Empirical Evaluation of Bone Extraction Protocols

Timothy P. Cleland1*, Kristyn Voegele2, Mary H. Schweitzer1,3

1 Department of Marine, Earth, Atmospheric Sciences, North Carolina State University, Raleigh, North Carolina, United States of America, 2 Biology Department, Concordia College, Moorhead, Minnesota, United States of America, 3 North Carolina Museum of Natural Sciences, Raleigh, North Carolina, United States of America

Abstract

The application of high-resolution analytical techniques to characterize ancient bone proteins requires clean, efficient extraction to obtain high quality data. Here, we evaluated many different protocols from the literature on ostrich cortical bone and moa cortical bone to evaluate their yield and relative purity using the identification of antibody-antigen complexes on enzyme-linked immunosorbent assay and gel electrophoresis. Moa bone provided an ancient comparison for the effectiveness of bone extraction protocols tested on ostrich bone. For the immunological part of this study, we focused on collagen I, osteocalcin, and hemoglobin because collagen and osteocalcin are the most abundant proteins in the mineralized extracellular matrix and hemoglobin is common in the vasculature. Most of these procedures demineralize the bone first, and then the remaining organics are chemically extracted. We found that the use of hydrochloric acid, rather than ethylenediaminetetraacetic acid, for demineralization resulted in the cleanest extractions because the acid was easily removed. In contrast, the use of ethylenediaminetetraacetic acid resulted in smearing upon electrophoretic separation, possibly indicating these samples were not as pure. The denaturing agents sodium dodecyl sulfate, urea, and guanidine HCl have been used extensively for the solubilization of proteins in non-biomineralized tissue, but only the latter has been used on bone. We show that all three denaturing agents are effective for extracting bone proteins. One additional method tested uses ammonium bicarbonate as a solubilizing buffer that is more appropriate for post-extraction analyses (e.g., proteomics) by removing the need for desalting. We found that both guanidine HCl and ammonium bicarbonate were effective for extracting many bone proteins, resulting in similar electrophoretic patterns. With the increasing use of proteomics, a new generation of scientists are now interested in the study of proteins from not only extant bone but also from ancient bone.

Introduction

The application of non-traditional, high-resolution analytical techniques to the study of ancient bone proteins holds great potential for increasing our understanding of the evolution, radiation, and ecology of extinct organisms. However, these analyses are challenging because they require the detection and interpretation of molecular components that are present at very low concentration and/or are highly altered. All of these high-resolution techniques require some form of protein extraction, and bone provides unique challenges for extraction of its protein components. Unlike soft tissues, proteins present in bone are secreted by osteoblasts and subsequently biomineralized with hydroxyapatite (Ca5(PO4)3OH; [1,2]). It has been proposed that the presence of these minerals, in addition to providing stability to the mineral lattice. By removing the calcium from the mineral, it releases the enzyme-antibody complexes on enzyme-linked immunosorbent assay and gel electrophoresis. Moa bone provided an ancient comparison for the effectiveness of bone extraction protocols tested on ostrich bone. For the immunological part of this study, we focused on collagen I, osteocalcin, and hemoglobin because collagen and osteocalcin are the most abundant proteins in the mineralized extracellular matrix and hemoglobin is common in the vasculature. Most of these procedures demineralize the bone first, and then the remaining organics are chemically extracted. We found that the use of hydrochloric acid, rather than ethylenediaminetetraacetic acid, for demineralization resulted in the cleanest extractions because the acid was easily removed. In contrast, the use of ethylenediaminetetraacetic acid resulted in smearing upon electrophoretic separation, possibly indicating these samples were not as pure. The denaturing agents sodium dodecyl sulfate, urea, and guanidine HCl have been used extensively for the solubilization of proteins in non-biomineralized tissue, but only the latter has been used on bone. We show that all three denaturing agents are effective for extracting bone proteins. One additional method tested uses ammonium bicarbonate as a solubilizing buffer that is more appropriate for post-extraction analyses (e.g., proteomics) by removing the need for desalting. We found that both guanidine HCl and ammonium bicarbonate were effective for extracting many bone proteins, resulting in similar electrophoretic patterns. With the increasing use of proteomics, a new generation of scientists are now interested in the study of proteins from not only extant bone but also from ancient bone.

These challenges have led investigators to propose many different protocols (20 or more) for demineralization and extraction of bone for protein analyses. Because of the many variants, choosing the best method for a protein of interest or particular analytical technique becomes challenging. Generally, investigations of bone protein use a weak inorganic acid [8,9], a diluted strong acid [10,11,12,13,14], or ethylenediaminetetraacetic acid (EDTA; [14,15,16,17,18,19,20,21,22,23,24,25]) for demineralization. However, the use of either weak inorganic acids or diluted strong acids may hydrolyze the proteins of interest, and therefore, cleave them into difficult to characterize fragments [26,27]. EDTA ligates calcium removing it from the mineral lattice. By removing the calcium from the mineral, it releases the phosphate into solution resulting in demineralization.

After mineral has been removed, bone proteins are typically extracted into solution for further analyses. In non-biomineralized tissues, this is usually accomplished by urea [28,29] or sodium dodecyl sulfate (SDS; [30,31]), but these methods have seldom been used in bone. These methods create challenges because they can modify proteins by forming adducts (SDS; [32]) or by carbamylation (urea; [29]). Instead, guanidine HCl [8,13,15,16,17,18,19,20,21,22,23,24,25] or ammonium bicarbonate [10,11,12] have been found to effectively solubilize bone proteins. Guanidine HCl functions by denaturing proteins into random coils, making
them more soluble [31], but many proteins are soluble in ammonium bicarbonate as well. Ammonium bicarbonate is widely used in proteomics-based techniques because it completely breaks down to ammonia and carbon dioxide and is directly compatible with typical digestive enzymes (e.g., trypsin) without the need for desalting [9,10,11].

