expression of particular proteins in uterine and nonuterine parchment (*53*), to understand animal-use and manufacturing practices of these key historic objects. While elevated expression levels of four proteins were observed in uterine parchments, none of these proteins were detected in archeological samples, limiting the application of this approach in this case. For bone, label-free quantification (*59*), whereby counts of spectra or signal intensity can be used to estimate protein abundance, was applied to bone to examine age dependence of these abundances (*60*). Jersie-Christensen (*61*) applied a quantitative analysis to metaproteomic (analysis of proteins from a substrate containing multiple taxa) samples of ancient dental calculus to identify oral disease and health states in medieval individuals. Using label-free quantification and tandem mass tag labeling, this analysis of protein expression revealed that certain individuals had notable contributions from bacteria known to be associated with periodontitis, and human immune protein expression could also be classified into healthy or diseased oral health states. This study demonstrates that rich and more refined insights can be gained into identifying disease processes using quantitative proteomic approaches, although there is still much to explore with understanding differential degradation rates of different proteins and whether protein abundance in archeological remains is an accurate reflection of biological processes occurring during life.

PROTEIN PRESERVATION AND AUTHENTICATION

Protein survival

Studies of protein survival and degradation, grounded in the field of organic geochemistry, represent some of the earliest research agendas in the field of paleoproteomics (*62*). Early studies provided important insight into the survival and degradation of ancient biomolecules, revealing that proteins undergo diagenesis into shorter, fragmented peptides—generating challenges for downstream analysis. These studies demonstrate the importance of strategies discriminating endogenous and modern sources of protein in archeological investigation—a major research strategy and concern that remains in present-day research (*1*).

Proteins can survive in archeological and paleontological materials for over 1 million years *(16)*. While the survival of proteins in dinosaur remains has been reported (*63*–*66*), but has also received previous criticisms (*67*–*69*), what is known is that the survival of ancient proteins in deep time has been linked to mineralized substrates. Biological substrates composed of a mineral component, such as bone, shell, and dental calculus, have been targeted as sources of ancient proteins, although rare, well-preserved organic remains sometimes found in cold or frozen, arid, or oxygen-deprived environments have also been targeted. For example, the survival of the bone protein collagen is associated with hydroxyapatite, the main mineral comprising bone structure (*70*). Similarly, the survival of proteins or peptides in eggshell has been linked to the binding of uterine proteins to the calcium mineral of egg shell, facilitating its long-term survival in the paleontological record (*21*). Recently, enamel has been explored as a well-preserved source of ancient proteins that can be analyzed to enable the phylogenetic analysis of extinct animals and humans (*71*, *72*), as well as the identification of sex based on sequence differences in the enamel protein amelogenin (*73*–*75*). While not as protein-rich as other skeletal tissues, enamel is the densest mineral in the human body, a trait that enables it to preserve well in archeological and even paleontological contexts. As these studies have made clear, different archeological substrates become subject to different degradation histories.

Authentication: Markers of degradation

In the field of aDNA, detecting markers of DNA degradation is integral for discriminating modern contamination from authentically old endogenous aDNA. Similarly, ancient proteins have been studied for their patterns of degradation and how these patterns can be used as markers for endogenous, ancient proteins as opposed to potential modern contaminants. Proteins undergo damage via bond cleavage through hydrolysis or enzymatic attack (particularly from the burial environment), resulting in protein fragmentation. As well as these patterns of fragmentation, MS-based protein identification is able to detect patterns of degradation owing to the detection of mass shifts that are the result of chemical changes to amino acid side chains. In biological protein synthesis, these changes are termed PTMs and include, for example, the addition of a phosphoryl group (phosphorylation) or the addition of a carbohydrate (glycosylation).

