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Influence of carboxylation on osteocalcin detection by mass spectrometry

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RATIONALE: Osteocalcin is a small, abundant bone protein that is difficult to detect using high-throughput tandem mass spectrometry (MS/MS) proteomic approaches from bone protein extracts, and is predominantly detected by non-MS immunological methods. Here, we analyze bovine osteocalcin and its post-translational modifications to determine why a protein of this size goes undetected.
METHODS: Osteocalcin was purified from cow bone using well-established methods. Intact osteocalcin or trypsin-digested osteocalcin were separated using an Agilent 1200 series high-performance liquid chromatography (HPLC) system and analyzed using a ThermoScientific LTQ-Orbitrap XL after fragmentation with higher-energy collision dissociation. Data were analyzed using Mascot or Prosight Lite.
RESULTS: Our results support previous findings that the cow osteocalcin has up to three carboxylations using both intact osteocalcin and digested forms. Using Mascot, we were able to detect osteocalcin peptides, but no fragments that localized the carboxylations. Full annotation using Prosight Lite of the intact (three carboxylations), N-terminal peptide (one carboxylation), and middle peptide (two carboxylations) showed complete fragmentation was present, but complete neutral loss was observed.
CONCLUSIONS: Osteocalcin carboxylation, and its associated neutral losses, makes high-throughput detection of this protein challenging; however, alternative fragmentation or limited purification can overcome these challenges.

Osteocalcin (OC) is an important protein in the extracellular matrix of bone that controls hydroxyapatite crystal properties[1,2] and plays a role in whole bone mechanics because of its interaction with both hydroxyapatite and bone collagen I.[3] Because of its multiple roles in bone and non-skeletal role in hormone promoting insulin production,[4] osteocalcin has been a protein of major interest in extant human[2,5–9] and model organism studies.[1,3,10–13] Additionally, because of its close association with hydroxyapatite, size, and acidity, it has been hypothesized to persist into the deep time making it a prime target for paleontological and archaeological investigations.[14–25] It has also been a target for fossil remains because the N-terminal residues are variable providing greater phylogenetically informative sequences than other commonly preserved proteins in bone (e.g., collagen I).[18] It is a small protein (~5800 Da) with three vitamin K dependent γ-carboxylated glutamic acid residues (Gla) at positions 17, 21, and 24[26] that allow for direct binding to calcium in hydroxyapatite.[27] In most species, all three residues are carboxylated; however, incomplete carboxylation has been observed in humans resulting from limited vitamin K uptake in the diet.[28] Additionally, OC derived from humans and other closely related primates has been shown to lack hydroxyproline (Hyp), which has been observed on OC from other mammals including *Bos taurus*.[16]

Traditionally, OC has been detected by radioimmunoassay[7,27,29] that can identify the proteins from whole bone protein extracts without additional purification; however, these antibody-based methods provide little or no information on the post-translational modifications (PTMs) of the protein (i.e., Gla, Hyp). Complimentary methods have been developed to assess whether osteocalcin is undercarboxylated (e.g., hydroxyapatite binding assay[27]), but these methods cannot indicate which Gla residues are modified or unmodified. These techniques remain the predominant way that OC is identified and levels of carboxylation are determined.[30–34] In contrast to the antibody-based methods, mass spectrometry (MS), especially using electrospray ionization (ESI), can provide direct measurement of the PTMs on OC; however, detection and/or characterization of this protein by MS or by Edman-type sequencing (i.e., for intact sequence characterization) has relied on extensive purification steps (e.g., high-performance liquid chromatography (HPLC)) before analysis.[16,28] This purification is also true for matrix-assisted laser desorption/ionization (MALDI)-based MS of OC, which has led to mass measurements but loss of Gla carboxylation precluding identification of osteocalcin carboxy-proteoforms.[35,36]

Using purified *B. taurus* OC, we analyze this protein and its PTMs, after ESI, to investigate why it is difficult to detect by high-throughput mass spectrometry of bone protein

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extractions. We additionally use a mixture of bovine collagen I and osteocalcin to show the effects of collagen I on osteocalcin ionization.

