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Solid Digestion of Demineralized Bone as a Method To Access Potentially Insoluble Proteins and Post-Translational Modifications

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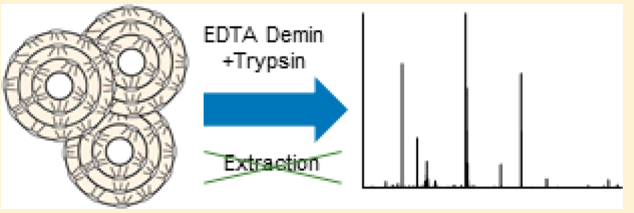
Supporting Information

ABSTRACT: Bone proteomics is an expanding field for understanding protein changes associated with disease as well as characterizing and detecting proteins preserved in fossil bone. Most previous studies have utilized a protocol with demineralization and extraction approach to isolate and characterize proteins from bone. Through near-complete EDTA demineralization, followed by solid digestion of the remaining bone pseudomorph, a total of 92 protein accessions were detected from dog bone. In the EDTA, 14 unique proteins were found, including osteocalcin, an important bone protein. Osteocalcin was not found in the solid digestion samples, demonstrating the importance of examining the demineralization supernatant. The solid-digestion samples were analyzed both with (11 unique accessions) and without (16 unique accessions) alkylation, resulting in a total of 78 protein accessions. In addition to the diversity of proteins detected, various post-translational modifications were observed, including phosphorylation and glycosylation. The solid-digestion approach will allow for characterization of proteins that are insoluble and would otherwise be missed by traditional bone protein extraction alone. All data are available at ftp://massive.ucsd.edu/MSV000081399.



KEYWORDS: *bone proteomics, solid digestion, collagen I, noncollagenous proteins*

INTRODUCTION

Bone proteomics has become an important avenue to evaluate protein changes associated with bone diseases[1−3] as well as to determine protein sequences and post-translational modifications (PTMs) from extinct taxa (i.e., paleoproteomics).[4−9] Although a diversity of questions pertaining to bone proteomics have been investigated, a limited number of approaches in accessing these bone proteins have been applied. The general approach of the majority of these studies is demineralization of the bone tissue, followed by solubilization of remaining proteins.[10,11] Because these extractions utilize multiple steps that have the potential to induce artifacts (e.g., loss of PTMs or increased deamidation of asparagine and glutamine[12]), recent studies have attempted to characterize what proteins are extracted across different methods (i.e., extractomics[11]) and which methods reduce laboratory-induced artifacts that negatively impact the results. Recently, novel extraction methodology has been developed to extract bone proteins without the use of demineralization,[13] thereby minimizing the number of steps required to have proteins accessible for proteomics. This demineralization-free approach has been successfully used for both extant remains[13] and fossil tissues,[5] suggesting that it is a useful extraction approach for a diversity of investigations. However, regardless of whether a demineralization step is included, many bone proteins remain in the tissue, insoluble even after chemical extraction. This precludes their identification by mass spectrometry and limits their accessibility for evaluations that monitor their changes with disease state or their potential to preserve in fossils.

Recent proteomics analyses of other extracellular-matrix-rich tissues (e.g., cartilage[14]) have applied enzymatic digestion directly to solid, insoluble tissue to maximize the diversity of proteins that can be gained from the sample, regardless of the extractability of these proteins. For example, Hsueh et al.[14] found 215 overlapping proteins between guanidine hydrochloride-extracted cartilage and in-situ-digested cartilage samples. Of these, 99 proteins were found exclusively in the solid digestion that would otherwise have been missed and which could potentially play an important role in cartilage disease. For bone, Cappellini et al.[7,15,16] digested bone pellets after traditional demineralization and solubilizing protein extraction and found 126 unique protein accessions; however, it is unclear how many additional proteins were identified from the postextraction digestion. To date, comprehensive shotgun proteomics of insoluble bone proteins by mass spectrometry after near-complete demineralization has not been attempted. Here, I apply digestion protocols designed for insoluble collagen preparation[17] after bone has been demineralized and evaluate both the demineralization supernatant and the subsequent solid-digest.