While PTMs can be the result of biological processes integral to protein function, modifications to amino acid side chains have also been linked to age-induced degradation, which may be a useful indicator of protein degradation or even authentically ancient proteins, although other factors, such as heat, may also induce modifications (*76*). The most well studied of these modifications is deamidation (the removal of an amide group) of glutamine and asparagine (a

process that transforms these amino acids into glutamic acid and aspartic acid, respectively), with glutamine deamidation more commonly applied as asparagine is subject to more influence from a protein's tertiary structure (*77*). While rates of deamidation vary with temperature, pH, secondary and tertiary structures, and laboratory procedures (*78*, *79*), deamidation can be a useful indicator of protein degradation, although still more work is needed to untangle contributing factors to these rates. Deamidation patterns have therefore been explored in multiple archeological substrates, such as collagen degradation in bone (*80*, *81*), keratin degradation in textiles (*82*), and food proteins from ancient dental calculus (*83*).

Deamidation rates are also influenced by the presence of adjacent amino acids, through steric hindrance and the presence of charged amino acids, with some amino acids adjacent to glutamine causing slower deamidation rates. On the basis of the previous work by van Doorn *et al.* (*84*) exploring deamidation rates based on the presence of other amino acids adjacent to glutamine (site-specific deamidation), Ramsøe *et al.* (*83*) developed a novel software tool (deamiDATE) to measure these site-specific deamidation rates. Such approaches help to discriminate authentically ancient proteins from potential modern contaminants. Other PTMs have also been detected and studied in relation to ancient protein degradation. For example, Hill *et al.* (*85*) identified hydroxylysine glucosyl galactosylation in 120,000-year-old bison bone remains, indicating the persistence of this biological modification involved in bone mineralization, as well as other modifications that may be the result of bone collagen taphonomy such as the loss of hydroxylation/glutamic semialdehyde and carboxymethyllysine (*86*).

APPLICATIONS OF ANCIENT PROTEIN ANALYSIS IN ARCHAEOLOGY

A diversity of methodologies, described above, has been developed and applied to archeological materials, including a focus on developing strategies of ancient protein authentication. After outlining these methodological approaches, this review will focus on outlining recent applications across four key themes in archeological research: understanding past subsistence strategies, human evolution, osteobiographies and past lifeways, and insights into historic and prehistoric material culture. Such applications touch on multiple fields in archeological research and demonstrate the diverse and ever-growing nature of ancient protein analysis.

Subsistence practices

Ecologies, environments, and animal-human interactions

ZooMS has been extensively applied to identifying and expanding faunal assemblages pertaining to ancient animal husbandry practices, in particular in the identification of taxa that can be morphologically challenging to discriminate, such as sheep and goat remains (*87*, *88*), as well as the expansion of zooarcheological datasets through the analysis of fragmented, unidentifiable remains. For example, ZooMS analysis has been developed and used to identify sheep and goat remains based on mass differences in collagen peptides between these two taxa (*89*). Following the identification of this differentiation by Buckley *et al.* (*89*), this approach has now been applied to understanding farming practices in Neolithic Turkey (*90*), Greece (*91*), the Chalcolithic Southern Levant (*92*), as well as differences in the use of sheep and goats in early pastoral Tanzania (*93*) and the relative importance of ruminant species in mixed-herd economies of Central Asia (*94*). The ability to distinguish between these two taxa enables greater insight into the choices made by farmers and pastoralists with regard to mixed-herd economies, variations in herd management strategies (such as culling, breeding, mobility patterns, and foddering), as well as differences in these animal's responses to environmental stress and vulnerability to disease.

ZooMS has also been applied to understanding past ecologies and environments through the expansion of zooarcheological identifications in archeological assemblages, an approach that can be particularly useful when examining highly fragmented bone assemblages (*95*–*97*). For example, Hofman *et al.* (*98*) applied to ZooMS to shell midden assemblages in the Californian Channel Islands, identifying the predation of at least three marine mammalian taxa and supporting the idea that the hunting of these animals supported the peopling of the Americas.

