Electrophoretic Analyses of Proteins Isolated From Mammalian, Avian, and Reptilian Bone Using a Pressure Cycling Technology

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1. Abstract

Comprehensive proteomic analysis of tissues is only possible when the total protein constituency of cells and extracellular matrices is effectively isolated. The efficiency of sample preparation is therefore a critical component of the analytical process and is essential to reliable proteomic analyses. Cortical bone is an extensively calcified osseous tissue and represents a specific challenge in sample preparation. Historically, bone requires prolonged acid demineralization over several days to enable complete penetration of histochemical reagents to cellular components. Mammalian, avian, and reptilian samples were used to model protein and peptide release from compact bone. Formic, acetic, and hydrochloric acid were evaluated as demineralization reagents prior to the extraction of proteins from bone using a pressure cycling technology (PCT) in which the sample was rapidly cycled 80 times between 35,000 psi and atmospheric pressure. Differences in proteins and peptides released during demineralization with different acids were analyzed by protein assay and electrophoresis and compared to those released by the downstream PCT process. Without prior acid demineralization, PCT yielded more protein than demineralized samples. The ability to extract proteins from bone without prior demineralization offers an important advantage in time savings. Moreover, when working with fossilized bone, where it is assumed that protein remnants will be fragmentary or highly cross-linked, extensive demineralization may further contribute to molecular damage, loss, or modification. Finally, since the carryover of Ca and PO_4 from these tissues interferes with IEF, several strategies for the removal of these substances prior to two-dimensional gel electrophoresis were investigated.

2 Materials and Methods

2.1 Acid Demineralization

Ostrich tibia was meticulously cleansed of connective tissue and pulverized into 1-2 mm fragments. Approximately 350 mg of pulverized bone was weighed into a tared microfuge tube with 475 uL 100 mM EDTA and 25 uL protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and incubated 30 min. An equal volume of 1M formic acid, acetic acid, or hydrochloric acid were added and the samples were incubated 36 h at room temperature. Alternatively, 350 mg of pulverized bone was incubated 36 h in IEF reagent consisting of 9M urea, 4% CHAPS supplemented with 100 mM DTT, 50 mM EDTA, and protease inhibitors. Samples were centrifuged and the supernatants were reserved. Bone fragments were resuspended in 1200 uL of IEF reagent supplemented with 100 mM DTT, 50 mM EDTA, and protease inhibitors. Bromophenol blue was added to the acid demineralized samples as a pH indicator. Concentrated Tris was added until a color transition to blue was obtained.

Bovine and alligator bone were processed without prior acid demineralization. Alligator bone was processed in (*i*) 9M urea, 4% CHAPS, (*ii*) 9M urea, 70 mM SDS, 40 mM dodecylsulfate (*iii*) 7M urea, 2M thiourea, 4% CHAPS, or (*iv*) 6M GuHCl. All extraction reagents contained 100 mM DTT and protease inhibitors.

2.2 Pressure Cycling Technology (PCT)

The suspensions were transferred to PULSE Tubes and processed in the Barocycler NEP-3229 (Pressure BioScience, West Bridgewater, MA) for 80 cycles. Each cycle consisted of 20s at 35,000 psi followed by 5s at atmospheric pressure. The supernatants were removed and the remaining bone fragments were subjected to an additional 80 cycles in fresh IEF reagent.

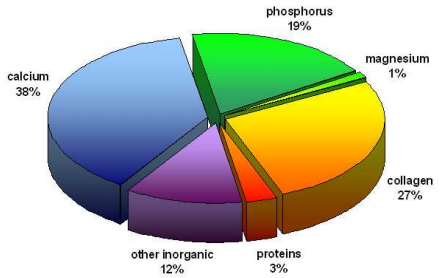


Figure 1. *Constituents of mammalian bone. Inorganic substances comprise 70% of the total bone mass.*

2.3 Sample Concentration and Removal of Interfering Substances

Bone samples cannot concentrated by acetone precipitation, since this also precipitates and concentrates phosphate and other salts [2]. Instead, the samples were transferred to ULTRA-4 ultrafiltration devices with 10 kDa MWCO (Millipore Corporation, Danvers, MA). The samples were ultrafiltered centrifugally and exchanged with 9M urea, 4% CHAPS until the final DDT concentration was 10 mM. Reduced and alkylated was performed directly in the ultrafiltration device by the addition of 5 mM tributylphoshine and 50 mM acrylamide [3]. The alkylation reaction was terminated after two hours by resuming centrifugation and ultrafiltrative exchange. Alternatively, samples were processed using ProteoSpin Total Protein Detergent Clean-Up Micro Kit (Norgen BioTek Corporation, Ontario, CA). Samples were passed through the spin columns centrifugally at 2000 RCF. The flow through was collected and recycled a second time through the column bed. The columns were washed twice with buffers provided in the kit, followed by a third wash with H2O. Proteins were eluted with 100 mM Tris pH 10, followed by a second elution with IEF Reagent. The two eluents were combined.

