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A method for whole protein isolation from human cranial bone

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ABSTRACT

The presence of the dense hydroxyapatite matrix within human bone limits the applicability of conventional protocols for protein extraction. This has hindered the complete and accurate characterization of the human bone proteome thus far, leaving many bone-related disorders poorly understood. We sought to refine an existing method of protein extraction from mouse bone to extract whole proteins of varying molecular weights from human cranial bone. Whole protein was extracted from human cranial suture by mechanically processing samples using a method that limits protein degradation by minimizing heat introduction to proteins. The presence of whole protein was confirmed by western blotting. Mass spectrometry was used to sequence peptides and identify isolated proteins. The data have been deposited to the ProteomeXchange with identifier PXD003215. Extracted proteins were characterized as both intra- and extracellular and had molecular weights ranging from 9.4 to 629 kDa. High correlation scores among suture protein spectral counts support the reproducibility of the method. Ontology analytics revealed proteins of myriad functions including mediators of metabolic processes and cell organelles. These results demonstrate a reproducible method for isolation of whole protein from human cranial bone, representing a large range of molecular weights, origins and functions.

1. Introduction

Together, the axial and the appendicular skeleton comprise a dynamic structure essential for structure, protection of vital organs, locomotion, muscle anchoring, maintenance of calcium and phosphate homeostasis, and acid/base regulation. Additionally, the marrow contained in long bones is the site of hematopoiesis. Depending on its composition, skeletal bone can be classified as cortical, compact bone or trabecular, spongy bone [1]. Both types have inorganic, organic, water and lipid components. The inorganic or mineral component is predominantly hydroxyapatite while collagens and proteoglycans comprise the organic portion [2]. The cellular constituents of bone include osteoblasts, osteoclasts and osteocytes [3,4]. These play important roles in bone remodeling that are essential for adaptation to changing biomechanical stresses, and for the turnover of old bone and subsequent replacement with new, stronger bone.

Dysregulation of osteoclasts and osteoblasts can give rise to diseases such as Paget disease of bone [5], osteopetrosis [6], or craniosynostosis [7]. However, our understanding of the pathophysiology underlying these and other bone disorders [8] is still limited. The development of analytical methods enabling accurate profiling of the bone proteome has immense potential to elucidate these disease mechanisms. Recent methods have shown potential in deriving bone proteins and characterizing them through liquid chromatography-mass spectrometry (LC-MS) platforms. For example, Jiang et al. isolated peptides from canine skull and analyzed them through mass spectrometry by first demineralizing the hydroxyapatite matrix of canine bone using hydrochloric acid [9]. While effective at the peptide level, this method was compromised at the protein level by the degradation of protein in strong acid.

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Mechanical processing of ancient human bone samples has been previously described by archaeologists as an alternative means to extract and analyze proteins [10]. In this method, samples were ground by hand with an agate mortar until powder formed. Isolated proteins of this class were identified following mass spectrometry and ranged in molecular weight from 20.1 to 269.2 kiloDalton (kDa). Applicability of this method to current biomedical research is limited by inherent differences between bone obtained from archeological and living specimens.

Recently, Alves et al. extracted protein from femoral trabecular bone of four patients undergoing total hip arthroplasty [11]. Collected specimens were mechanically fragmented using a dismembrator and proteins were subsequently homogenized and denatured before being analyzed using nanoflow LC-MS/MS. Notably, 3038 unique proteins were detected with 844 present in all four femoral samples.

The extraction of protein specifically from human skull bone poses additional challenges due to the high density of cortical bone which hinders the extraction of protein from its hydroxyapatite matrix [12]. The heat or chemicals needed to break down this matrix compromise protein stability. To date, there has been no method developed for extraction and analysis of proteins from human skull without extensive protein damage. In our laboratory, we are interested in elucidating differential protein expression between patent and prematurely fused human cranial sutures. Therefore, our research objectives call for a protein extraction technique of high yield, resolution and protein integrity. Here we describe a method of protein extraction from human cortical bone that minimizes thermal injury to protein. Using LC-MS/MS platforms, we detected proteins with a wide range of molecular weights that are derived from both intra- and extracellular milieus. Our methods can be utilized for detecting protein-level changes in diseases such as craniosynostosis.

2. Materials & methods

2.1. Materials

This study was approved by the University of Chicago Institutional Review Board (IRB #15-0539). Both patent and pathologically fused (craniosynostotic) human cranial suture samples stripped of periosteum were extracted from patients undergoing cranial vault reconstruction at the University of Chicago Medicine Hospital from January 2013 through September 2014. As a pair of suture types (patent/fused) was harvested during surgery, each patient served as their own internal control. Samples were immediately labeled, frozen in liquid nitrogen and then stored at −80 °C until use. Ethylenediaminetetraacetic acid (EDTA), aprotinin, leupeptin and pepstatin, sodium orthovanadate (Na_3VO_4), ammonium bicarbonate (NH_4HCO_3), and dithiothreitol (DTT) were acquired from Sigma Aldrich (St Louis, MO).