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MATERIALS AND METHODS

Samples and Demineralization

Control Bovine Insoluble Collagen I (tendon) was purchased from Alfa Aesar and used as a control insoluble collagen I to confirm that the insoluble digestion method applied here works regardless of demineralization effects. Adult dog diaphyseal, humeral cortical bone (88.3 mg) received post-mortem with limited information on its skeletonization from the North Carolina College of Veterinary medicine and stored at room temperature was demineralized in 883 μL of 500 mM tetrasodium EDTA for 5 days at 4 °C, followed by incubation at 37 °C for an additional 6 days. To complete demineralization at a faster rate, heat was applied after one change of EDTA in the middle of the demineralization procedure. Bone samples were cut into approximately 2 × 2 mm cubes and washed three times (15 min each) in 1 mL aliquots of 50 mM ammonium bicarbonate to remove residual EDTA. The 4 °C EDTA extraction was buffer -exchanged into 50 mM ammonium bicarbonate using an Amicon Ultra 3000 MWCO spin column (Millipore). To clearly distinguish between the EDTA and insoluble fractions, only the 4 °C EDTA supernatants were analyzed here. No treatment was made to remove residual periosteum or bone fatty content.

von Kossa Stain

Adjacent demineralized bone pieces were stained with von Kossa stain after extensive washing.[18] In short, washed and demineralized bone pieces were incubated in 1% silver nitrate for 45 min under a Fiber-Lite High Intensity Illuminator series 180 (Dolan-Jenner Industries) set to high intensity, followed by washing and incubation in 5% sodium thiosulfate for 5 min. Samples were imaged with a Hirox KH-8700 at medium magnification under a MXG-2500REZ, 35−2500× zoom lens.

Digestion of the Solid Collagen Preparation and Demineralized Bone

After demineralization (i.e., when little or no evidence of von Kossa staining was present (Figure S1)), 1 and 1.5 mg aliquots of demineralized bone (mass assumed to represent protein content) and 1.9 mg of the tendon control were taken for digestion. Both the bone sample and tendon were digested following a protocol modified from Terajima et al.[17] In short, samples were resuspended in 500 μL of 50 mM ammonium bicarbonate and heated for 15 min at 65 °C to denature the tissue. After heat denaturation, 1:200 trypsin (w/w; Promega-modified trypsin) was added to each and incubated overnight at 37 °C. After overnight digestion, the sample was heated for an additional 10 min at 65 °C, then digested with an additional aliquot of trypsin (1:200) for 3 h at 37 °C (Figure 1). Aliquots of each solid digest sample were analyzed either (1) without reduction and alkylation or (2) after digestion, followed by reduction with 10 mM dithiothreitol for 1 h at room temperature, followed by alkylation with 30 mM iodoacetamide. Whereas Terajima et al.[17] reduced with sodium borohydride prior to digestion, this predigestion reduction was not applied here because examination of collagen I cross-linking was not a goal of this study. No additional desalting was performed precolumn. Additionally, 20 μg (as determined from the Pierce BCA assay (ThermoScientific) following manufacturer's instructions) of protein extracted by the 4 °C EDTA (demineralization) fraction was reduced and alkylated as above, then digested with trypsin (1:20) overnight at 37 °C.

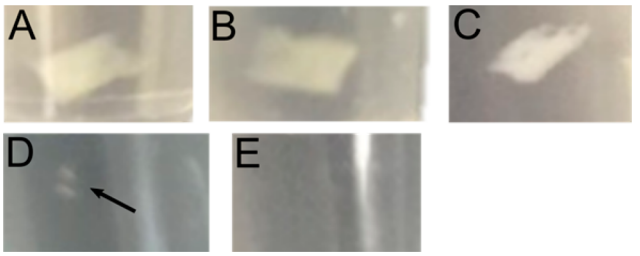

Figure 1. Solid digestion of 1 mg demineralized bone sample. (A) Predigestion, post demineralization. (B) After heat denaturation for 15 min at 65 °C. (C) Immediately post-overnight trypsin digestion and second 65 °C heat denaturation. (D) Post-overnight trypsin digestion and second 65 °C heat denaturation, 10 min wait time; arrow indicates residual solid organic bone matrix. (E) Post-final trypsin digestion; no visible bone remains.