Once the mineral has been removed and proteins extracted, previous investigators have characterized these proteins using molecular biological techniques [19,20,21,33], mass spectrometry [10,11,21,34], amino acid analysis [11,35,36,37], and/or assessment of stable isotopes [14,38,39]. Each technique requires different concentrations of the proteins of interest, different sample preparation, and differing degrees of sample purity. For example, molecular methods utilizing antibodies can detect very small concentrations of a protein, either in situ or in solution, based on epitope identification [7,40]; however, these techniques do not provide primary sequence information, and therefore, only allow for crude phylogenetic inference [40,41,42,43,44,45,46,47]. Mass spectrometry, on the other hand, requires greater concentrations of proteins and can provide primary sequence, but high concentration of salts can interfere with ionization and interpretation [48]. Amino acid analysis has been used extensively for racemization studies to determine kinetics of amino acid changes within bone collagen, crudely determining protein content, and assessing the amount of protein degradation (e.g., [11,35,36,49,50]); however, this method results in the loss of primary sequence information and cannot address all amino acids from a sample because some amino acids are not stable under necessary acidic hydrolysis conditions [51]. Stable isotope studies also do not provide primary sequence information, but can provide additional environmental and ecological information for the organisms studied (e.g., [38,39,52]).

Bone is composed of a variety of proteins and other molecules, but archaeologists and paleontologists have focused on collagen I (e.g., [10,11,19,20,21,34,53,54]) and osteocalcin (e.g., [55,56,57,58]) because they are the two most abundant proteins in extant bone and both have high potential for preservation [6]. Collagen I is, by far, the most dominant protein, making up ~85–90% of the organic constituents in bone [2]. Because collagen is vital for bone structure and formation, its sequence is highly conserved across taxa, making it less useful for determining relationships of extinct organisms [41]. It has, however, been used to determine vertebrate relationships at the species [21,59] and supraspecific level (e.g., being able to identify bone from elephantidae but not from a specific taxon; [53]). Non-collagenous proteins (NCPs) have sequences with greater sequence variation providing potentially greater phylogenetic resolution than collagen I. However, identifying these proteins in bone extracts is difficult, even in extant bone, because they make up a relatively small fraction of the total protein content. If these proteins are not preferentially collected or concentrated, their signal can be overwhelmed by much more abundant proteins. Recently, investigators have used mass spectrometry to study NCPs [8,9,13], which may be used to provide a better understanding of relationships between extinct organisms, and in addition may elucidate diagenetic processes present within bone.

Here, we compare previously described methodology for demineralizing and extracting proteins from bone. We use extant ostrich (Struthio camelus) bone as a baseline for the expected protein composition of bone, and extant moa bone as an exemplar for what is expected for ancient material. Both electrophoretic separation and enzyme-linked immunosorbent assay (ELISA) are employed to compare these different methods for yield and purity.

Results and Discussion

Modern Bone Protein Extraction Methods

Even though many different protocols (Tables 1, 2, 3, 4) have been used for preparing ostrich bone samples, only a few show some amount of protein solubilization, resulting in a white powder after lyophilization, with yields ranging from negligible to 29.5% (Table 5). Urea/thiourea and SDS have seldom been used for solubilization of protein in bone, but they have both been shown to be effective in this study on both gels and in ELISA. Both methods show at least some antibody binding in ELISA (C+C and Rabilloud, Table 6) and a few bands on gels (Fig. 1). SDS has additional post-extraction benefits including usage in polyacrylamide analyses (e.g., in gel digestion for mass spectrometry, Western blotting) without additional sample cleanup. It can, however, impact mass spectrometry by forming adducts to proteins/peptides making characterization more difficult [32].

Like SDS, urea can be used in polyacrylamide gel electrophoresis without removal [29,60] and is especially good for low molecular weight proteins [60]. Buffers containing urea can also be used directly for mass spectrometry (e.g., [61]), but care must be taken not to overheat the samples leading to carbamylation [29]. Urea does not precipitate in the presence of SDS, unlike guanidine HCl [62], so less desalting is necessary for urea extractions than for guanidine HCl ones. However, we found that neither SDS or urea was as effective as methods utilizing guanidine HCl or ammonium bicarbonate based on ELISA and gel data, but they still could be used for bone. Further methodological development is necessary to evaluate benefits of SDS or urea in characterization of the bone proteome, especially with the usage of HCl for demineralization. The ammonium bicarbonate extraction (Buckley 2) and guanidine HCl extraction (Jiang 2) following HCl demineralization show very few differences. Both methods show collagen I detection by ELISA (Table 6) and very little difference on gel (Fig. 1). The Jiang method results in a smaller yield than the Buckley method for approximately the same starting amount of bone, so if greater yield is necessary, the Buckley method may be better. The yield in the Jiang steps is very small because the overall volume is small per step, but enlarging the volume may help overcome protein saturation levels for the solvents. Larger volumes give larger yields based on comparable methods and their yield percentages. For example, the Schweitzer method uses a total of four volumes of buffer per step and the Franzen and Heinegård method uses ten volumes of buffer per step. The Franzen and Heinegård method has over 17 times more yield than the Schweitzer method (Table 5). These large yields likely include residual salt, however, because the yields are greater than the protein content in bone (~20%; [63]). The increase in yield allows for multiple assays to be performed without being material limited resulting in additional extraction periods. Future optimization of buffer capacity and volume should allow for high yield.

We also compared the yield of precipitated samples to that of dialyzed samples and found that precipitation gives anomalously high yield values. For example, Wendel 2 and Schweitzer 1 methods produce greater yield than the original bone mass. This implies that the precipitation method causes precipitation of a large amount of salt, in addition to the proteins of interest. The tested precipitation method is designed for low concentration salt or detergents, so it may not be optimal for these extraction types.

Acetone precipitation was not used because we found that many of the tested solutions precipitate, even though it has been used on bone extractions previously [13]. The current data suggest that dialysis is a better method of desalting for these highly concentrated salt solutions; although, ethanol precipitation of
guanidine HCl solutions [62] may be a more effective means of protein concentration and purification than either the chloroform-methanol-water precipitation or dialysis used here.

