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Bone protein extraction without demineralization using principles from hydroxyapatite chromatography

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ABSTRACT

Historically, extraction of bone proteins has relied on the use of demineralization to better retrieve proteins from the extracellular matrix; however, demineralization can be a slow process that restricts subsequent analysis of the samples. Here, we developed a novel protein extraction method that does not use demineralization but instead uses a methodology from hydroxyapatite chromatography where high concentrations of ammonium phosphate and ammonium bicarbonate are used to extract bone proteins. We report that this method has a higher yield than those with previously published small-scale extant bone extractions, with and without demineralization. Furthermore, after digestion with trypsin and subsequent high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) analysis, we were able to detect several extracellular matrix and vascular proteins in addition to collagen I and osteocalcin. Our new method has the potential to isolate proteins within a short period (4 h) and provide information about bone proteins that may be lost during demineralization or with the use of denaturing agents.

The study of bone proteins and their modifications has emerged as a promising method to better understand and identify bone diseases (e.g., osteoporosis) [1–6] as well as provide molecular information for extinct taxa [7–23]. However, because bone is mineralized, analyzing the protein content in bone is more challenging than analyzing the proteins in other non-mineralized tissue. In particular, protein extraction protocols rely on demineralization of bone followed by protein solubilization (reviewed in Ref. [24]). Consequently, the protocols are typically slow, taking days to weeks to perform [24], or may result in unknown breakdown of proteins by hydrolysis. Through these traditional extraction protocols, approximately 1% or less of the original bone mass is extracted [24], and most of this is composed of collagen I.

In contrast to the above, several protocols have extracted proteins without the demineralization step [25–29], but the total yield has been limited to approximately 3 mg protein/g bone or less [27]. Jiang and coworkers [27] suggested that demineralization is a critical step for bone protein extraction; however, bone protein extraction with only acid-labile surfactant allowed for extensive bone proteome coverage using mass spectrometry [28]. Salmon and coworkers [28] further suggested that the method does not fully release mineral-specific proteins but may allow recovery of non-collagenous proteins without demineralization. In fact, Pastorelli and coworkers [26] identified more than 200 gel spots from extraction using only a low-concentration phosphate buffer for extraction [26]. Thus, a large number of proteins could be extracted from bone without extensive demineralization.

In hydroxyapatite chromatography, proteins are eluted from the hydroxyapatite column with increasing phosphate concentrations [30]. Because bone is a composite of hydroxyapatite and protein, we have incorporated the use of higher concentration phosphate buffers, similar to the final concentration used in hydroxyapatite chromatography, to develop a novel bone protein extraction protocol without the use of demineralization.

Materials and methods

Bone samples

Tibial cortical bone samples were sampled from seven Caucasian cadavers (23F, 25M, 48M, 56M, 79M, 81F, and 82M). All samples were previously diagnosed to be free from metabolic bone diseases, HIV, and hepatitis B (National Disease Research Interchange and International Institute for the Advancement of Medicine). No live human subjects were involved in this research

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Protein extraction

Using phosphate elution principles from hydroxyapatite chromatography [30,31], we made either 400 mM ammonium phosphate dibasic (Sigma–Aldrich) or 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate (Sigma–Aldrich). To determine differences between the two extraction solutions, we performed a number of initial tests on bone obtained from a 48-year-old male donor. Bone samples (100 mg each, fragmented to $\sim 1\ mm^3$) were extracted in 600 μL of solutions of 400 mM ammonium phosphate dibasic or 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate after homogenization using stainless-steel beads in a Bullet Blender (Next Advance). Because this is a tube-based homogenization method, particle size was not measured. Aliquots were taken at 4, 8, and 24 h to evaluate the amount of time necessary to extract protein for each solution.