LC−MS

Peptides were trapped on a 3 cm in-house packed Thermo BioBasic C18 column (75 μm i.d., 5 μm particle size), followed by separation on a 21 cm in-house-packed Thermo BioBasic C18 column analytical column using a Dionex Ultimate 3000 at 300 nL/min. An initial run of bone and tendon was performed utilizing the initial gradient in Table 1. Buffer A is 0.1% Optima-grade formic acid in Optima-grade water and buffer B is 99.9% Optima-grade acetonitrile and 0.1% Optima-grade formic acid. Subsequently, the gradient for each tissue type was optimized using the Gradient Optimization and Analysis Tool (GOAT[19]), and the final analyses were performed in triplicate, all using optimized gradients (Table 1). Peptides were nanosprayed into a ThermoScientific LTQ Orbitrap Velos and analyzed with the

Table 1. LC Gradients Used for Analysis of SDT and SDB[a]

time (min)	initial gradient (%B)	bone GOAT gradient (%B)	tendon GOAT gradient (%B)
0	2	2	2
8	2	2	2
13		8.48	14.96
18		9.66	16.13
23		10.83	16.72
28		12.01	17.90
33		13.19	19.08
38		14.37	20.26
43		15.54	21.43
48		16.72	22.61
53		17.90	24.38
58		19.08	25.56
63		20.84	26.73
68		22.02	28.50
73		23.79	29.68
78		24.97	31.44
83		27.32	33.21
88		29.09	36.74
93		32.62	39.10
98	55	35.57	48.52
100	90	90	90
103	90	90	90
104	2	2	2
120	2	2	2

[a]Initial gradient was linear between 8 and 98 min with no intermediary time points.

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following parameters: MS1:60k resolution, 100 ms ion time, 1E6 AGC target; MS2: top 8 peaks fragmented with higher-energy collisional dissociation (HCD; 30% NCE), 15k resolution, 5 m/z isolation width, 250 ms AGC, 5E5 AGC target.

Data Analysis

All RAW data were searched against either a *Bos taurus* or *Canis familiaris* database downloaded from Uniprot with PEAKS 8.0. The following parameters were used for each node: (1) Denovo − 10 or 15 ppm parent mass error tolerance, 0.02 Da fragment mass tolerance, fixed modifications − none, variable modifications − deamidation (NQ), oxidation (M), oxidation or hydroxylation (RYFPNKD), up to three variable PTMs per peptide, five peptides per spectrum, enzyme specified by each sample; (2) PEAKS DB − 10 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, enzyme set to trypsin, up to three missed cleavages, one nonspecific cleavage end, fixed modifications − carbamidomethyl (C) when alkylated, variable modifications − deamidation (NQ), oxidation (M), oxidation or hydroxylation (RYFPNKD), up to three variable modifications per peptide; (3) PEAKS PTM − 10 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, enzyme set to trypsin, up to three missed cleavages, one nonspecific cleavage end, fixed modifications − carbamidomethyl (C) when alkylated, all other Unimod PTMs set to variable, up to seven variable PTM per peptide, FDR estimation enabled, 15 ALC% threshold, 30.0 peptide hit threshold; and (4) SPIDER − 10 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, enzyme set to trypsin, up to three missed cleavages, one nonspecific cleavage end, fixed modifications − carbamidomethyl (C) when alkylated, variable modifications − deamidation (NQ), oxidation (M), oxidation or hydroxylation (RYFPNKD), up to three variable modifications per peptide, 15 ALC% threshold, 30.0 peptide hit threshold, L equals I, Q equals K. All peptides were filtered at a 1.0% FDR and proteins −10lgP ≥ −20 with at least 1 unique peptide. All data are available at ftp://massive.ucsd.edu/MSV000081399.

Protein and peptide matches were analyzed and statistics were generated in RStudio 1.0.143. All contaminant accessions were removed (i.e., keratins, trypsin, cytochrome c (column peptide standard)). UpSet plots were generated using UpSetR.[20] R script and associated files are included in the Supporting Information.

Individual spectra from example glycopeptides characterized by PEAKS were deconvoluted using Xtract in Qual Browser for Xcalibur 4.0.27.21 and subsequently annotated using Prosight Lite v1.4 (Build 1.4.6) with 10 ppm accuracy. P-score and PCS were reported for each characterized peptide.

RESULTS

Tendon Standard

The bovine tendon collagen I standard (SDT) was used to confirm that this solid digestion method is effective and functional at 1:200 trypsin concentration before applying it to the demineralized bone samples. Here both reduced and nonreduced aliquots of the tendon were tested. Reduced samples produced 157 total protein accessions (24 unique; Table S1), nonreduced samples produced 201 total protein accessions (68 unique; Table S2), and together a total of 225 protein accessions were identified (133 overlapping between reduced and nonreduced; Figure 2). Collagen I alpha 1 and alpha 2 were the highest scoring proteins, but multiple other collagen types (e.g., collagen VI) and proteins associated with collagen I were detected (e.g., decorin).