All tested protocols on ostrich bone, with the exception of the Gurley 1 method, show silver staining on SDS-PAGE gels (Fig. 1). All of these methods have been found to solubilize proteins to varying degrees; however, some methods provide better apparent quality on gels. For example, intense bands are visible for the Buckley 1,2 and Jiang 1,2 methods whereas the Schweitzer 1 and Embery 2 methods show smears for ostrich extractions. The cause of the smearing has not been investigated here; however, smearing may relate to residual EDTA left after dialysis because methods using HCl as a demineralizing agent show distinct, intense bands indicating a more pure extraction of intact proteins. This alternative, however, is unlikely because smears occur across the entire molecular weight range in Schweitzer 1, and bands are present on other methods indicating EDTA is most likely interfering with electrophoresis. The most intense bands occur on procedures that do not utilize EDTA or EDTA is a minor component in demineralization.

The method of solubilization is important depending on the protein of interest. Almost all of the extractions tested, with the exception of the SDS extraction, showed detection, based on light absorbance, of binding for anti-collagen I antibodies (Table 6). High molecular weight bands or smears are visible on gels for guanidine HCl, urea/thiourea, and ammonium bicarbonate extractions supporting the ELISA data (Fig. 1). Antibody binding for anti-hemoglobin was also detected for many extractions, including the SDS extraction, but was not detected for the ammonium bicarbonate extraction following HCl demineralization (Table 6). This is despite being observed in all subsequent Jiang extractions (guanidine HCl, EDTA/guanidine HCl, and HCl). This result was unexpected and may indicate that ammonium bicarbonate is not a good buffer for extraction of hemoglobin from bone and a different denaturing buffer may be required to collect this molecule. Very few extractions showed binding for antibodies to osteocalcin (Table 6) for ostrich; however, apparent osteocalcin bands are present in many different

**Table 1.** Summary of Franzén and Heinegård methods.

| Citation | Sample Name | Solution | Purpose | Volume | Incubation Time | Sample treatment* | Additional notes |
|----------|-------------|----------|---------|--------|-----------------|-------------------|------------------|
| [16,17]  | FH1         | Chilled 4 M GuHCl | Remove free protein | 10 volumes | 6 hr | D/L or precip |                       |
|          | FH2         | 250 mM disodium EDTA in 4 M GuHCl in 50 mM Tris pH 7.4 | Demineralize and extract protein | 3 × 10 volumes | 3 × 24 hr | D/L or precip |                       |
| Modified [16,17] | FH1 6 M 50 mM | Chilled 4 M GuHCl | Remove free protein | 10 volumes | 6 hr | D/L |                       |
| Modified [16,17] | FH2 6 M 50 mM | 250 mM disodium EDTA in 6 M GuHCl in 50 mM Tris pH 7.4 | Demineralize and extract protein | 3 × 10 volumes | 3 × 24 hr | D/L |                       |
| Modified [16,17] | FH1 6 M 100 mM | Chilled 4 M GuHCl | Remove free protein | 10 volumes | 6 hr | D/L |                       |
| Modified [16,17] | FH2 6 M 100 mM | 250 mM disodium EDTA in 6 M GuHCl in 100 mM Tris pH 7.4 | Demineralize and extract protein | 3 × 10 volumes | 3 × 24 hr | D/L |                       |

*Dialysis and lyophilization is abbreviated D/L. Precip represents chloroform:methanol:water precipitation performed on half of the supernatant. Volumes correspond to the number of milliliters of buffer multiplied by the grams of bone powder.

doi:10.1371/journal.pone.0031443.t001

**Table 2.** Summary of Gurley method and Wendel method.

| Citation | Sample Name | Solution | Purpose | Volume | Incubation Time | Sample treatment* | Additional notes |
|----------|-------------|----------|---------|--------|-----------------|-------------------|------------------|
| [18]     | 2 M HCl     |          | Demineralize | 20 volumes | 48 hr | D/L | Neutralized with NaOH, lyophilized and extracted in Gurley 1 |
|          |             |          | Extract protein | 3 × 6.5 mL | 3 × 1 hr | D/L | On neutralized salt powder |
|          |             |          | Extract protein | 3 × 6.5 mL | 3 × 1 hr | D/L | On pellet |
| [25]     |             |          | Remove free protein | 10 volumes | 6 hr | D/L or precip | White precipitate formed during incubation. This was resolubilized at 60°C for 1–2 hr |

*Dialysis and lyophilization is abbreviated D/L. Precip represents chloroform:methanol:water precipitation performed on half of the supernatant. Volumes correspond to the number of milliliters of buffer multiplied by the grams of bone powder.

doi:10.1371/journal.pone.0031443.t002
extraction types on gel (Fig. 1, Table 6). The difference between the ELISA and gel data may relate to the amount of serial dilution required for ELISA that is not necessary for gels, and/or the difference in solubility of osteocalcin in the dilution buffers between the two assays. In addition to solubility, osteocalcin may not adhere to the ELISA plates or is prevented from sticking to the plates by the abundant collagen in the samples preventing detection by antibody. The data suggest that the best methods for osteocalcin extraction, like collagen extraction, are ones utilizing HCl for removing mineral instead of EDTA. Given the suggested relationship between osteocalcin and collagen I [64], the greatest dissociation of osteocalcin and acid-insoluble collagen I should occur at low pH, in the presence of aqueous phosphate and calcium, as would be expected to occur during demineralization of bone.

The best protein extraction methods appear to be those that follow an HCl demineralization step, which induces ‘swelling’ of the collagen matrix [65] and increases the ability of both collagen I and collagen-associated proteins to go into solution. The use of 0.6 M HCl in the Buckley method [10,11] is optimized for reduction of acid-induced hydrolysis for archaeological bone [65] and retention of acid-insoluble collagen I in the pellet for subsequent extraction. EDTA is a less optimal method for demineralization because it is slow [65] and requires a greater number of washing steps (~15 washes; [14]) to fully desalt.

Based on the apparent quality of ostrich bone extractions, the methods in [10,11] utilizing HCl for demineralization and [16,17] utilizing EDTA for demineralization were performed on the moa bone. The methods in [10,11] were chosen because they use less harsh conditions (i.e., lower concentration HCl) for demineralization than [13]. The methods used in [16,17] also allow for investigation of exogenous protein because they utilize a pre-demineralization extraction step that could be beneficial for taphonomic studies of archaeological and paleontological bone. These two methods were chosen because one appears to be very clean [11] and the other has close to the highest yield value [16,17], although it likely contains residual salt. These two methods also represent each of the common demineralization protocols tested throughout.