EXPERIMENTAL

Bos taurus osteocalcin and digestion

OC from *B. taurus* bone was purified following Gundberg *et al.*[37] In short, cleaned, marrow-free bovine bone was ground and then pulverized to <100 μm in size with a magnetically driven mill (Spex Industries, Methuchen, NJ, USA). The resultant bone powder was rinsed with methanol and then ether, dried, and extracted twice with 20 vol. 0.5 M EDTA, pH 7.2, containing protease inhibitors. The EDTA extract was exhaustively dialyzed, lyophilized, redissolved in 50 mM NH_4HCO_3, and then fractionated on Sephadex G-75. A large peak, which eluted near the expected size of osteocalcin (~6 kDa), was subjected to DEAE ion exchange. The DEAE-purified material was further purified by HPLC using a preparative C_{18} column with a 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid. Rechromatography of the central portion of a large trailing peak yielded a single symmetrical peak which was intact osteocalcin. For intact analyses, OC was reduced with 10 mM dithiothreitol for 20 min at 55°C followed by acidification with an equal volume of 1% formic acid. Prior to digestion, OC was reduced with 10 mM dithiothreitol for 1 h at room temperature, alkylated with 30 mM iodoacetamide for 1 h in the dark, and digested overnight at 37°C with Trypsin Gold (Promega, Madison, WI, USA) at a ratio of 1:100 (trypsin/protein). All intact and digested OC were concentrated using Empore (3 M, Saint Paul, MN, USA) C18 stage tips.[38] Peptides were eluted from the tip using 20 μL of 80% acetonitrile, 0.1% formic acid. After evaporation of the acetonitrile, peptides were resuspended in 0.1% formic acid.

To evaluate the effect of collagen I peptides on osteocalcin detection by MS, we also digested a sample with 100:1 bovine collagen I (BD Biosciences)/OC as described above. We chose 100:1 to reflect a similar ratio of collagen to OC in bone, and clearly show the effect of collagen I on osteocalcin detection.

Mass spectrometry

OC peptides, collagen I/OC mixture, and intact OC were separated using a 1200 Series HPLC system (Agilent, Santa Clara, CA, USA) with a BioBasic C18 column (2.1 mm ID, 150 mm length, 5 μm particle size; Thermo Scientific) for 75 min using the following gradient: 2% B 0–5 min, 30% B 5–35 min, 60% B 35–60 min, 95% B 60.01–64 min, 2% B 64.01–75 min where A is 0.2% formic acid and B is 100% acetonitrile, 0.2% formic acid. Eluted peptides were characterized in positive mode on a LTQ-Orbitrap XL (Thermo Scientific) with the following parameters: top three peaks fragmented with higher-energy collision dissociation (HCD) (35 NCE) with a 3 *m/z* isolation window; 30,000 resolution for precursor and fragment scans; precursor scan range 300–2000 *m/z*. The three most abundant peaks were fragmented using HCD in the Orbitrap.

Data analysis

To identify OC peaks, raw files were converted into peak lists (mgf) using MassMatrix Mass Spectrometric File Conversion Tools version 3.2 and searched against Swissprot and a decoy database using Mascot 2.3 (Matrix Science, Boston, MA, USA). The following parameters were set for each search: taxonomy was set to Mammals; enzyme = trypsin; up to 3 missed cleavages; variable modifications: carbamidomethyl (CAM) N-terminus, CAM lysine, deamidation of asparagine and glutamine, carboxy glutamic acid, oxidation of methionine and proline; static modifications: CAM cysteine; peptide tolerance =10 ppm; fragment tolerance =0.5 Da; and peptide charge =2+, 3+, 4+. Peptide results were filtered using a Mascot score cutoff of 45.

Additional, *in silico* digestion of *B. taurus* OC was performed with either ProteinMass[39] or Protein Prospector version 5.14.3.[40] Averaged MS/MS spectra corresponding to these peaks were extracted with a 1.5 S/N in Qualbrowser (ThermoScientific) and annotated using Prosight Lite[41] with error tolerance set to 10 ppm and fragmentation type HCD. Proline 9 was modified to hydroxyproline (+15.994915 Da), and Glu 17, 21, 24 were modified to Gla (+43.989829) manually or left unmodified. For the digested peptides, cysteine or the protein N-terminus was modified by carbamidomethylation (+57.021464).