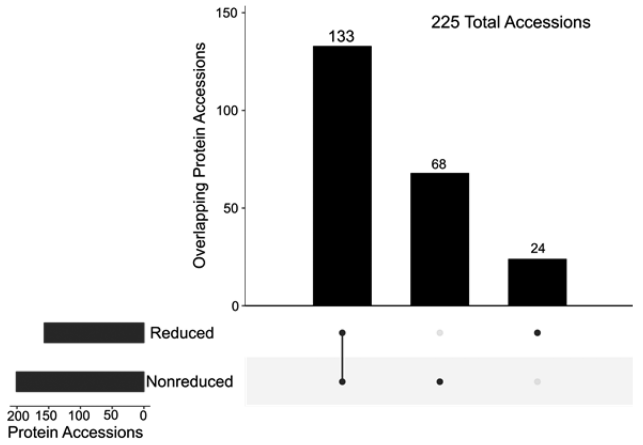

Figure 2. UpSet of solid-digested insoluble preparation collagen I from bovine tendon. Dots indicate sample with containing accessions; connecting bar indicates multiple samples.

Bone Samples

A total of 92 protein accessions were detected across the reduced and nonreduced solid digested bone and EDTA extraction samples. The EDTA extraction produced a total of 44 accessions, 14 of them unique (Figure 3, Table S3).

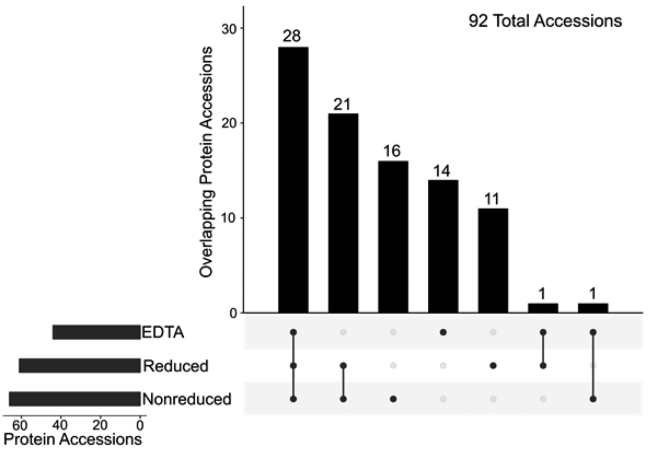

Figure 3. UpSet plot of EDTA and solid-digested bone samples. Dots indicate sample with containing accessions; connecting bar indicates multiple overlapping samples.

Osteocalcin was only detected in the EDTA fraction and was not present in the solid-digested bone (SDB) samples based on extracted ion chromatograms of m/z 1036.50−1036.54 corresponding to the N-terminal peptide (YLDSGLGAPVPY-PDPLEPK; Figure 4A). The nonreduced SDB aliquots had a total of 66 accessions, after removal of contaminant accessions, with 16 unique accessions (Table S4). The reduced SDB aliquots had a total of 61 accessions, after removal of contaminant accessions, with 11 unique accessions (Table S5). The lowest scoring accessions represent most of the unique accessions for the SDB samples, suggesting that the main differences are stochastic (Tables S3, S4, and S5). Together, the reduced and nonreduced samples contained 49 overlapping accessions. Collagen I was detected in all three analyses; the greatest sequence coverage for alpha 1 was