Through the analysis of another substrate, ZooMS is also revealing temporal and geographic differences in the use of animals in medieval economies (*52*–*54*). Parchments, animal skins used for manuscript production until the invention and adoption of paper, can be viewed as biomolecular archives with tight chronologies of animal use and geographic specificity (*99*). For example, by examining 72 pocket bibles from Europe, Fiddyment *et al.* (*53*) were able to identify differences in animal exploitation for these objects in different regions, reflecting potentially available livestock and preferences over the use of certain animals for parchment production, for example, sheep in England, goat in Italy, and calf in France.

Food and cuisine

While ZooMS has been extensively applied to enriching zooarchaeological datasets on subsistence practices and the environment, LC-MS/MS–based approaches have yielded insight into ancient foods and culinary practices. Where environmental contexts have enabled good organic preservation, proteins have been extracted and identified from whole foodstuffs to identify their constituents, for example, from food remains found at cemetery sites in the Tarim Basin, China, including whole cheese curds (*100*–*102*) and bread (*103*), and in Egyptian tombs (*104*), where dry conditions may have slowed biomolecular deterioration. Similarly, artifacts found in frozen and cold contexts also harbor protein preservation that enables a detailed insight into food remains. For example, Colonese *et al.* (*105*) identified proteins from wheat from a residue sticking to the surface of a well-preserved wooden box in the Swiss Alps. Proteomic analysis has also been applied to the stomach contents of "Ötzi the Iceman," in combination with aDNA, reconstructing Ötzi's "last meal," revealing evidence of meat and cereals (*106*).

As well as identifying taxa and particular plant or animal tissues used for food, ancient proteins are also being used to study food processing and culinary techniques, owing to the fact that the proteins differ in the abundance, type, and chemical modifications when subject to different culinary processes. For example, Yang *et al.* (*101*) examined preserved remains of putative cheese curds associated with mummified individuals from Bronze Age Xinjiang. After identifying that proteins in the substrate derived from milk, the authors examined one specific protein identified in detail, kappa-casein, which plays a key role in cheese curd formation. Kappa-casein undergoes specific cleavage of the protein chain when subjected to coagulation by the enzyme rennet. The team observed that such a cleavage pattern was absent, suggesting that these cheese curds were not formed as a result of rennet coagulation but were likely created by using acid or microbial-based dairy processing. This example

demonstrates the level of insight possible with the analysis of proteins, generating insight into the taxa identified (cow), the specific food product (dairy), and its potential processing technique (acid/microbial coagulation).

Beyond these exceptional examples, dietary analysis using proteomics has been applied to ancient dental calculus to identify food consumption practices directly from past human mouths. Dental calculus is mineralized tooth plaque, also known as dental tartar, which accumulates on teeth during life and is often preserved on skeletal teeth. Ancient proteins derived from dietary sources have been extracted from this reservoir, revealing evidence of a range of consumed foods. While informative on the consumption of particular foodstuffs, it is clear that this approach may not capture the diversity of foods consumed by that individual, and there is a bias toward certain food groups (*107*). Where this technique has been particularly informative is in exploring past patterns of dairy consumption (*108*–*112*). In contrast to other biomolecular approaches to study past dairying, ancient protein analysis is able to identify the taxa consumed, acting as a "zooarchaeology by proxy," enabling the identification of animal taxa that may have been important in local economies, environments, and cuisines. In addition, this technique enables an exploration of individual dietary patterns that may be tied to other information gained from osteological or archeological analyses, such as indications of status and health.

Ceramics have also been turned to understand food preparation practices. While the analysis of fats, oils, and waxes is typically applied to this ceramic material culture [e.g., (*113*–*116*)], the analysis of proteins has the potential to yield further insights into the taxa used, as well as insights into food mixing (*116*). While some success from extracting proteins from ceramics has been reported (*117*–*120*), challenges have arisen in the removal of protein from the silica matrix and protein preservation in this context is not fully understood (*7*, *121*, *122*).