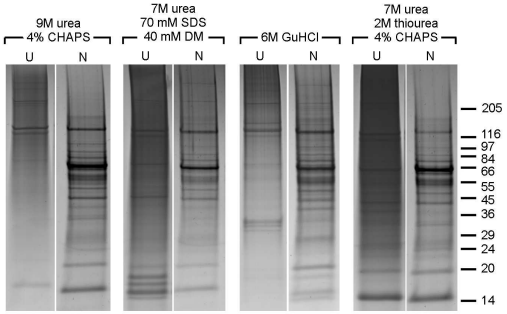
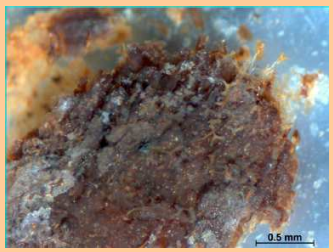


Figure 2. *SDS PAGE of bovine femur bone proteins isolated by PCT with various reagents, then concentrated by ultrafiltration (U). The ultrafiltrates were then passed twice through Proteospin columns to remove interfering substances from the protein fractions (N).*



Why Ostrich Bone?

Of all living taxa today, ostrich is probably most closely related to theropod dinosaurs. Recently, an extraordinarily well-preserved femur from *Tyrannosaurus rex* was discovered [1]. If the eventual goal is to apply this technology to dinosaur bone, a database must be derived from extant animals that bracket this organism. Because cortical bone is so dense, it is better protected from environmental influences than trabecular bone and is most likely to be preserved in fossilized specimens. Cortical bone has produced dinosaur cells and vessels, so it stands to reason that microenvironmental conditions may be appropriate for recovery.

Figure 1. *Vessels and other fine structure revealed after demineralization of compact dinosaur bone.*

TABLE 1: Milligrams protein from OSTRICH TIBIA BONE with or without prior acid demineralization and sequential PCT [a]

process	formic acid	HAc	HCl	9M urea 4% CHAPS
demineralization	0.23 ± 0.01	0.25 ± 0.01	0.24 ± 0.02	0.41 ± 0.01
PCT 1 [b]	0.40 ± 0.04	0.28 ± 0.04	0.48 ± 0.03	0.42 ± 0.02
PCT 2 [c]	0.13 ± 0.03	0.08 ± 0.01	0.22 ± 0.02	0.22 ± 0.06
total	**0.77 ± 0.04**	**0.60 ± 0.06**	**0.95 ± 0.06**	**1.05 ± 0.11**

[a] *Duplicate PULSE Tubes containing 345 ± 15 mg fragmented bone (n = 9).*

[b] *80 pressure cycles (35,000 psi maximum pressure).*

[c] *Additional 80 pressure cycles of residual bone following buffer replacement.*

2.4 LC-MS/MS

Gel bands from 35 kDa, 45 kDa, 120 kDa, and 125 kDa were in-gel digested using trypsin; Cys residues were reduced and alkylated using iodoacetamide. Each digest was separated using reverse-phase liquid chromatography coupled directly to a linear ion trap (LTQ XL from Thermo-Fisher) operating in the data-dependent acquisition mode. Tandem-MS data was analyzed using both Sequest and Mascot database search engines.