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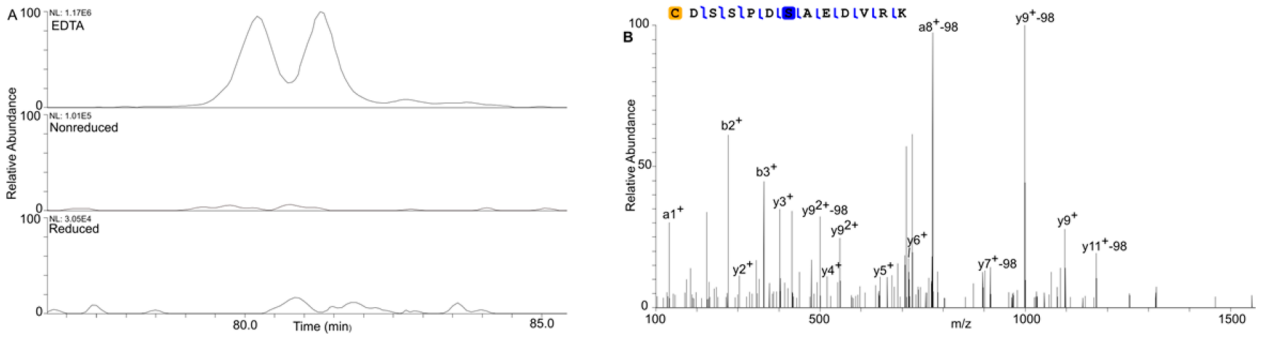

Figure 4. (A) Extracted ion chromatograms (m/z 1036.50−1036.54) of the carboxylated osteocalcin peptide (YLDSGLGAPVPYPDPLEPK) showing its presence in EDTA and not in either solid-digestion sample. (B) Alpha-2-HS-glycoprotein phosphopeptide (CDSSPDSAEDVRK) showing phosphorylation neutral losses (−98 Da), carbamidomethyl cysteine (yellow box), and phosphorylation (blue box). Annotation follows PEAKS determination.

detected in the nonreduced SDB aliquots, and there was equal coverage of alpha 2 in the reduced and nonreduced SDB aliquots. In all three aliquots, both N- and C- telopeptides of collagen I were detected, including the N-terminal glutamine of collagen I alpha 1 (including the pyroglutamate and unmodified forms). The C-terminal alanine of collagen I alpha 1 was not detected in any fraction as a result of tryptic digestion. In both SDB samples for collagen I alpha 2, the C-terminal alanine on collagen I alpha 2 was detected. The N-terminal glutamine of collagen I alpha 2 was only detected in the nonreduced SDB. Furthermore, various post-translational modifications (PTMs) were detected in the SDB samples including phosphorylation (Figure 4B). In addition to collagen I, various proteoglycans (e.g., biglycan) and proteins associated with collagen I (e.g., decorin) were detected (Table S2). Peptide sizes were statistically larger ($p < 0.001$; Figure 5) for both the reduced and nonreduced demineralized bone aliquots than in the EDTA extraction. The nonreduced SDB peptides were also statistically ($p < 0.05$) longer than the reduced SDB peptides.

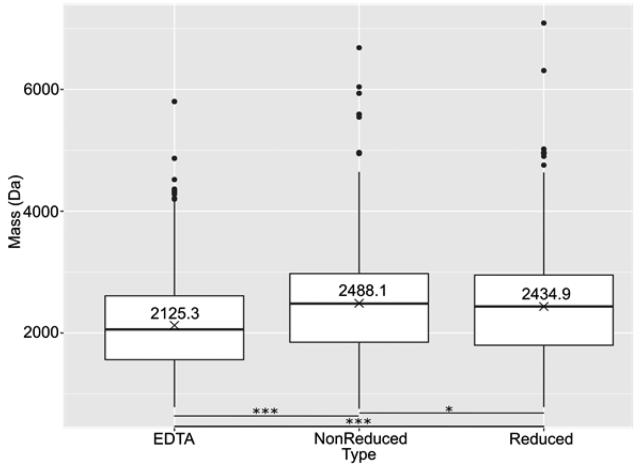

Figure 5. Peptide sizes of EDTA and nonreduced and reduced solid-digested bone. × indicates the mean of each peptide set. * $p < 0.05$; *** $p < 0.001$.

DISCUSSION

Study of bone extractomics has been able to show a large diversity in proteins identified by different methods,[11] but the insoluble proteins lost during the extraction process limits the overall scope of what is possible to detect. The solid digestion approach applied here has major advantages to previously applied extract and digest because the level of protein solubility is not a concern. One caveat is its usage of more trypsin than typical mass spectrometric analyses; however, this can be mediated with smaller demineralized bone pieces. Despite this caveat, the advantages may be especially true for collagen I that has reduced solubility as a result of advanced glycation end-products that form during the in vivo aging process.[21] Furthermore, the maturation of enzymatic collagen cross-links also reduces the solubility of collagen I.[22,23] Unknown processes affecting bone proteins during diagenesis may also decrease their solubility, potentially leading to their preservation, so extraction-based methodology would then be ill-suited to extract and identify them. Therefore, applying this solid digestion protocol on fossil bone may provide more direct evidence of the preservational mode of the proteins than extraction-based approaches alone. Few diagenetic modifications have been hypothesized based on traditional extraction methodology,[4] but many more might be evident after directly digesting the proteinaceous component of fossil bones.