Paleoproteomics has also been applied to understanding animal subsistence. For example, Tsutaya *et al.* (*123*) examined the rib bones of a neonate dog, revealing the preservation of dog milk proteins and suggesting that food proteins derived from the animal's stomach contents have the potential to be preserved in adjacent tissues. Investigations of animal foddering strategies have long been examined by stable isotope analysis and other approaches, e.g., (*124*–*126*), and with the development of proteomic strategies, this is a likely avenue of future research.

Future work in the analysis of ancient proteins to understand past food use will be in uncovering biases in the entrapment and preservation of food-derived biomolecules in different archeological substrates. For example, Hendy *et al.* (*107*) reported that in samples of ancient dental calculus there appears to be a bias toward the detection of milk proteins over other foods. In addition, future work may be in the integration of protein analysis with other biomolecular approaches, such as lipids, to develop a more well-rounded picture of food contributions, as well as the development and adoption of strategies for protein authentication.

Material culture and personal objects

Paleoproteomics is also applied to organic objects representing personal material goods, such as jewelry and other adornments, clothes, and other material objects. Such insights help to understand manufacturing practices, resource use and object movement, and cultural exchange. For example, Jensen *et al.* (*127*) analyzed an osseous ring from early Neolithic Denmark, revealing that the ring was made from locally available red deer (*Cervus elaphus*), an observation not possible from morphological analysis of the artifact alone. Where cold, arid, or oxygen-deprived environments have permitted exceptional organic survival, paleoproteomics has also been applied to understand manufacturing of clothing, garments, and textiles such as leather and skin garments (*57*, *128*, *129*), woolen textiles, and other fibers (*130*–*132*) and silks (*133*, *134*). Other forms of adornment have also been explored using proteomic approaches, in contexts where such objects survive. Mai *et al.* (*135*), using several molecular characterization tools, analyzed "cosmetic sticks" found in Xiaohe Cemetery from the Early Bronze Age, revealing evidence of muscle proteins from cattle, which suggests that, in part, these cosmetic sticks were made from animal tissue.

Such analyses have the potential to inform on broader questions of cultural contact and exchange. Viking combs, highly personal objects intimately connected with personhood and social status, have been analyzed using ZooMS to reveal insights into historic cultural contact. For example, von Holstein *et al.* (*56*) examined combs from Orkney, an island archipelago in northeastern Scotland, to examine the history of contact between Atlantic Scotland and Scandinavia. The analysis revealed that comb types native to Orkney were composed of locally available red deer, suggesting that people were using local resources for the creation of objects with their own cultural style, and called into question the degree of cultural contact between these two regions of the Viking world. In contrast, ZooMS analysis of combs from the southern North Sea region and the Scandinavian peninsula revealed evidence of contact between urban centers (*136*). Similarly, objects from the medieval town of Odense, including a gaming piece, have been analyzed using ZooMS, demonstrating that some objects are manufactured from animal species well out of their immediate geographic range, reflective of long-distance trading, while other objects showed evidence of local resource use and manufacturing (*137*).

The paleolithic and human evolution studies

Paleoproteomics holds great potential for understanding human evolution through use of ZooMS as a screening tool for identifying hominin remains within fragmentary faunal assemblages and in understanding hominin phylogeny through the identification of discriminating single amino acid polymorphisms (SAPs) between hominin taxa. The application of ancient proteins to the study of human evolution has recently been reviewed by Welker (*2*), and its application to evolutionary inference alongside other biomolecules has been explored by Cappellini *et al.* (*3*).

ZooMS has been applied to rapidly and cost-effectively detect hominin remains among fragmentary faunal assemblages (*138*–*140*). While this has typically focused on cold or temperate environments (*2*), future research will seek to apply this approach to subtropical or tropical zones (*141*). As well as providing data to enrich the faunal assemblage in its own right, ZooMS analysis enables screening for hominin remains ahead of further downstream biomolecular analysis such as radiocarbon dating (*142*) or aDNA analysis (*143*).