Carboxylation level was quantified using areas from extracted ion chromatograms in Qualbrowser. The following masses were used for area calculations based on the intact protein mass extracted across the mass envelope: 0 carboxylations (1143.93–1145.58 [5+]), 1 carboxylation (1153.00–1154.92 [5+]), 2 carboxylations (1161.62–1163.53 [5+]), and 3 carboxylations (1170.28–1172.46 [5+]), and for the chromium-adducted intact form:[35,42] 0 carboxylations (1155.05–1156.29 [5+]), 1 carboxylation (1163.97–1164.84 [5+]), 2 carboxylations (1172.57–1174.02 [5+]), and 3 carboxylations (1180.83–1183.48 [5+]). For the N-terminal peptide we used the following masses: 0 carboxylations (1097.96–1099.13 [2+]), 1 carboxylation (1119.95–1122.60 [2+]), 0 carboxylations +1 CAM (1126.48–11283.61 [2+]), 1 carboxylation +1 CAM (1148.46–1151.11 [2+]), 1 carboxylation +2 CAM (1176.97–1179.11 [2+]), 1 carboxylation +3 CAM (1205.48–1208.13 [2+]), and for the middle peptide: 0 carboxylations +2 CAM (979.37–981.14 [3+]), 1 carboxylation +2 CAM (994.03–995.82 [3+]), 1 carboxylation +3 CAM (1013.01–1014.85 [3+]), 2 carboxylations +2 CAM (1008.70–1010.51 [3+]), and 2 carboxylations +3 CAM (1027.66–1029.56 [3+]).

RESULTS AND DISCUSSION

Osteocalcin is the second most abundant bone protein,[43] and through its carboxyglutamic acids binds tightly to bone hydroxyapatite.[27] In humans, this protein is found in various states of undercarboxylation as a result of limited vitamin K in human diets.[27] This important variation in carboxylation state may impact the mechanical properties of bone, and the levels have been extensively measured using plate-based assays; however, these methods provide incomplete characterization of the various osteocalcin proteoforms that exist in bone.[27] Here, we chose to analyze bovine OC

because it is a typically fully carboxylated exemplar of what to expect for carboxylated OC, without the complexity of undercarboxylation from human bone. For intact OC, we were able to detect proteoforms possessing between 0–3 carboxylations. Additionally, we identified chromium-adducted (+51.9) forms with the 0–3 carboxylations.[42] This adduction has been hypothesized to derive from the ESI emitter capillary; however, based on our chromatography and later elution, it appears this adduction is likely from earlier in the HPLC run (Supplementary Fig. S1, Supporting Information). In total (i.e., combined chromium-adducted and non-adducted OC), we found fully carboxylated *B. taurus* OC (81.5 ± 0.2%) with hydroxyproline (Fig. 1(A); Supplementary Fig. S2) as has been previously reported from *B. taurus* bone;[26] however, the doubly (15.7 ± 0.5%), singly (1.9 ± 0.3%), and uncarboxylated (0.9 ± 0.1%) proteoforms were also detected (Fig. 1(A)). The chromium-adducted forms show a more consistent level of carboxylation (Supplementary Table S1; for 3 carboxy: 84.0 ± 0.9%) compared to the non-adducted form (Supplementary Table S1; for 3 carboxy: 77.0 ± 12.4%), suggesting that the chromium is stabilizing the osteocalcin carboxylation, and that some of the carboxylation variation that we detect is the result of decarboxylation during or before LC. This loss of carboxylation must be considered in future MS carboxylation quantitation experiments for OC from all species, especially humans where undercarboxylation is common.[28] Additionally, fragmentation of the triply carboxylated bovine OC shows the distinct neutral losses of carboxylated glutamic acids (Fig. 1(B)).