After the initial set of tests, we repeated the extraction on approximately 50 mg of bone with 400 mM ammonium phosphate, 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate, and 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate/4 M guanidine hydrochloride (GuHCl)[1] for a fixed period of 24 h only. Temperature was varied at 4 °C, room temperature, or 75 °C to determine the effects of temperature on extraction. Lastly, an additional approximately 50 mg of bone was extracted at 75 °C with 200 mM ammonium bicarbonate for 24 h for comparison with the ammonium phosphate extractions. The 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate/4 M GuHCl extraction was tested only at 75 °C.

After establishing the method with the highest yields, approximately 50 mg of bone from other cadaveric donors was extracted using the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate extraction for 24 h at 75 °C. Protein concentration was determined using a Coomassie (Bradford) Assay Kit (Thermo Scientific) with bovine serum albumin as a protein standard, and all samples were desalted using microdialysis (3500 MWCO [molecular weight cutoff] regenerated cellulose, Fisher Scientific) against nanopure water [32] for 4 days.

To evaluate whether proteolysis occurs during the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate or 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate/4 M GuHCl extraction process, additional 50 mg aliquots of the 48M samples were homogenized with the inclusion of 10 μg/ml Halt Protease Inhibitor (Thermo Scientific) and incubated for 24 h at 75 °C.

Mass spectrometry

The 400 mM ammonium phosphate dibasic extraction and all 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate samples were reduced with 10 mM dithiothreitol for 1 h at room temperature followed by alkylation using 30 mM iodoacetamide for 1 h in the dark. Proteins were digested overnight with Trypsin Gold (Promega) at 37 °C (1:100 trypsin/protein). Peptide samples were prepared for mass spectrometry using a C18 stage tip [33]. After binding to the C18 disk, samples were washed with 50 μl of 0.1% formic acid and eluted using 20 μl of 80% acetonitrile and 0.1% formic acid. Samples were partially dried in air to remove excess acetonitrile and resuspended to a final volume of 15 μl in 0.1% formic acid. Prepared peptides were separated using an Agilent 1200 Series HPLC (high-performance liquid chromatography) system with a Thermo Scientific BioBasic C18 column (2.1 mm i.d., 100 mm length, 5 μm particle size) for 75 min using either of the following gradients: (i) 2% B for 0 to 5 min, 30% B for 5 to 15 min, 60% B for 15 to 60 min, 95% B for 60 to 64 min, and 2% B for 64 to 75 min or (ii) 2% B for 0 to 5 min, 30% B for 5 to 35 min, 60% B for 35 to 60 min, 95% B for 60 to 64 min, and 2% B for 64 to 75 min, where A is 0.1% formic acid and B is 100 acetonitrile and 0.1% formic acid. Eluted peptides were characterized on an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). The top two peaks were fragmented using either collision-induced dissociation (CID) or higher energy collisional dissociation (HCD) in the Orbitrap or the top five peaks were fragmented with CID and analyzed in the ion trap. All samples were analyzed by mass spectrometry in triplicate.

Peak lists (MGF) were created in MassMatrix Mass Spectrometric File Conversion Tools version 3.2. Peak lists were searched against Swiss–Prot and a decoy database using Mascot 2.3 (Matrix Science). The following parameters were set for each search: taxonomy was set to *Homo sapiens;* enzyme = trypsin; up to 3 missed cleavages; variable modifications: carbamidomethyl (C), deamidation (NQ), carboxy (E), oxidation (MKP); static modifications: none; peptide tolerance = 10 ppm; fragment tolerance = 0.5 Da; and peptide charge = 2+, 3+, 4+. Peptide results were filtered using Percolator at $P < 0.05$. Peptides with nonsensical post-translational modifications (e.g., carboxyglutamic acid [Gla] on non-Gla-containing proteins) were filtered by hand.

Statistics

To evaluate the differences in protein yield between extraction types, one-way analysis of variance (ANOVA) was performed in SigmaStat for Windows 2.03 (SPSS). Significance was set at $P < 0.05$.