While both biomolecular classes are subject to degradation and taphonomic processes, ancient proteins offer an alternative to aDNA for understanding hominin phylogeny, especially in scenarios where aDNA preservation may be insufficient to be informative (*144*). Proteins harbor SAPs between homologous proteins of different taxa. These SAPs originate from nucleotide substitutions on protein-coding genes, causing variation to the protein sequence of amino acids, and therefore, this amino acid variation facilitates phylogenetic

analysis. This approach was adopted in the identification of Neanderthal remains using paleoproteomics, through the identification of distinct SAPs in the bone protein COL10a1 (*145*). Similarly, distinct SAPs in the bone protein COL1a2 enabled the identification of Denisovan remains found in the Tibetan plateau, the first Denisovan-like individual found outside of Denisova cave (*144*).

Noncollagenous bone proteins, as well as proteins from other tissues, have also been explored as potential candidates for use in paleoproteomic phylogenetic studies (*146*). For example, Welker *et al.* (*71*) explored paleoproteomic analysis on tooth remains of *Gigantopithecus*, an extinct giant ape. While no surviving proteins were detected in dentine, 409 unique peptides matching to six proteins were identified in enamel. Comparison with enamel protein sequences from Hominoidea revealed that *Gigantopithecus* represents a pongine clade and a sister taxon to *Pongo* spp. While requiring reliable detection of SAPs, facilitated in part by full ion coverage of peptides, these deep-time studies demonstrate the longevity of protein survival to yield phylogenetically informative data. This approach has also been applied to the study of extinct faunal taxa, including *Stephanorhinus* (an extinct genus of rhinoceros) (*72*), *Macrauchenia* and *Toxodon* (South American ungulates) (*147*, *148*), *Castoroides ohioensis* (giant beaver) (*149*), and *Plesiorycteropus* (Malagasy aardvark) (*150*), and will continue to yield insights into other ancient taxonomies.

Osteobiographies and paleopathologies

Ancient protein analysis is also contributing to the study of human osteology and paleopathology through the identification of proteins informative on physiology, biological processes, and disease in human remains, including skeletal material and mummified remains.

Estimating age and sex are key facets of osteological identification. Recently, several studies have examined the preservation of amelogenin, an enamel protein whose gene is found on the X and Y chromosomes. Protein sequences of amelogenin differ in the X and Y variants, meaning that it is possible to use this protein as a marker of biological sex. Building on forensic approaches for sex identification using enamel, this concept was first applied by Nielsen-Marsh *et al.* (*151*), who also applied the technique to identifying the sex of Neanderthal remains. More recently, more sensitive MS techniques have enabled increased protein sequence coverage (*74*, *75*, *152*). This approach enables an expansion of osteoarcheological datasets where sexing individuals through osteological analysis alone may not be possible due to ambiguous morphology or fragmentary remains. Using label-free analysis to indicate protein abundance, Sawafuji *et al.* (*60*) examined the abundance of alpha-2-HS-glycoprotein as a function of biological age, showing a significant negative correlation in this protein's abundance with biological age, building off observations from modern bone studies that bone proteomes alter with age (*153*). Similarly, Procopio *et al.* (*81*) observed alpha-2-HS-glycoprotein declining during animal aging in bovine remains.

Bone proteomes have also been explored in terms of understanding ancient disease processes. Archeological bone can contain proteins associated with immune responses, such as leukocyte-derived proteins (*60*), although their use in antibody-based testing for the detection of ancient infectious disease has been questioned (*154*). Dental calculus has also been shown to contain a proteome indicating an acute immune response (*155*), a reflection of its composition as an oral biofilm. Ancient protein analysis has also been applied to paleopathological lesions; Bona *et al.* (*156*) explored extracted proteins to identify an osteogenic sarcoma, and identified protein biomarkers of a tumor. Paleoproteomic approaches, while in their infancy for the study of ancient disease, may find particular application in discovering disease processes where osteological evidence of disease is lacking. The proteinaceous content from well-preserved mummified material has also been explored to shed light on immune responses and disease processes at the time of death. For example, buccal swabs from 500-year-old mummified remains from the Andes revealed evidence of an immune response to an active bacterial infection at the time of death (*157*) and proteins extracted from the brain of Ötzi the Iceman also suggested evidence of wound healing (*158*). Exploring pathways that may lead to the differential degradation of different proteins [e.g., (*159*)] will be key in understanding whether immune-associated proteins found in archeological contexts are an accurate reflection of those that occurred during life. As well as examining human proteome profiles, shotgun proteomics has also been applied to ancient disease identification through the detection of proteins derived from pathogenic bacteria. These include studies to examine the oral microbiota preserved in ancient dental calculus, identifying evidence of respiratory and oral taxa (*155*), dental pulp, investigating evidence of *Yersinia pestis* (*160*), and mummified remains for identifying evidence of tuberculosis (*161*, *162*).