2.2. Lysis buffer preparation

A solution of 2 ml of 1.2 M tris-acetate (pH 6.8), 1 ml 10% SDS, 50 μl glycerol, 100 μl 100 mM EDTA, and 7 ml H_2O was mixed and placed on ice. Immediately before use, 10 μl each of 1 mg/ml aprotinin, leupeptin and pepstatin were added. Additionally, 100 μl 1 M β-glycerol phosphate, 50 μl 200 mM Na_3VO_4, and 500 μl 1 M DTT were added. The solution was mixed in a conical tube by hand for approximately 30 s, placed on ice, and subsequently aliquoted into 500 μl Eppendorf tubes (each) and kept on ice.

2.3. Sample preparation

The suture samples were removed from the freezer and wrapped in several layers of autoclaved aluminum foil. A hammer was used to break up the sample and the fragments were placed into a mortar and pestle kept on dry ice. The fragments were ground into a fine powder before being placed into Eppendorf tubes. The tubes were vortexed for one minute or until the ground suture went into solution. If necessary, more lysis buffer was added to solubilize the ground suture.

The solution was boiled for ten minutes (ensuring that the eppendorf tops were screwed on tightly) and then frozen at −80 °C. The samples were thawed and then centrifuged at 14,000 rpm for 10 min. The supernatant was removed and placed into two ml tubes. The supernatant was sonicated for a total of ten seconds and then needle-sheared using a 25G needle. It was then aliquoted and frozen at −80 °C.

2.4. Protein digestion

1D gel electrophoresis was performed on sample eluates using 20 μg for a gel plug digest. IP eluates were loaded onto a 12% MOPS buffered SDS-PAGE gel (Invitrogen, Thermo Scientific, San Jose, CA), and run for 10 min at 200 V resulting in a ~2 cm “gel plug.” The gel was stained with 25 ml Imperial Stain (Thermo Scientific, San Jose, CA) at room temperature, and de-stained overnight in deionized H2O at 4 °C.

The gel plugs for each sample to be analyzed were excised by sterile razor blade and chopped into ~1 mm^3 pieces. Each section was washed in deionized H_2O and destained using 100 mM NH_4HCO_3 pH 7.5 in 50% acetonitrile. A reduction step was performed by addition of 100 μl 50 mM NH_4HCO_3 pH 7.5 and 10 μl of 200 mM tris(2-carboxyethyl)phosphine HCl at 37 °C for 30 min. The proteins were alkylated by addition of 100 μl of 50 mM iodoacetamide prepared fresh in 50 mM NH_4HCO_3 pH 7.5 buffer and allowed to react in the dark at 20 °C for 30 min. Gel sections were washed in water, then in acetonitrile, and vacuum dried. Trypsin digestion was carried out overnight at 37 °C with 1:50-1:100 enzyme–protein ratio of sequencing grade-modified trypsin (Promega, Madison WI) in 50 mM NH_4HCO_3 pH 7.5, and 20 mM $CaCl_2$. Peptides were sequentially extracted with 5% formic acid, then 75% acetonitrile with 5% formic acid, combined and vacuum dried.

2.5. LC-MS/MS analysis

All fused and patent samples were re-suspended in Burdick & Jackson HPLC-grade water containing 0.2% formic acid (Fluka), 0.1% trifluoroacetic acid (TFA, Pierce, Waltham, MA), and 0.002% Zwittergent 3–16 (Calbiochem, Billerica, MA), a sulfobetaine detergent that contributes the following distinct peaks at the end of chromatograms: MH^+ at 392, and in-source dimer $[2M + H^+]$ at 783, and some minor impurities of Zwittergent 3–12 seen as MH^+ at 336. The peptide samples were loaded to a 0.25 μl C_8 OptiPak trapping cartridge custom-packed with Michrom Magic (Optimize Technologies, Oregon City, OR) C8, washed with Mobile phase A solution, then switched in-line with a 20 cm by 75 μm C_{18} packed spray tip nano column packed with Michrom Magic C18AQ, for a 2-step gradient. Mobile phase A was water/acetonitrile/formic acid (98/2/0.2) and mobile phase B was acetonitrile/isopropanol/water/formic acid (80/10/10/0.2). Using a flow rate of 350 nl/min, a 90 min, 2-step LC gradient was run from 5% B to 50% B in 60 min, followed by 50%–95% B over the next 10 min, hold 10 min at 95% B, back to starting conditions and re-equilibrated.

The samples were analyzed via electrospray tandem mass