Even though, we tested these methods on moa bone, it was not our intent to demonstrate endogeneity or to make claims for advancing our understanding of moa biology or evolution. We

### Table 3. Summary of Rabilloud method, Craig and Collins method, Embery method, and Schweitzer method.

| Citation | Sample Name | Solution | Purpose | Volume | Incubation Time | Sample treatment* |
|----------|-------------|----------|---------|--------|-----------------|-------------------|
| [28] Rabilloud | 500 mM disodium EDTA | Demineralize | 10 volumes | Overnight | Combined with next step |
| [30] C+C | 2% SDS in 500 mM disodium EDTA | Demineralize and extract protein | 2×10 volumes | 48 hr then 24 hr | D/L or precip |
| [15] Embery 1 | 10% disodium EDTA | Demineralize | 10 volumes | 7 days | D/L |
| Embery 2 | 4 M guanidine HCl in 50 mM sodium acetate (pH 5.8) | Extract protein | 10 volumes | 72 hr | D/L |
| [20,21] Schweitzer 1 | 500 mM disodium EDTA | Demineralize | 4 volumes | Overnight | Discarded |
| Schweitzer 2 | 6 M guanidine HCl in 100 mM Tris pH 7.4 | Extract protein | 2×2 volumes | 72 hr | Combined with next step |
| Schweitzer 2 | 500 mM tetrasodium EDTA in 6 M guanidine HCl in 100 mM Tris pH 7.4 | Demineralize and extract protein | 6.5 mL | 72 hr | D/L |

*Dialysis and lyophilization is abbreviated D/L. Precip represents chloroform:methanol:water precipitation performed on half of the supernatant. Volumes correspond to the number of milliliters of buffer multiplied by the grams of bone powder.

doi:10.1371/journal.pone.0031443.t003

### Table 4. Summary of Jiang method and Buckley method.

| Citation | Sample Name | Solution | Purpose | Volume | Incubation Time | Sample treatment* | Additional notes |
|----------|-------------|----------|---------|--------|-----------------|-------------------|-----------------|
| [13] Jiang 1 | 1.2 M HCl | Demineralize | 6.5 mL | Overnight | D/L |
| Jiang 2 | 6 M guanidine HCl in 100 mM Tris pH 7.4 | Extract protein | 6.5 mL | 72 hr | D/L |
| Jiang 3 | 500 mM tetrasodium EDTA in 6 M guanidine HCl in 100 mM Tris pH 7.4 | Demineralize and extract protein | 6.5 mL | 72 hr | D/L |
| Jiang 4 | 6 M HCl | Extract protein | 6.5 mL | Overnight | D/L |
| [10,11] Buckley 1 | 0.6 M HCl | Demineralize | 10 volumes | 4 hr | D/L |
| Buckley 2 | 50 mM NH₄HCO₃ | Extract protein | 24.7 mL | 5 hr at 65°C | D/L | Pellet neutralized with water before addition of ammonium bicarbonate |

*Dialysis and lyophilization is abbreviated D/L. Volumes correspond to the number of milliliters of buffer multiplied by the grams of bone powder.

doi:10.1371/journal.pone.0031443.t004
used this bone to show that these methods can also be applied with equal efficiency to fossil and subfossil bone. For all studies employing molecules recovered from ancient bone to determine evolutionary relationships, rates, or direction, environmental controls, consisting of at least depositional sediments and laboratory buffers, treated in tandem with test samples, is critical for evaluation of potential contamination in the extractions.

**Ancient Bone Protein Extraction Methods**

Efficient extraction of ancient proteins is imperative to their study; especially, if only a small amount of original protein remains in the bones. The two extractions performed on the moa bone follow a similar pattern to the ostrich extractions, except that the resultant powders are a light brown color. This coloration may correspond to the appearance of the bone pre-extraction or other diagenetic products that co-extract (e.g., humics), but additional characterization is needed to determine the color’s origin. Of the two methods tested, the Franzen and Heingård method yields a greater amount of material than the Buckley method (Table 5), yet this may be a product of sample purity as observed in the ostrich extractions. Antibodies to collagen I show detectable binding, as measured by absorbance, in three of the four extraction parts (Table 7). Smearing at high molecular weights (Fig. 2) is visible for these three as well, consistent with other studies of ancient material [20,21,66]. Unexpectedly, the Buckley 1 method does not show high molecular weight silver binding, but instead only has binding below 50 kDa (Fig. 2) supporting that it is appropriate for demineralization with minimal extraction of high molecular weight species for this previously unprocessed/discarded step. The solubilization of proteins in HCl without high molecular weight species, like collagen, may allow for characterization of NCPs from ancient bone. In this case, the extraction method (Buckley) yields a better signal than the FH method for all proteins assayed. Other analytical techniques (e.g., mass spectrometry) are required to fully characterize these samples and determine their protein content. Because mass spectrometry gives primary sequence information, it makes molecular data obtained from ancient bone more useful. The primary sequence allows for formation of molecular phylogenies, comparisons of molecular evolution in deep time, and determination of endogeneity. The addition of molecular information to morphological phylogenies may help elucidate relationships that otherwise would not be resolved. Determination of endogeneity is extremely important for ancient bone because, without sequence information, all molecular information could be considered contamination.