Future work exploring quantitative protein expression in ancient remains may help to further untangle evidence of disease and health states. For example, Jersie-Christensen *et al.* (*61*) analyzed the metaproteome of 20 individuals from medieval Denmark, using a combination of shotgun proteomics with quantification methods. On the basis of bacterial taxa, the study identified two groups of individuals: one showing evidence of microbiome dysbiosis (a disruption of a healthy state oral microbiome) and one group showing a healthy microbiome. In addition, future work may see the application of metaproteomics (the analysis of proteomes from multiple taxa in one sample) to ancient microbiomes. Such work has already been applied to ancient dental calculus (*61*, *155*, *163*) but may also be applied to other microbiome sites in the future, such as the analysis of the gut microbiome through paleofeces or coprolite analysis. Here, studies may focus on understanding microbial community function, rather than the roles of individual taxa, as is the focus of many modern microbiome studies (*164*). Moreover, while previous studies on microbiota in archeological remains have focused on bacteria, future work may focus on identifying viruses, for example, in identifying envelope or capsid proteins.

FUTURE RESEARCH AGENDAS

Some emerging analytical and data analysis strategies have been highlighted above; this section explores potential key general future directions in paleoproteomics research. While Hendy *et al.* (*1*) highlighted the need for adequate data curation and sharing practices, peer reviewing of raw data, and future reanalysis of data with more refined or updated bioinformatic strategies, here I argue that future research could encompass the development and application of nondestructive or minimally invasive methodologies, the integration of paleoproteomics with other biomolecular tools in archaeology, and the continued exploration of protein preservation and degradation.

Nondestructive methodologies

The analysis of proteins, as is typical of most biomolecular analysis, is often destructive. However, several strategies have been developed to

mitigate artifact damage, including minimally invasive sampling strategies and the use of buffers that enable sample reuse. With the rise of minimally destructive or nondestructive sampling techniques, analysis of rare, unique, or elaborate organic objects may open new avenues into understanding object manufacture and use, which previously may have been inappropriate to subsample or damage. Recently, McGrath *et al.* (*165*) developed and applied a nondestructive sampling technique to worked bone points from Iroquoian village sites in southern Quebec. Here, ZooMS extractions were performed on the bags the objects were stored in, rather than the objects themselves, as friction contact from the plastic with the object allowed loose collagen molecules to be separated from the bone point. This novel approach resulted in taxonomic identifications of these bone artifacts, revealing a diversity of species, including bear, human, and deer. Strategies for analyzing bone that do not require full demineralization of the bone mineral have also been pioneered and adopted, including protein extraction with ammonium bicarbonate (*166*) and ammonium phosphate (*167*). To non-osseous material, nondestructive methodologies have also been applied. Fiddyment *et al.* (*53*) developed and applied minimally invasive techniques to study parchments (manuscripts) made of animal skin by gently rubbing parchments with an eraser, which pulls off loose collagen fibers, and extracting collagen proteins from the eraser shavings. To artworks, manuscripts, and other cultural heritage objects, applied polymer discs have also been explored as a potential route to identifying proteinaceous components with minimal damage (*168*).