Results

Protein extraction

The 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate had a significantly greater yield than the 400 mM ammonium phosphate dibasic alone for all times ($P < 0.001$; see Fig. 1). No variation in yield was observed between times.

Temperature change resulted in a significant increase ($P < 0.001$) in protein concentration for both the 400 mM ammonium phosphate dibasic and 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate solutions (Fig. 2A). Very

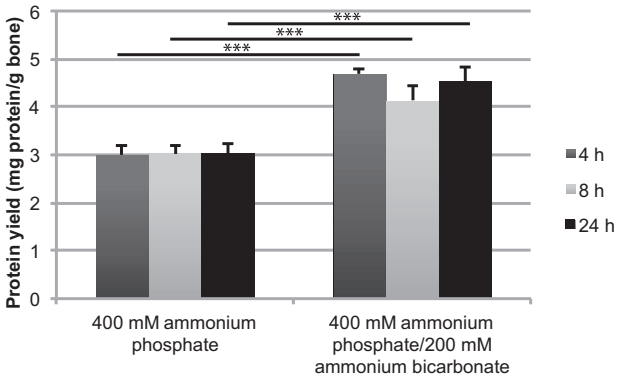

Fig.1. Time series (4, 8, and 24 h) for 400 mM ammonium phosphate and 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate extracted at room temperature. ***$P < 0.001$.

[1] *Abbreviations used:* GuHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; CID, collision-induced dissociation; HCD, higher energy collisional dissociation; ECM, extracellular matrix.

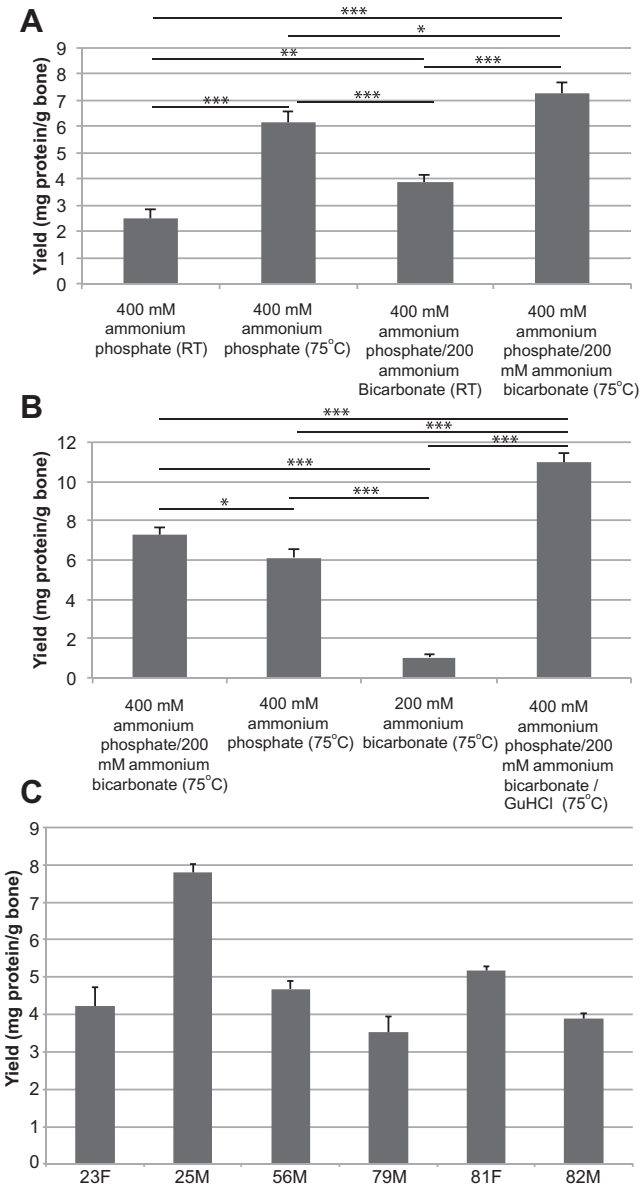

Fig.2. (A) Protein yields from a 48-year-old Caucasian male donor bone for 400 mM ammonium phosphate dibasic at room temperature (RT) and 75 °C and for 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate at RT and 75 °C. (B) Protein yields at 75 °C for 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate, 400 mM ammonium phosphate dibasic, 200 mM ammonium bicarbonate, and 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate/4 M GuHCl. (C) Protein yields at 75 °C for all other human samples extracted using 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