The incomplete carboxylation is also observed after digestion of the *B. taurus* OC with trypsin on both the N-terminal peptide (94.9 ± 0.1% singly carboxylated; Fig. 2(A))

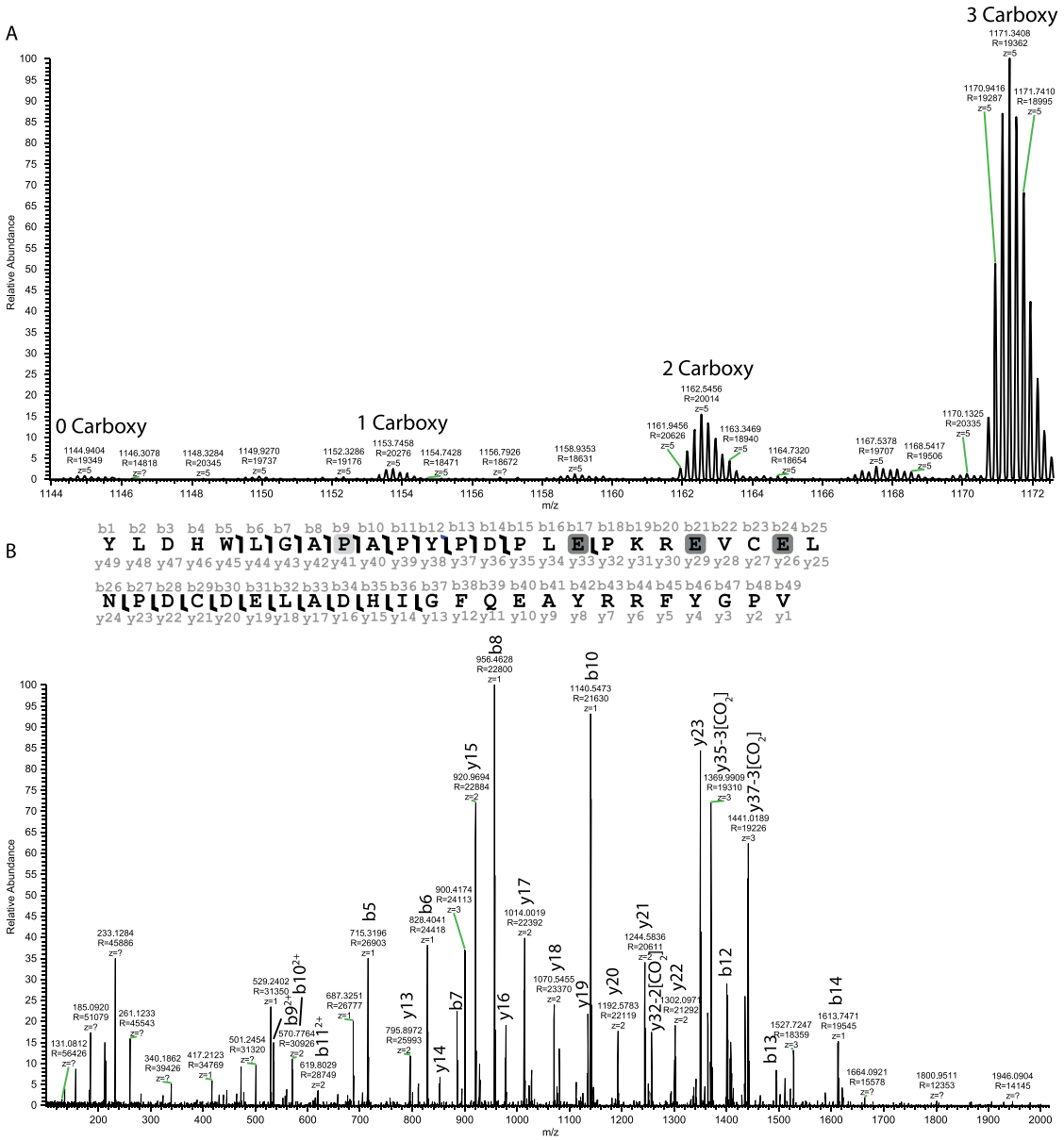

Figure 1. (A) Intact *Bos taurus* OC showing between 0 and 3 carboxylations. Most are triply carboxylated. (B) Fragmentation of *m/z* 1171.3408 showing as many as three CO_2 losses. Light gray box = hydroxylation; dark gray box = carboxylation.

