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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

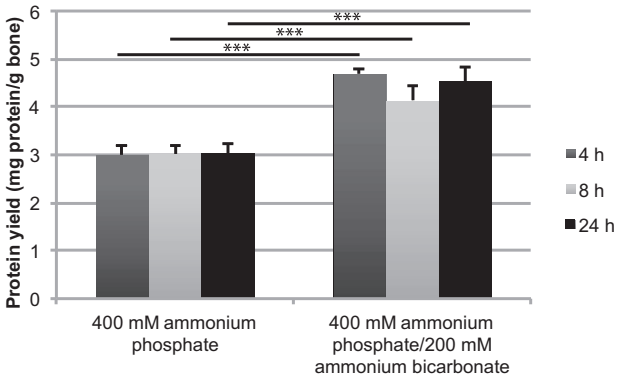

Fig.1. Time series (4, 8, and 24 h) for 400 mM ammonium phosphate and 400 mM ammonium phosphate dibasic/200 mM ammonium bicarbonate extracted at room temperature. ***$P < 0.001$.

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