Electrophoretic Analyses of Proteins and Peptides Isolated From Cortical 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 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. Ostrich tibia was used as a model for the release of protein and peptide from compact bone. Several acids (formic, acetic, and hydrochloric acid) were evaluated as demineralization reagents prior to the extraction of proteins from bone by pressure cycling technology (PCT). Bone samples were rapidly cycled 80 times between 35,000 psi and atmospheric pressure in a Barocycler. 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 fossil tissues, it may be assumed that protein remnants will be fragmented, highly cross-linked, or extensive demineralization of the bone has occurred. These conditions may have contributed to molecular damage, loss, or modification of proteins. In addition, 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 cortical bone 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 of 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 suspended 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.

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 20 s at 35,000 psi followed by 5 s at atmospheric pressure. The supernatants were removed and the remaining bone fragments were subjected to an additional 80 cycles in fresh IEF reagent.

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 (Millipore Corporation, Danvers, MA) and reduced and alkylated as previously described [3]. 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 H_2O. Proteins were eluted with 100 mM Tris pH 10, followed by a second elution with IEF Reagent. The two eluents were combined.

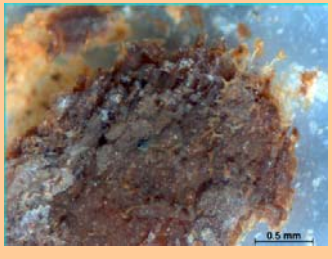
Figure 2. *Interference in IEF from carryover of PO_4. The concentration of PO_4 and the anodic extreme of the immobilized pH gradient increases electroendoosmosis and the transport of water causing the gel to collapse in one region and swell in another.*

2.4 Electrophoresis

SDS PAGE was performed on 4-12% NuPAGE (Invitrogen, Carlsbad, CA) polyacrylamide gradient gels. IPGs pH 4-7 (Proteome Systems, Woburn, MA) were hydrated with sample for 4 h, followed by IEF for 100,000 Volthours.

2.5 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.



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 bone with or without prior acid demineralization and sequential PCT [(a)]

process	formic acid	HAc	HCl	ProteoSOLVE
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.*

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

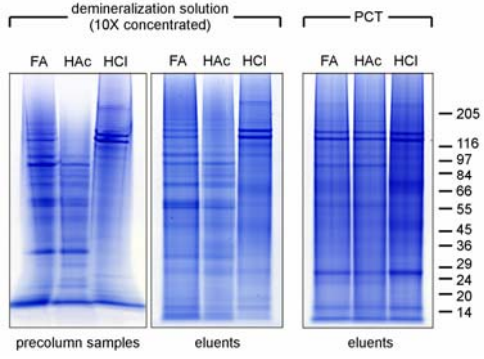


Figure 3. *Interference of salts with electrophoresis (left) and their removal using Norgen ProteoSpin columns (middle). Acid solutions were reserved and concentrated 10X to show the loss of proteins resulting from demineralization in formic, acetic, or hydrochloric acid. Proteins extracted by PCT shown on right.*

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 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, as shown in Figure 3 (*left and center panels*).

The precursor ion scans for two of the gel bands (35 kDa and 45 kDa) are shown in Figure 4 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 either 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, the data we have obtained from the 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 – this is an area for which we are directing our efforts. Furthermore, given these results, we are in process of demonstrating the effectiveness of PCT using bone from humans and other organisms with available databases.

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 are important because they offer insight into evolution at the molecular level. Developing extraction protocols is the first step in such an effort, hence the present work on extant bone tissues, which represent one of the most difficult 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 aspects of dinosaur biology and evolution.

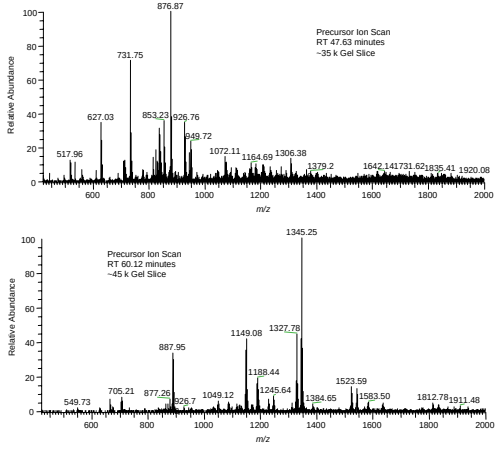


Figure 4. *Representative precursor ion scans from the in-gel digestion products of the 35 kDa and 45 kDa bands by RP-HPLC coupled to a linear ion trap mass spectrometer. Tandem-MS data (not shown) exhibited characteristics of tryptic fragments (2+ and 3+ charge states, sequence tags, elution time). However, given the lack of an Ostrich database, protein identification and homology searching remains elusive.*

4. References

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

[2] Smejkal GB, Li C, Robinson MH, Lazarev A, Lawrence N, Chernokalskaya E. *J. Proteomic Res.* 2006, 5, 983- 987.

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