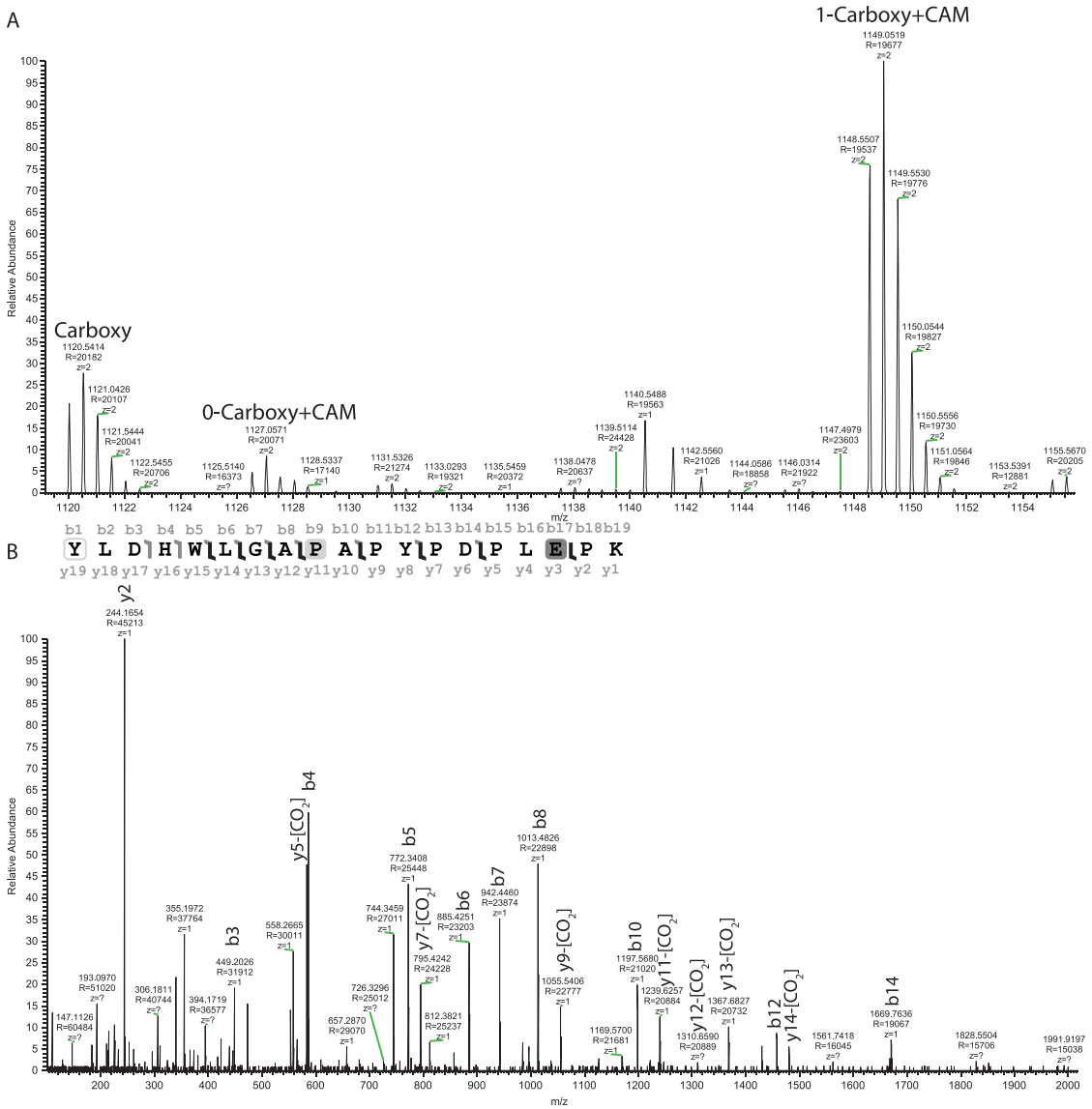

Figure 2. (A) Spectrum of the N-terminal tryptic peptide showing single carboxylation with carbamidomethyl N-terminus (1-Carboxy + CAM) and uncarboxylated (0-Carboxy + CAM) forms. (B) Fragmentation pattern of *m/z* 1149.05 in (A). No carboxylated y-ion peaks are present; all detected peaks show CO_2 loss except y2, which does not overlap the carboxylation. Light gray box = hydroxylation; dark gray box = carboxylation; empty gray box = carbamidomethylation of the N-terminus. Gray fragment markers indicate fragments detected by Mascot.