**Conclusions**

Collecting the protein content in bone follows a very standard pattern of demineralization and solubilization independent of assay type. We have shown that all of the tested methods for

### Table 5. Yields from ~1.3 g of bone powder of each extraction protocol described in Table 1.

| Lyophilized Sample | Total mass (mg) | %Yield | Lyophilized Sample | Total mass (mg) | %Yield |
|--------------------|----------------|--------|--------------------|----------------|--------|
| FH2 6 M 50 mM      | 385.3          | 29.5   | MOD FH50 Total     | 385.3          | 29.5   |
| FH2                | 361.8          | 27.8   | Jiang Total        | 372.5          | 28.7   |
| Wendel 2           | 352.5          | 27.3   | FH Total           | 361.8          | 27.8   |
| Jiang 4            | 354            | 27.3   | Wendel Total       | 356.5          | 27.6   |
| Rabilloud          | 222.1          | 17.0   | MOD FH100 Total    | 220.3          | 16.9   |
| FH2 6 M 100 mM     | 220.3          | 16.9   | Schweitzer Total   | 56.3           | 4.3    |
| C+H                | 124.3          | 9.8    | Buckley Total      | 20.3           | 1.6    |
| Embry 1            | 41.5           | 3.2    | Schweitzer Total   | 20             | 1.6    |
| Jiang 3            | 15.9           | 1.2    | Buckley Total      | 15.6           | 0.0    |
| Embry 2            | 14.8           | 1.1    |                    |                |        |
| Buckley 1          | 10.9           | 0.8    | Moa Buckley 1      | 18.9           | 2.1    |
| Schweitzer 2        | 10.3           | 0.8    | Moa Buckley 2      | 35.6           | 3.9    |
| Schweitzer 1        | 9.7            | 0.8    | Moa FH1           | 5.4            | 0.6    |
| Buckley 1          | 9.4            | 0.7    | Moa FH2           | 186.6          | 20.7   |
| Wendel 1           | 4              | 0.3    |                    |                |        |
| Jiang 1            | 2.6            | 0.2    | Moa Buckley Total  | 54.5           | 6.0    |
| Gurley 2           | 14.7           | 0.0    | Moa FH Total       | 192            | 21.3   |
| Gurley 1           | 0.9            | 0.0    |                    |                |        |
| FH1 6 M 100 mM     | B.D.           | B.D.   | Precipitated Samples Total mass (mg) | %Yield |
| FH1                | B.D.           | B.D.   | Wendel 2           | 3188.6         | 246.8  |
| FH1 6 M 50 mM      | B.D.           | B.D.   | Schweitzer 1       | 1293.2         | 100.3  |
| Jiang 2            | B.D.           | B.D.   | C+H               | 731            | 57.5   |
|                   |                |        | FH2               | 642.2          | 49.3   |
|                   |                |        | Rabilloud Precip  | 569.6          | 43.6   |
|                   |                |        | Schweitzer 2       | 70.1           | 5.4    |

B.D. refers to below the limit of detection for the balance used (0.1 mg). Total values correspond to lyophilized samples only and are calculated by addition of each step of an individual protocol. doi:10.1371/journal.pone.0031443.t005
solubilization, including those typically used on non-biomineralized tissues, can work for bone proteins. The choice of buffers used to extract proteins will ultimately depend on the protein of interest and/or analytical application. Protocols utilizing HCl for demineralization result in some of the purest extractions, but may result in unwanted hydrolysis, whereas those utilizing EDTA usually leave residual salt and therefore require an additional purification step. Both ammonium bicarbonate and guanidine HCl extract bone proteins well and either is suitable for use in recovering protein from bone. However, it is necessary to modify these to increase yield, concentration, and purity while decreasing artifact or contamination opportunities, particularly when working with ancient samples. Some protocols have been optimized to increase yields for a particular protein (e.g., collagen I; [10,11]), but still result in low total yields. The purest samples only result in 1–2% yield, which is only 5–10% of the total bone protein (assuming ~20% of the total bone mass is protein; [63]). Moderate increases in protein recovery may allow for greater characterization of non-collagenous proteins from both extant and ancient samples.

### Table 6. Ostrich enzyme-linked immunosorbent assay results showing means plus or minus one standard deviation.

|           | Collagen | Osteocalcin | Hemoglobin |
|-----------|----------|-------------|------------|
| Buckley   | 0.29±0.02 (+) | 0.05±0.07 (+) | 0.24±0.02 (+) |
| Buckley 2 | 0.12±0.02 (+) | 0.00±0.03 (*) | 0.01±0.01 (-) |
| C/C      | 0.03±0.01 (-) | 0.03±0.01 (*) | 0.19±0.03 (+) |
| Embry 1  | 0.04±0.00 (-) | 0.03±0.00 (*) | 0.06±0.00 (+) |
| Embry 2  | 0.02±0.03 (-) | 0.01±0.03 (-) | 0.01±0.00 (-) |
| FH 1     | 3.25±0.01 (+++) | 0.04±0.03 (-) | 0.19±0.01 (+) |
| FH 2     | 0.88±0.02 (+++) | 0.07±0.01 (+) | 0.85±0.03 (+++) |
| FH1 6 M 100 mM | 0.95±0.02 (+++) | 0.02±0.01 (-) | 0.14±0.00 (+) |
| FH2 6 M 100 mM | 0.13±0.03 (+) | 0.02±0.01 (*) | 0.07±0.02 (+) |
| FH1 6 M 50 mM | 1.64±0.08 (+++) | 0.00±0.01 (-) | 0.28±0.05 (+) |
| FH2 6 M 50 mM | 0.23±0.26 (+) | 0.04±0.01 (*) | 0.05±0.01 (+) |
| Gurley 1  | 0.05±0.04 (-) | 0.03±0.00 (-) | 0.04±0.00 (-) |
| Gurley 2  | 3.02±0.15 (+++) | 0.03±0.01 (-) | 0.24±0.08 (+) |
| Jiang 1   | 0.50±0.09 (+) | 0.04±0.03 (*) | 0.18±0.02 (+) |
| Jiang 2   | 0.60±0.01 (+) | 0.00±0.00 (*) | 0.20±0.01 (+) |
| Jiang 3   | 0.42±0.05 (+) | 0.03±0.01 (*) | 0.18±0.06 (+) |
| Jiang 4   | 0.23±0.03 (+) | 0.08±0.02 (-) | 0.12±0.04 (+) |
| Rabilloud | 0.11±0.01 (+) | 0.00±0.01 (*) | 0.05±0.00 (+) |
| Schweitzer 1 | 2.30±0.07 (+++) | 0.08±0.02 (-) | 0.90±0.03 (+++) |
| Schweitzer 2 | 1.72±0.11 (+++) | 0.05±0.02 (-) | 0.43±0.03 (+++) |
| Wendel 1  | 2.32±0.27 (+++) | 0.03±0.00 (-) | 0.21±0.09 (+) |
| Wendel 2  | 0.88±0.07 (+++) | 0.03±0.00 (-) | 0.53±0.06 (+++) |

Values correspond to absorbance at 405 nm. – represents no detected absorption. + represents between two and ten times the average absorbance of buffer controls, ++ represents 10 and 20 times, and +++ represents >20 times. *Represents bands on gels consistent in molecular weight with osteocalcin.

doi:10.1371/journal.pone.0031443.t006

![Figure 1. Sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels of ostrich extractions. Arrowheads indicate bands observed in many different extractions. The bands at ~12 kDa likely correspond to osteocalcin, which can act as a dimer under these conditions. The bracket next to the Schweitzer 1 lane indicates smearing with no apparent banding.](https://doi.org/10.1371/journal.pone.0031443.g001)
ancient bone and will allow for a greater understanding of protein preservation into deep time.