Recently, Cappellini and coworkers[7,15,16] have devised and successfully used a hybrid method of digesting the pellet after partial demineralization with EDTA instead of strictly digesting proteins in the supernatant after extraction. However, they still recovered osteocalcin,[16] which is highly associated with bone mineral, so their approach does not appear to result in the same degree of demineralization applied to the SDB here. The detection of osteocalcin and other unique proteins in only the EDTA extraction and not in the SDB is consistent with Cairns and Price,[24] where they found that osteocalcin is fully extracted in EDTA. This finding shows the importance of characterizing the EDTA soluble proteins (also suggested by Schroeter et al.[11]), which has historically been shown to be different from the insoluble matrix by lower resolution methods (e.g., gel filtration[25]), in addition to the SDB peptides to form a comprehensive analysis of the bone proteome. Despite the effectiveness of the SDB in characterizing portions of the bone proteome that may be missed by extraction methods, characterization of the EDTA fraction remains critical despite its discard in many bone proteomic and paleoproteomic studies. In addition to testing the demineralization solution, tracking the depletion of osteocalcin in the samples could provide a direct means to evaluate the level of demineralization without needing to apply computed tomography measures of the bone mineral density.

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Previous bone proteomic analyses have applied reduction and alkylation, following traditional proteomic approaches to reduce the complexity of tandem mass spectra generated by disulfide bonds.[26] Because collagen I does not have any disulfide bonds, this solid-digestion protocol did not originally include these steps; however, both reduced and nonreduced peptides were characterized here to detect potential differences with the addition of reduction and alkylation. The postdigestion reduction and alkylation likely impacts the results obtained here by reducing the access of trypsin to disulfide bound areas of the proteins; however, the results here show that, even without predigestion reduction and alkylation, digestion following this method is effective at obtaining a large number of bone proteins without extraction. Alkylation increases the detection of peptides with cysteine and post-translational modifications (e.g., Figure 4B). A similar number of protein accessions were detected for both reduced (61) and nonreduced (66) SDB samples (Figure 3) after the removal of contaminant accessions, but different regions of the same proteins were detected as a result of the retained disulfide bonds. The application of other advanced algorithms (e.g., Byonic) that can directly detect retained disulfides will maximize the sequence coverage and linked peptides retained in nonreduced SDB samples. The nonreduced bone peptides were longer ($p < 0.05$) than the reduced ones (Figure 5), consistent with possible disulfides still present in the samples. This size difference likely is also the result of incompletely digested proteins from the solid digestion. Increasing the maximum number of missed cleavages (currently up to three for the largest peptides) allowed by the algorithm may also increase the size of detected peptides to a middle down size, allowing for a greater diversity of diagenetic modifications to be detected, especially those associated with protein backbone cleavage.

Post-translational modifications (PTMs) of bone proteins remain a problem in bone proteomics because retention of labile PTMs (e.g., carboxylation on osteocalcin under acidic conditions;[27,28] phosphorylation in strongly basic solutions[29] including those used to remove humic substances in paleoproteomics[30]) after extraction may not be possible. When these PTMs are lost, the implications of protein changes associated with bone diseases and bone fragility[31−34] may be obscured, and preserved in vivo PTMs and PTMs that form as a result of diagenesis in fossil samples may be missed.[4,35] The large majority of PTMs detected from bone extractions are hydroxylated proline in collagen I molecules.[4,5,7,9,16,36] Other PTMs on bone proteins (e.g., glycosylation, phosphorylation, carboxylation) may or may not be detected depending on the extraction and sample preparation approaches. For example, gamma carboxylation of glutamine has previously been shown to be labile and removed in acidic solutions precluding evaluation of the levels of carboxylation,[27] potentially leading to the incorrect determination of undercarboxylation (an important factor in bone fragility[37,38]). Using the SDB approach, detection of phosphorylation of serine-137[39] on alpha-2-HS-glycoprotein (A2HSG) was possible (Figure 4B). This phosphopeptide was not detected in the EDTA extraction, although A2HSG was present in the EDTA extraction as well. Peptide spectral matches containing glycosylation (i.e., the following modifications in alphabetical order: glucosylgalactosyl hydroxylysine, Hex1HexNAc1NeuAc1, Hex1HexNac1NeuAc2, Hex1HexNAc2, Hex1HexNAc 2dHex1Pent1, Hex1HexNAc2-Pent1, Hex1HexNAc2Pent1, HexNAc 2dHex1, HexNAcylation [N], HexNAcylation [ST], Hexosamine, Hexose, Hexose [NSY]) on collagen I and collagen V (Figure 6) were much more numerous for the SDB samples than the EDTA extraction