and middle peptide (65.8 ± 1.6% singly carboxylated, 34.2 ± 0.8% doubly carboxylated; Fig. 3(A)); however, no evidence of chromium adduction is present on the tryptic peptides. Consistent with the intact proteoform, neutral losses were observed for all positions of carboxylation for the N-terminal and middle peptides. Mascot (Supplementary Tables S2–S4, Supporting Information) was able to consistently detect the N-terminal peptide and variably detect the middle tryptic peptide of the bovine OC (Figs. 2(B) and 3(B)); however, fragments localizing the carboxylations were not detected, instead only b-ion fragments of the N-terminal peptide or y-ion fragments of the middle peptide were detected. The fragmentation pattern differences between Prosight Lite and Mascot reflect the presence of fragments corresponding to the neutral loss peaks associated with the loss of carboxylation after fragmentation (Figs. 2(B) and 3(B)). The digestion of this *B. taurus* OC shows that, even for peptides with good HCD fragmentation, carboxylation can limit identification of fragments that localize carboxylation positions. We also observed extensive overalkylation of the cysteine-free N-terminal peptide (86.1 ± 0.1%) and the di-cysteine middle OC peptide (61.1 ± 1.4%) regardless of carboxylation state, making the addition of the variable N-terminal CAM modification in Mascot critical for detection of these peptides. Routine inclusion of N-terminal CAM may not be feasible with the increase in database search space; however, rapid identification of overalkylation for accurate searching is available in various software packages, including Protein Metrics Preview.[44] Missing identifications through database searching, as a result of neutral loss and overalkylation, becomes a major problem for global bone proteome studies where this important protein may not be identified.