**Materials and Methods**

**Bone Samples**

Ostrich cortical bone fragments were degreased using a 10% Zout solution (Dial Corporation) to more closely approximate ancient bone. They were then frozen in liquid nitrogen and powdered using mortar and pestle. Very dark brown to black cortical bone fragments from an 800–1000 yr old moa (MOR OFT255, courtesy J. Horner) were powdered using mortar and pestle. MOR OFT255 has been briefly described as originating from cave deposits in New Zealand [67].

**Bone Extractions**

All extractions were performed at room temperature on approximate ancient bone and will allow for a greater understanding of protein preservation into deep time.

**Materials and Methods**

**Bone Samples**

Ostrich cortical bone fragments were degreased using a 10% Zout solution (Dial Corporation) to more closely approximate ancient bone. They were then frozen in liquid nitrogen and powdered using mortar and pestle. Very dark brown to black cortical bone fragments from an 800–1000 yr old moa (MOR OFT255, courtesy J. Horner) were powdered using mortar and pestle. MOR OFT255 has been briefly described as originating from cave deposits in New Zealand [67].

**Bone Extractions**

All extractions were performed at room temperature on ~1.3 g of bone, and solutions were centrifuged at 6000 rcf for 15 minutes between each step and supernatants were collected, unless otherwise noted (see Tables 1, 2, 3, and 4). After collection of the supernatants, each extraction was dialyzed for 4 days against pure water in a 2000 MWCO Pierce Slide-A-Lyzer Dialysis Cassette to remove salt and lyophilized to completion. Because some evidence suggests that degraded organic material may be adhering to dialysis membranes, we compared recovery rates between dialysis and protein precipitation. Half of the supernatant from [16,17,20,21,24,25,28,30] were dialyzed while the second half was precipitated using a chloroform:methanol:water precipitation method [68]. The yield for each desalting protocol was kept separate in Table 5. The resultant lyophilates were weighed, and yield was calculated by dividing the mass in milligrams of lyophilate by the original mass of bone powder and multiplying by 100 according to equation 1.

\[
\% \text{Yield} = \frac{\text{lyophilate}(mg)}{\text{bone}(mg)} \times 100
\] (1)

The total yield of individual procedures was calculated by adding the yield of each step. For brevity, all extractions are described in Tables 1, 2, 3, and 4. Yield values in Table 5 are given for each step of each protocol. Multipart protocols are added subsequently to give a total yield value.

**Extraction of Moa**

Approximately 0.9 g of powder were aliquoted and, based upon the results from extant bone samples, extracted using the [10,11,16,17] methods. The HCl supernatant from [10,11] and supernatants from [16,17] were dialyzed against water and lyophilized as described above; the ammonium bicarbonate supernatant [10,11] was dried using a speed vacuum. Yield was calculated following equation 1.

**Enzyme linked immunosorbent assay (ELISA)**

Each ostrich extract was resuspended in 1× phosphate buffered saline (PBS) to a final concentration of 1 μg/μl. Because it was expected that some moa proteins would be degraded, each moa extract was resuspended to a final concentration of 10 μg/μl. Fifty microliters of each extract and PBS blanks were aliquoted to Immulon 2HB UBottom (Thermo Scientific) 96-well ELISA plates and allowed to incubate for four hours at room temperature. Wells and plated antigen were incubated for four hours at room temperature or overnight at 4°C with an antibody dilution buffer. This buffer consisted of 5% bovine serum albumin (BSA) in 1× PBS solution with Tween 20 and Thimersol. It was used to saturate the well with protein to inhibit spurious binding of primary or secondary antibodies and reduce background signal. This buffer was removed, and 200 μl of primary antibodies (polyclonal anti-ostrich hemoglobin [custom antibodies produced by Genscript], polyclonal anti-chicken collagen I [United States Biological], diluted 1:400 in the above antibody dilution buffer, and monoclonal anti-osteocalcin [Abcam] diluted 1:100) were allowed to incubate with plated antigen for four hours at room temperature or overnight at 4°C. Primary antibodies were removed and wells were washed 10 times in an ELISA wash buffer, consisting of 1× PBS with 0.1% Tween 20. After washing, each well was incubated in 100 μl of secondary antibody (alkaline phosphatase conjugated goat anti-rabbit IgG [ZYMED], diluted 1:2000 in dilution buffer) for two hours, then washed 10 additional times. Antibody-antigen complexes were detected using a p-nitrophenylphosphate tablet (Sigma) diluted in a substrate buffer consisting of 9.8 mM diethanolamine and 10.5 M MgCl2. Positive

---

**Table 7. Moa enzyme-linked immunosorbent assay results showing means plus or minus one standard deviation.**

|                  | Collagen I | Hemoglobin | Osteocalcin |
|------------------|------------|------------|-------------|
| Moa Buckley 1    | 0.35±0.25  | −0.15±0.071| 0.38±0.43   |
| Moa Buckley 2    | 2.34±0.02 (+)| 0.48±0.01 (+)| 1.37±0.152 (+) |
| Moa FH1          | 2.04±0.12 (+)| 0.11±0.06 (-) | 0.71±0.10 (+) |
| Moa FH2          | 1.54±0.15 (+)| 0.19±0.08 (-) | 0.05±0.58 (-) |

Values correspond to absorbance at 405 nm. − represents no detected absorption. + represents at least two times the average absorbance of buffer control.

doi:10.1371/journal.pone.0031443.t007

---

**Figure 2. SDS-PAGE gel of moa extractions.** Arrowheads indicate faint bands visible through the smearing.

doi:10.1371/journal.pone.0031443.g002
binding was quantified at 405 nm using a Molecular Devices THERMOmax microplate reader for ostrich samples and a Molecular Devices Spectra Max Plus for moa samples. Data were acquired in Softmax Pro 4.8.