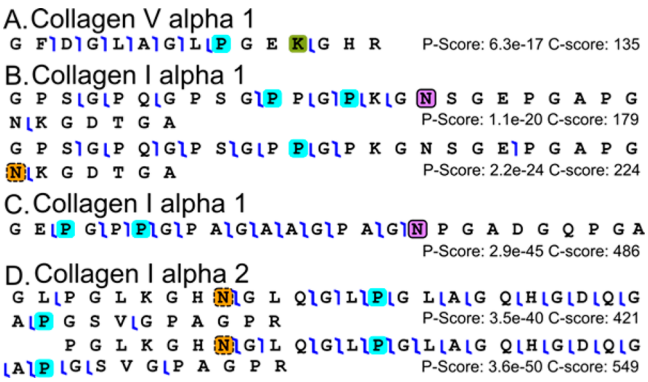

Figure 6. Example fragment maps of collagen I and V peptides with glycosylations. (A) Peptide from collagen V alpha 1 with glucosylgalactosylhydroxylysine. (B) Peptide spectra matches from collagen I alpha 1 showing either hexose or hexosamine of asparagine. (C) Peptide from collagen I alpha 1 with hexose modification of asparagine. (D) Peptide spectral matches from collagen I alpha 2 showing hexosamine modification of asparagine regardless of the n-terminal amino acids. Light blue boxes, hydroxylation of proline; green box, glucosylgalactosylhydroxylysine; purple with black border, hexose; orange boxes with dashed border, hexosamine.

(nonreduced SDB: 224; reduced SDB: 310; EDTA: 25; All peptides are present in the R supplementary peptide tables in the Supporting Information: 10_peptide61417.xls [SDB non-reduced], 29_peptide61317.xls [SDB reduced], 36_peptide61317.xls [EDTA]). This solid digestion can better access these modifications of collagens I and V that may be missed during traditional extraction, making it a critical tool for fully understanding the post-translational state of bone proteins. With future refinements to this SDB approach, optimization of mass-spectrometry parameters, and use of alternative digestion protocols, critical PTMs from a diversity of bone proteins can be fully characterized. For example, osteopontin is known to be phosphorylated, but the locations of phosphorylation in bone are not known. Instead, our understanding of phosphorylation sites on osteopontin is known solely from milk.[40]

The results of the solid preparation of tendon as a collagen I standard provide a cautionary point in choosing standards for other assays where sensitivity/selectivity is important. While collagen I represented the vast majority of the detected peptide spectra matches, finding a multitude of other proteins in this standard may lead to false-positives for other assays (e.g., Western blots, enzyme-linked immunosorbent assays) when it is assumed, as part of the experimental design, that only collagen I is present.

CONCLUSIONS

Utilization of solid-digestion of bone pseudomorphs after demineralization provides access to a large diversity of proteins and PTMs that may otherwise be missed using traditional extraction methodology. Application of this approach on fossil remains will provide vital information on how proteins preserve in the rock record.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00670.

Figure S1. von Kossa staining of demineralized bone. (PDF)

Table S1. PEAKS proteins detected from the reduced collagen I standard from bovine tendon. (XLSX)

Table S2. PEAKS proteins detected from the nonreduced collagen I standard from bovine tendon. (XLSX)

Table S3. PEAKS proteins detected from EDTA extraction. (XLSX)

Table S4. PEAKS proteins detected from the nonreduced solid-digested bone samples. (XLSX)

Table S5. PEAKS proteins detected from the reduced solid-digested bone samples. (XLSX)

R script and associated csv files. (ZIP)

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Notes

The author declares no competing financial interest.

All data are available at ftp://massive.ucsd.edu/MSV000081399.

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