Integration of multiple biomolecular techniques

When destructive methodologies do occur, a future approach should be to maximize sample information obtained from that damage through the application of multiple biomolecular techniques where relevant. For example, complementary aDNA and ancient protein analysis has proven useful for studies of evolution and phylogeny, where one can be used to verify the findings of the other (*3*). Similarly, the more specific taxonomic resolve of aDNA analysis can be used in tandem with the functional information gained from ancient proteins. For example, Warinner *et al.* (*155*) used both aDNA and ancient protein approaches in the analysis of ancient dental calculus, where aDNA yielded insights into the taxonomic diversity of the ancient oral microbiome and protein analysis demonstrated the survival of biofilm functional profiles. The use of multiple methodologies from the same sample is also of value in the study of ancient food and diets, where the analysis of fats, proteins, and microfossils, alongside macroscopic and microscopic analyses, can generate a more well-rounded picture of food sources and use (*105*, *120*). For example, in the analysis of a residue adhering to the interior of a wooden box found in the Swiss Alps, Colonese *et al.* (*105*) detected the presence of alkylresorcinols found at appreciable levels in wheat and rye. The presence of this "cereal biomarker" detection was further supported by the presence of cereal proteins from the same sample. Future work may also focus on the integration of different biomolecular techniques within laboratory procedures, such as the dual extraction of aDNA and ancient proteins (*169*), the simultaneous extraction of DNA, protein and microfossil analysis from samples of dental calculus (*170*, *171*), as well as the integration of ZooMS, stable isotope analysis, and radiocarbon dating during collagen preparation (*142*, *172*).

Mechanisms of survival and taphonomy

Ancient biomolecules undergo degradation over time, including fragmentation into smaller constituents and chemical alteration. Understanding this degradation is critical for study feasibility and identifying ancient protein authenticity. Unlike, for example, C to T transitions occurring at the ends of DNA reads, there is currently no single identified marker of ancient protein degradation. Deamidation of glutamine and asparagine has been extensively explored (described above), and tools have been developed to identify patterns of deamidation associated with time (*83*); however, there is still much to uncover with regard to other influences beyond time on these rates of deamidation (such as predepositional influences, like the effects of cooking on food proteins). Nonetheless, such a tool is a step toward developing strategies in the field of examining protein endogeneity. Other strategies to examine protein authenticity include modeling the mechanisms of protein survival. For example, Demarchi *et al.* (*21*) examined the survival of eggshell peptides, showing that those surviving into deeper time demonstrated mineral binding.

Moreover, possible future avenues of research also include the development of screening methodologies using minimally or non-invasive techniques ahead of any more destructive and/or more costly downstream analysis to generate a sense of protein preservation. Recently, FTIR has been proposed as an in-field screening ahead of downstream ZooMS analysis through the detection of amide bonds that may be the result of the presence of amino acids (*173*). For some studies, MALDI-TOF–based analyses have been used as a screening tool for LC-MS/MS analysis (*174*). In future work, techniques such as MALDI imaging MS (MALDI-IMS) may be used to examine protein preservation within objects to optimally select areas for sampling (*5*), a strategy to prevent unnecessary sample destruction.

CONCLUSION

On the back of changes and improvements in methods of detection and analysis, the information gained from ancient protein analysis has evolved over time. Early approaches focused on the detection of protein content to assess ancient protein survival; now, the use of MS is enabling the detection of sequence information in samples of greater complexity. While assessments of ancient protein survival are still fundamental to ancient protein research, more recent approaches are focusing on gaining insight into cellular processes and biological functions from ancient samples, mechanisms of protein degradation, and evolutionary insights beyond the reach of aDNA preservation. In the last several years, ancient protein analysis has gained particular attention (*175*–*177*), especially with regard to the technique's reach into deeper time and the generation of new conclusions unattainable using other biomolecular approaches. With the rise in this field, there is the opportunity for lessons to be learned from other fields of archeological science with regard to minimizing sample damage, integration with other biomolecular techniques, appropriate data sharing, and sample curation.

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