Gel Electrophoresis

Lyophilates resulting from the above extractions listed in tables 1, 2, 3, and 4 were resuspended in 1 x Laemmli buffer (Bio-Rad) with 20 mM DTT (Hoefer) to a stock concentration of 80 μg/μl. 5 μl of each sample was diluted in an additional 27 μl of 1 x Laemmli buffer (final concentration 0 μg/μl) and 5 μl was added to the wells of 15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). Samples were electrophoresed at a constant voltage of 240 V for 1 hour to separate components by size and/or charge. Silver staining was performed at room temperature following [69].

Acknowledgments

We would like to thank P. Hauschka for helpful comments in the process; E. Schroeter, J. Green, E. Vaughn, E. Cadena, A. Moyer, K. Dzikiewicz, B. Riddell, M. Buckley and several anonymous reviewers for helpful critiques on the manuscript; and J. Horner for access to the moa specimen.

Author Contributions

Conceived and designed the experiments: TPC MHS. Performed the experiments: TPC KV. Analyzed the data: TPC MHS. Contributed reagents/materials/analysis tools: TPC MHS. Wrote the paper: TPC. Revisions: TPC KV MHS.

References

1. Zylberberg L (2004) New data on bone matrix and its proteins. Comptes Rendus Palevol 3: 591–604.
2. Weiner S, Wagner HD (1998) The material bone: Structure-mechanical function relations. Annual Review of Material Science 28: 271–298.
3. Trueman CN, Marrill DM (2002) The long term survival of bone: the role of bioerosion Archaeeology 44: 371–392.
4. Collins MJ, Riley MS, Child AM, Turner-Walker G (1995) A Basic Mathematical Simulation of the Chemical Degradation of Ancient Collagen. Journal of Archaeological Science 22: 173–183.
5. Semal P, Orban R (1995) Collagen Extraction from Recent and Fossil Bones: Quantitative and Qualitative Aspects. Journal of Archaeological Science 22: 463–467.
6. Collins MJ, Nielsen-Marsh CM, Hiller J, Smith CI, Roberts JP, et al. (2002) The survival of organic matter in bone: A review. Archeometry 44: 383–394.
7. Child AM, Powell AM (1992) A review of the applications of immunochrometry to the study of the bone collagen. Journal of Archaeological Science 19: 39–47.
8. Gerstenfeld LC, Feng M, Gotoh Y, Glümcner JM (1998) Selective extractability of non-collagenous proteins from chicken bone. Calcified Tissue International 55: 230–235.
9. Schrives MA, Butler JP, Kalkarni NK, Knierman MD, Higge RE, et al. (2007) A proteomic analysis of adult rat bone reveals the presence of cartilage/ chondrocyte markers. Journal of Cellular Biochemistry 101: 466–476.
10. Buckley M, Collins M, Thomas-Oates J, Wilson JC (2009) Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation mass spectrometry. Rapid Communications in Mass Spectrometry 23: 3843–3854.
11. Buckley M, Whitcher Kanna S, Howard S, Campbell S, Thomas-Oates J, et al. (2010) Distinguishing between archaeological sheep and goats bones using a simple collagen peptide. Journal of Archaeological Science 37: 13–20.
12. Dobberstein B, Collins M, Craig O, Taylor G, Penkman K, et al. (2009) Archaeological collagen: Why worry about collagen diagenesis? Archaeological and Anthropological Sciences 1: 31–42.
13. Feng M, Ye M, Liu G, Feng S, Cui L, et al. (2007) Method development of efficient protein extraction in bone tissue for proteome analysis. J Proteome Res 6: 2257–2294.
14. Turos N, Fogel ML, Hare PE (1988) Variability in the Preservation of the Isotopic Composition of Collagen from Fossil Bone. Geochimica et Cosmochimica Acta 52: 929–935.
15. Embury G, Milner AC, Waddington RJ, Hall RC, Langley MS, et al. (2003) Identification of proteinaceous material in the bone of the dinosaur Iguanodon. Connective Tissue Research 44: 41–46.
16. Franken A, Heinegard D (1984) Extraction and purification of prolylglycans from mature bone. Biochemistry 122: 47–58.
17. Franken A, Heinegard D (1985) Isolation and characterization of two sialoproteins present only in bone calcified matrix. Biochemistry 232: 713–724.
18. Gurley LR, Valdez JG, Spall WD, Smith BF, Gillette DD (1991) Proteins in the fossil bone of the dinosaur, seismosaurus. Journal of Protein Chemistry 10: 73–90.
19. Schrivers M, Hill CL, Asara JM, Lane WS, Fincus SH (2002) Identification of Immunoreactive Material in Mammoth Fossils. Journal of Molecular Evolution 55: 696–705.
20. Schrivers MH, Soro Z, Avcis R, Asara JM, Allen MA, et al. (2007) Analyses of Soft Tissue from Tyrannosaurus rex Suggest the Presence of. Science 316: 277–280.
21. Schrivers MH, Zheng W, Organ CL, Avcis R, Soro Z, et al. (2009) Biomolecular Characterization and Protein Sequences of the Campanian Hadrosaur B. canadensis. Science 324: 626–631.
22. Terminie JD, Belcourt AB, Christjer PJ, Conn KM, Nylen MU (1980) Properties of dissociatively extracted fetal tooth matrix proteins. I. Principal molecular species in developing bovine enamel. Journal of Biological Chemistry 255: 9760–9768.
23. Terminie JD, Belcourt AB, Conn KM, Kleinman HK (1981) Mineral and collagen-binding proteins of fetal cell bone. J Biol Chem 256: 10403–10408.
24. Wendel M, Sommarin Y, Bergman T, Heinegard D (1995) Isolation, characterization, and primary structure of a calcium-binding 63-kDa bone protein. J Biol Chem 270: 6125–6133.