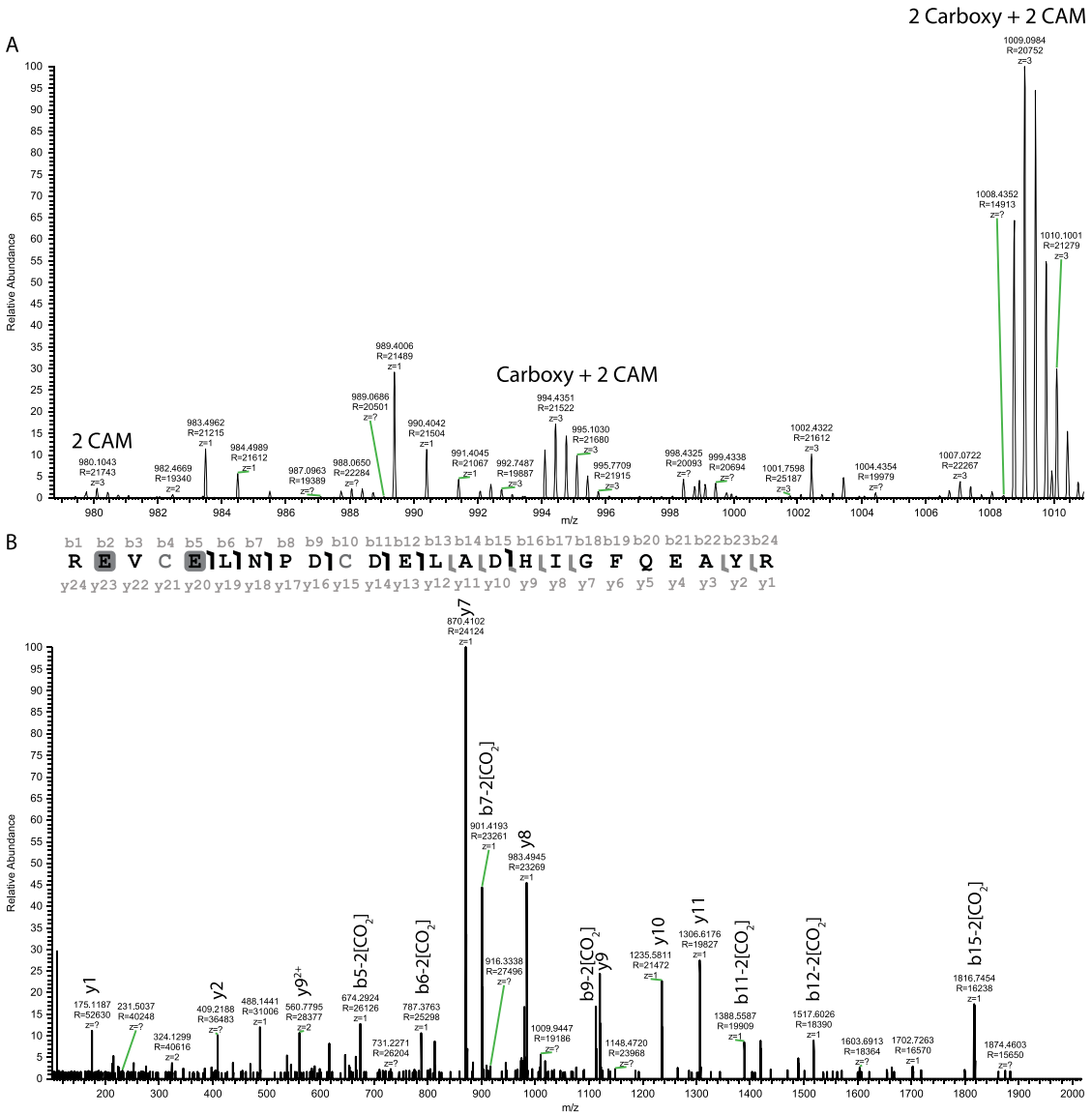

Figure 3. (A) The middle peptide (REVCELNPDCDELADHIGFQEAYR) showing 0–2 carboxylations. (B) Fragmentation of *m/z* 1009.09 in (A) showing two CO_2 losses for all b-series ions. No y-series ions overlapping the carboxylation were detected. Dark gray box = carboxylation; gray C = carbamidomethylation of cysteine. Gray fragment markers indicate fragments detected by Mascot.

It is noteworthy that detection of osteocalcin peptides by MS is hindered by two major factors: (1) γ-carboxylation of one or more glutamic acids, and (2) the acidity of OC. γ-Carboxylation of glutamic acid results in neutral loss after fragmentation using CID or HCD (Figs. 1–3). Consequently, these neutral losses make identification of the modified peptides more difficult.[45,46] CO_2 loss during fragmentation gives b- or y-ion series consistent with the unmodified protein (Fig. 1(B)) or peptide (Figs. 2(B) and 3(B)) depending on the position of the carboxylations instead of the modified forms, resulting in missed identifications or limited fragment matches using Mascot. Other fragmentation methodology (e.g., electron transfer dissociation,[45] electron capture dissociation,[42,46] ultraviolet photodissociation[47]), methylation of the glutamic acid carboxyl groups,[45] or mass spectrometry immunoassay (MSIA[35]) or other osteocalcin purification methods (e.g., preparative HPLC,[37] gel filtration[48]) may be necessary to better identify the carboxylated OC. However, the inclusion of additional modifications through methylation may further complicate the analysis by inducing unknown numbers of methylations or further splitting the peptide/protein signal into a greater number of forms. MSIA or HPLC purification provides an opportunity to isolate OC from bone extracts in quantitative amounts lending itself to a complete characterization of the bone OC proteoforms present.

Furthermore, the acidity of OC may impact its detectability, especially in bone extractions. In positive ionization mode, acidic peptides show reduced ion signals[47] compared to comparatively basic ones (e.g., those from collagen I). While we do not have problems electrospraying the *B. taurus* OC by itself, in the presence of bovine collagen I, we were unable

to detect the bovine OC. Mascot searching only resulted in detection of collagen I alpha 1 and alpha 2 peptides. This result is similar to our previous results detecting only the N-terminal peptide of OC from human samples without prior purification in the presence of a large number of collagen I peptides.[49] Removal/depletion of the collagen I or enrichment of osteocalcin by purification[37] may help to better identify this protein from bone samples by MS.

CONCLUSIONS

With mass spectrometry and HCD fragmentation, we are able to detect variably carboxylated *B. taurus* OC, yet technical challenges remain to detect modified OC in an automated or high-throughput way. Alternative fragmentation methodology and/or additional search strategies may overcome the challenges of characterizing carboxylated OC by MS.

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