little yield (1.05 ± 0.16 mg protein/g bone) was detected after extraction with 200 mM ammonium bicarbonate at 75 °C (Fig. 2B), much less than after either ammonium phosphate extraction ($P < 0.001$). The highest yield was obtained with the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate/4 M GuHCl extraction ($P < 0.001$).

Extractions from bones obtained from the other cadaveric donors resulted in yields between 3.53 ± 0.42 and 7.79 ± 0.23 mg protein/g bone (Fig. 2C).

Mass spectrometry

For both of the initial extractions from the 48-year-old donor, peptides from collagen I were the most abundantly detected (Table 1; see also Table S1 in online Supplementary material). Osteocalcin and ceruloplasmin were detected only in the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate extractions using the top two CID method (Table 1), and actin, serum albumin, and apolipoprotein A-1 were detected only in the 400 mM ammonium phosphate dibasic extraction also using the top two CID method. Hemoglobin, vimentin, and fibrinogen gamma chain peptides were detected for both the 400 mM ammonium phosphate dibasic and the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate (Table 1). Osteocalcin was detected in the 400 mM ammonium phosphate dibasic extraction when using the top five CID fragmentation method.

Although as short a time as 4 h of extraction is possible for the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate method, we used 24 h for the individual ages to maximize the amount and types of protein extracted for mass spectrometry. In all samples for the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate extraction fragmented using the top five method, collagen I alpha-2 and alpha-1 were consistently the most abundant and second most abundant protein chains detected, respectively (see Table S2 in Supplementary material). Osteocalcin was detected by Mascot for all samples (Table S1). Several other proteins were also detected (e.g., vitronectin, lumican, biglycan; see Table S2). For all samples, 7.3 ± 2.4 proteins, 939.1 ± 185.8 total peptides, and 128.4 ± 19.1 unique peptides were detected using this extraction and mass spectrometry method. After using protease inhibitors, 9 proteins were identified in the 48M sample, whereas only 5 were identified in the non-inhibited sample.

The 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate/4 M GuHCl extraction resulted in the greatest number of protein identifications (as many at 20 unique accession numbers; see Table S2). Collagen I alpha 1 and alpha 2 and osteocalcin were the highest scoring proteins for this extraction, consistent with the other 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate extraction. Other matrix proteins (e.g., lumican, biglycan, collagen III, vitronectin, osteomodulin) were also detected (Table S2).

Discussion

Because extraction remains at a low level of total yield for extant bone [24,27], bone protein extraction remains a major challenge in fully understanding the proteome of bone beyond the isolation and characterization of individual proteins [34–37]. Using methodology derived from hydroxyapatite chromatography for elution, we have developed a small-scale technique to extract protein from extant bone that has yields higher than other small-scale, previously reported non-demineralization and demineralization extractions. This result is especially evident when heating was included during incubation. However, unlike other non-demineralization extractions [26,27,29], our methodology does not require the use of denaturing agents (e.g., GuHCl [27], Rapigest [28]), potentially leading to a better understanding of bone proteins in a more native conformation or without the loss of denatured cross-links. However, the addition of 4 M GuHCl to the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate buffer resulted in an increase in the number of protein identifications by increasing the solubility of matrix proteins that might not be soluble in the extraction without denaturation. The inclusion or exclusion of GuHCl to the extraction buffer can provide the flexibility to examine proteins in more native or completely denatured states, broadening the application of this extraction methodology.