25. Wendel M, Sommarin Y, Heinegard D (1996) Bone matrix proteins’ isolation and characterization of a novel cell-binding keratin sulfate proteoglycan (osteodentin) from bovine bone. The Journal of Cell Biology 141: 839–847.
26. Fountoulakis M, Lahn H-W (1998) Hydrolysis and amino acid composition analysis of proteins. Journal of Chromatography A 826: 109–114.
27. Simo G, Cifuentes A (2003) Capillary electrophoresis-mass spectrometry of peptides from enzymatic protein hydrolysis: Simulation and optimization. Electrophoresis 24: 834–842.
28. Rabilloud T (1998) Use of thiol reagents to increase the solubility of membrane proteins in two-dimensional electrophoresis. Electrophoresis 19: 758–760.
29. McCarthy J, Hopwood F, Oxley D, Laver M, Castagna A, et al. (2003) Carbamylation of Proteins in 2-D Electrophoresis-Myth or Reality? Journal of Proteome Research 2: 239–242.
30. Craig OE, Collins MJ (2002) The Removal of Protein from Mineral Surfaces: Implications for Result Analysis of Archaeological Materials. Journal of Archaeological Science 29: 1077–1082.
31. Greene RF, Pace CN (1974) Urea and Guanidine Hydrochloride Denaturation of Ribonuclease, Lysozyme, β-Chymotrypsin, and β-Lactoglobulin. Journal of Biological Chemistry 249: 5380–5393.
32. Fritidikson EK, Baird B, McLafferty FW (1999) Electrospray mass spectra from protein electroeluted from sodium dodecylsulfate polyacrylamide gel electrophoresis gels. Journal of the American Society for Mass Spectrometry 10: 453–455.
33. Collins MJ, Gernaey AM, Nielsen-Marsh CM, Vermeer C, Westbrook P (2000) Slow rates of degradation of osteocalcin: Green light for fossil bone protein? Geology 28: 1139–1142.
34. Asara JM, Schweitzer MH, Freimark LM, Phillips M, Cateley LC (2007) Protein Sequences from Mammot and Tyrannosaurus R Rex Revealed by Mass Spectrometry. Science 316: 290–295.
35. Heffman PM, Bada JL (1975) Aspartic acid racemization in tooth enamel from living humans. Proceedings of the National Academy of Science USA 72: 2891–2894.
36. Bada JL (1983) Amino Acid Racemization Dating of Fossil Bones. Annual Review of Earth and Planetary Sciences 13: 241–260.
37. Buckley M, Andering C, Proctor K, Ryan BJ, Goldhstrom A, et al. (2008) Comparing the survival of osteocalcin and mtDNA in archaeological bone from four European sites. Journal of Archaeological Science 35: 1756–1764.
38. Clemenz MT, Fox-Dobbs K, Wheatley PV, Koch PL, Doak DF (2009) Revisiting old bones: coupled carbon isotope analysis of bisapogen and collagen as an ecological and palaeoecological tool. Geological Journal 44: 605–620.
39. Germonpre M, Sablin MV, Stevens RE, Hedges REM, Hofreiter M, et al. (2009) Revisiting old bones: coupled carbon isotope analysis of bisapogen and collagen as an ecological and palaeoecological tool. Geological Journal 44: 605–620.
40. Schweitzer MH, Avcis R, Collier T, Goodwin MB (2008) Amino Acid Racemization in Bone Collagen. Science 36: 473–490.
41. Schweitzer MH, Avcis R, Collier T, Goodwin MB (2008) Microscopic, chemical and molecular methods for examining fossil preservation. Comptes Rendus Palevol 7: 159–184.
42. Lowenstein JM (1981) Immunological Reactions from Fossil Material. Philosophical Transactions of the Royal Society of London Series B, Biological Sciences 292: 143–149.
42. Lowenstein JM, Sarich VM, Richardson BJ (1981) Albumin systematics of the extinct mammoth and Tasmanian wolf. Nature 291: 409–411.
43. Rainey WE, Lowenstein JM, Sarich VM, Magor DM (1984) Sirenian molecular systematics — including the extinct Steller’s sea cow (Hydrodamalis gigas). Naturwissenschaften 71: 586–588-588.
44. Lowenstein JM, Ryder OA (1985) Immunological methods in molecular Palaeontology [and Discussion]. Philosophical Transactions: Biological Sciences 315: 375–380.
45. Borja C, Garcia-Pacheco M, Olivares EG, Scheuenstuhl G, Lowenstein JM (1997) Immunoreactivity of albumin detected in 1.6 million-year-old fossils from Venta Micena in Orce, Granada, Spain. American Journal of Physical Anthropology 103: 433–441.
46. Annesley TM (2003) Ion Suppression in Mass Spectrometry. Clinical Chemistry 49: 1041–1044.
47. Buckley M, Larkin N, Collins M (2011) Mammoth and Mastodon collagen sequences; survival and utility. Geochimica et Cosmochimica Acta 75: 2007–2016.
48. Richter KK, Wilson J, Jones AKG, Buckley M, van Doorn N, et al. (2011) Fish ‘n chips: Mass spectrometry peptide mass fingerprinting in a 96 well plate format to identify fish bone fragments. Journal of Archaeological Science 38: 1502–1510.
49. Shoshani J, Lowenstein JM, Walz DA, Goodman M (1985) Proboscidean Origins of Mastodon and Woolly Mammoth Demonstrated Immunologically. Paleobiology 11: 429–437.
50. Hedges REM, Millard AR, Pike AWG (1995) Measurements and Relationships of Diagenetic Alteration of Bone from Three Archaeological Sites. Journal of Archaeological Science 22: 201–209.
51. Schweitzer MH, Wittmeyer JL, Horner JR (2007) Soft tissue and cellular preservation in vertebrate skeletal elements from the Cretaceous to the present. Proceedings of the Royal Society B: Biological Sciences 274: 183–197.
52. Wessel D, Flugge UI (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 130: 141–143.
53. Shvchenko A, Wilm M, Vorm O, Mann M (1996) Mass Spectrometric Sequencing of Proteins from Silver-Stained Polyacrylamide Gels. Analytical Chemistry 68: 4550–4558.