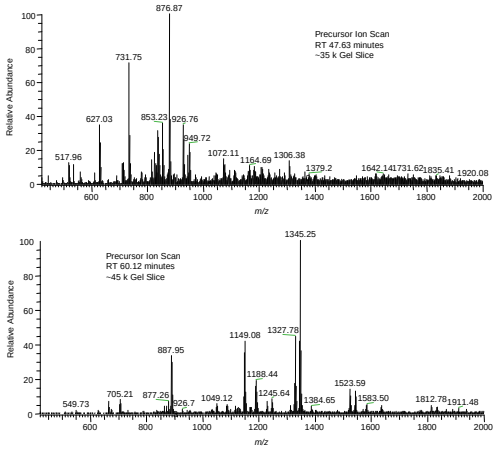


Figure 3. *Representative precursor ion scans from the in-gel digestion products of 35 kDa and 45 kDa ostrich bone proteins by RP-HPLC coupled to a linear ion trap mass spectrometer. Tandem-MS data exhibited characteristics of tryptic fragments (2+ and 3+ charge states, sequence tags, elution time). Given the lack of an ostrich database, protein identification and homology searching remains elusive.*

3. Results and Discussion

Ostrich bone samples were exposed to different acids to determine whether prior demineralization would effectively soften bone fragments resulting in increased protein yield. Table 1 shows there was no increase in protein yield when samples were demineralized with acid prior to PCT extraction. Samples pretreated with HCl yielded more protein than samples pretreated with either formic or acetic acid. To a possible disadvantage, measurable protein was released in the demineralization solutions.

The precursor ion scans for two of the gel bands (35 kDa and 45 kDa) are shown in Figure 3 for a specific retention time. The molecular species present in the precursor ion scans where tryptic peptides typically elute were determined to be 2+ and 3+ charge-states. This is consistent with the behavior of tryptic fragments (i.e., elution time, charge-states).

The tandem-MS spectra also have characteristics of the dissociation of tryptic peptides (i.e., amino acid sequence tags); however, using database search engine and a plethora of databases, no identities or homologous proteins were found to be significant. This presents a major issue when working with organisms without genomic or proteomic databases. We are currently in the process of carrying out *de novo* sequencing on these data. Importantly, data obtained from 1D gel bands is clearly indicative of tryptic peptides, and thus, we are confident it is derived from proteins. Unfortunately, none of the LC-MS/MS data gave rise to confident protein identifications for ostrich bone. Given these results, we are in process of demonstrating the effectiveness of PCT using bone from bovine and other organisms with available databases.

From bovine femur, 7M urea, 2M thiourea, 4% CHAPS yielded more protein (2.33 ± 0.43 mg/mL) than 9M urea, 70 mM SDS, 40 mM dodecylsulfate (1.32 ± 0.20 mg/mL) or 6M GuHCl (0.58 ± 0.16 mg/mL). In comparison, 7M urea, 2M thiourea, 4% CHAPS yielded more than twice the protein (6.26 ± 1.24 mg/mL) from alligator phalanges than from bovine femur.

Because of the high sensitivity and maximal recovery of the PCT method, it seems ideal for working with molecules derived from fossils, which are likely to be highly altered, cross-linked, and in very low concentrations. Molecules recovered from fossils would be worth the effort because they offer insight into evolution at the molecular level. Developing protocol is the first step in such an effort, hence the present work on extant bone tissues, which represent the most difficult of sample preparation challenges. Phylogenetic estimation of divergence times and the direction and rate of evolutionary change has been limited to the extant members of crown clades. Crocodiles and birds are the two crown group clades representing archosauria, but both are highly derived and specialized and may not be the best models for predicting certain aspects of dinosaur biology and evolution.

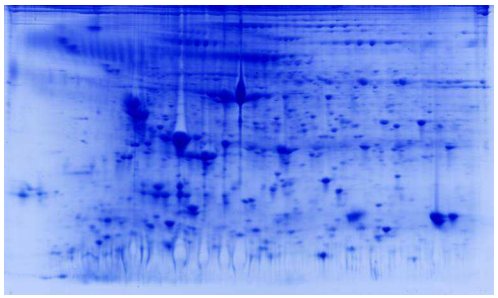


Figure 4. *2DGE of alligator bone proteins isolated by PCT and concentrated by ultrafiltration. Dried immobilized pH gradients (IPGs) pH 4-7 (from left to right) were hydrated for five hours. IEF was performed for 100,000 volt-hours followed by equilibration and second dimension PAGE on 8-16% gradient gels.*

4. References

[1] Schweitzer MH, Whittmeyer JL, Horner JR. *Science* 2005, 308, 1456-1460.

[2] Smejkal GB, Robinson MH. *Electrophoresis.* 2007, in press.

[3] Smejkal GB, Li C, Robinson MH, Lazarev A, Lawrence N, Chernokalskaya E. *Journal Proteomic Research* 2006, 5, 983- 987.

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