Electrophoretic Analyses of Proteins and Peptides Isolated From Cortical Bone Using Pressure Cycling Technology (PCT)

Extraction of proteins from extensively calcified osseous tissue, such as cortical bone has been particularly challenging for traditional methods of sample preparation. However, a comprehensive proteomic analysis of bone is only possible when the total protein constituency is effectively isolated. The efficiency of sample preparation is therefore a critical component of the analytical process. Historically, extraction of protein from bone required prolonged acid demineralization over several days to enable complete penetration of histochemical reagents to cellular components. Here we describe a method for the extraction of protein from ostrich tibia, which was used as a model sample to develop an extraction process that uses pressure cycling technology (PCT) and also which obviates the need for acid demineralization prior to extraction. The ability to extract proteins from bone without prior demineralization offers important advantages in efficient representative extraction of protein and significant time savings during sample preparation.

Pressure Cycling Technology (PCT)

PCT uses alternating cycles of high and ambient pressures to induce cell lysis. Cell suspensions or tissues, such as bone, are placed in specially designed, single-use processing containers (PULSE Tubes) and are subsequently subjected to alternating cycles of high (up to 35,000 PSI) and ambient pressures in a pressure-generating instrument (Barocycler©) – together, the PCT Sample Preparation System (PCT SPS). Maximum and minimum pressures, the time at each pressure level, and the number of cycles are defined using a programmable logic controller. The reaction chamber of the Barocycler instrument can be temperature controlled using a peripheral circulating water bath. Safety features in the design of the PCT SPS significantly reduce risk of exposure to the researcher to pathogens and prevent cross-contamination of samples [5]. The PCT SPS offers a safer, more efficient method for protein extraction than other methods in use today. [1].

Methods

Evaluation of Extraction Buffers

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 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 was 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. The extraction buffers are listed in Table 1. Samples were centrifuged and the supernatants were retained. 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.

TABLE 1: Milligrams of 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.*

PCT-treatment

The suspensions were transferred to PULSE Tubes and processed in a Barocycler NEP3229 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.

Concentration and Removal of Interfering Substances

Bone samples cannot be concentrated by acetone precipitation, since this also precipitates and concentrates phosphate and other salts [3]. In this experiment, samples were transferred to ULTRA-4 ultrafiltration devices (Millipore Corporation, Danvers, MA) and reduced and alkylated as previously described [2]. 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.

Electrophoresis

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

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.

Results and Discussion

Ostrich bone samples were exposed to different acids to determine whether demineralization prior to PCT would result in increased protein yield. Samples pretreated with HCl yielded more protein than samples pretreated with either formic or acetic acid. However, significant amounts of protein was released, and potentially lost for analysis, in the demineralization process (See Figure 1).

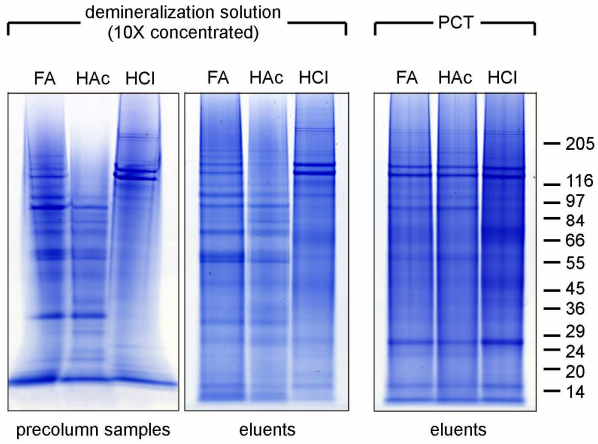

Figure 2. *Interference of salts with electrophoresis (left) and their removal using Norgen ProteoSpin columns (middle). Acid solutions were reserved and concentrated 10X to show the loss of proteins resulting from demineralization in formic, acetic, or hydrochloric acid. Proteins extracted by PCT shown on right.*

Figure 3 shows precursor ion scans and specific retention time of these two bands in the gel (35 kDa and 45 kDa) for a specific retention time. The molecular species present in the precursor ion scans are where tryptic peptides typically elute an area determined to be 2+ and 3+ charge-states. This is consistent with the behavior of tryptic fragments (i.e., elution time, charge-states). These data are indicative of tryptic peptides and the tandem-MS spectra also have characteristics of the dissociation of tryptic peptides (i.e., amino acid sequence tags). However, no identities or homologous proteins with either band were found to be significant after extensive search of protein data bases. This presents a potential problem when working with organisms without genomic or proteomic databases.

The PCT SPS is an efficient system for the release of proteins from extensively calcified osseous tissue, such as cortical bone. The combination of the biophysical process of cycling pressure and the use of the extraction buffer ProteoSolve proved to be more effective in releasing proteins for analysis than pre-treatment of the bone in acid buffers. The elimination of the acid-treatment also shortened the processing-time used in traditional extractions from several days to minutes.

In addition to being faster than traditional processing methods, the PCT SPS enables the researcher to extract more protein from cortical bone, which may allow for a more comprehensive a more comprehensive analysis of the proteome from bone previously considered difficult to process.

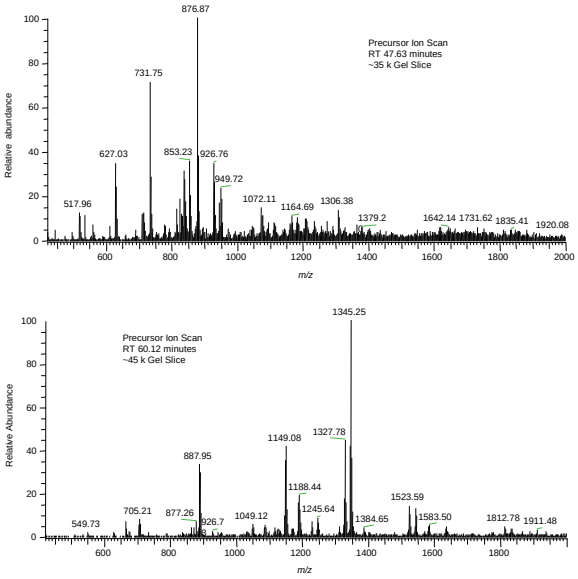

Figure 3. *Representative precursor ion scans from the in-gel digestion products of the 35 kDa and 45 kDa bands by RP-HPLC coupled to a linear ion trap mass spectrometer. Tandem-MS data (not shown) exhibited characteristics of tryptic fragments (2+ and 3+ charge states, sequence tags, elution time).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

Acknowledgements

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