In accordance with other bone proteome and extraction studies [24–27,29], peptides from collagen I were the most commonly

Table 1
Proteins detected for the 48-year-old male donor bone for all temperatures and times on the initial tests of 400 mM ammonium phosphate dibasic and ammonium phosphate dibasic/200 mM ammonium bicarbonate in alphabetical order.

400 mM ammonium phosphate dibasic	400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate
Actin, aortic smooth muscle	Ceruloplasmin
Apolipoprotein A-1	Collagen alpha-1(I) chain
Collagen alpha-1(I) chain	Collagen alpha-2(I) chain
Collagen alpha-2(I) chain	Fibrinogen gamma chain
Fibrinogen gamma chain	Hemoglobin subunit alpha
Hemoglobin subunit beta	Hemoglobin subunit beta
Histone H2A	Osteocalcin
Serum albumin	Vimentin
Vimentin	

detected (total: alpha-1, 376 ± 50.7 peptides; alpha-2, 469.6 ± 63.4 peptides; unique: alpha-1, 95.7 ± 10.8 peptides; alpha-2, 94.9 ± 9.0 peptides) by mass spectrometry because collagen I is by far the most abundant protein in bone [38]. The detection of collagen I peptides still remains a problem in understanding the bone proteome because it can block the detection of lower abundance proteins from the extracellular matrix (ECM), bone cells, and vasculature. However, osteocalcin (the second most abundant protein [38]), several proteins derived from vasculature (e.g., hemoglobin, serum albumin, fibrinogen), and vimentin from cells within the bone were still detected, suggesting that the use of ammonium phosphate dibasic and ammonium bicarbonate can extract a variety of proteins beyond collagen I and allow for their detection. This observation is further bolstered by samples of various ages that show additional proteins from the ECM (e.g., biglycan, collagen III, lumican, vitronectin, tenascin, osteomodulin, chondroadherin). Osteocalcin [36] and osteomodulin were the only mineral-specific proteins we detected (Tables 1, S1, and S2). However, this result is promising because it implies that our extraction methodology can interact with the hydroxyapatite surface sufficiently to dissociate mineral proteins. Because osteocalcin was detected consistently only in the extraction containing ammonium bicarbonate, our results suggest that bicarbonate can disrupt the carboxyl interaction between osteocalcin and the mineral surface. Future pre-HPLC fractionation (e.g., strong ion exchange, phosphopeptide enrichment) might be necessary to fully characterize these protein samples beyond a basic shotgun approach. This is especially true for peptides from the acidic mineral associated proteins (e.g., osteocalcin, osteopontin) that may coelute with more basic peptides (e.g., from collagen I), resulting in limited ionization [39]. In addition, although we observe only a few additional identifications with the addition of protease inhibitors (Table S2), these may be critical to more widely characterize the bone proteome using this methodology without loss of post-translational modifications or production of non-tryptic peptides resulting in more complicated database searching.

Our study has limitations. Although we detected fewer total proteins than some previous studies [26,27], this may have been a result of our chromatographic separation and peptide fragmentation rather than the extraction method itself. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels (data not shown) indicate a large amount of protein across the molecular weight range for the heated extractions using both the 400 mM ammonium phosphate dibasic and the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate. Thus, other proteins are also present in these extractions beyond those that are detected by mass spectrometry. We were also unable to detect osteopontin, but this might be the product of phosphorylation resulting in peptide suppression during coelution with other non-phosphorylated peptides [40]. Additional sample processing (e.g., immobilized metal affinity chromatography [40]) might be necessary to identify this important ECM protein. Our total protein yields for both the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate and the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate/4 M GuHCl are among of the highest reported for any bone type (e.g., long bones, skull bones), especially at 75 °C for the 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate, without demineralization and are at a similar level to previously published methods including demineralization on canine parietal bone [27].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.12.006.

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