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Journal of Chromatography A, 962 (2002) 95–103

JOURNAL OF CHROMATOGRAPHY A

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High-performance liquid chromatography–mass spectrometry and electron-capture dissociation tandem mass spectrometry of osteocalcin
Determination of γ-carboxyglutamic acid residues

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Received 11 January 2002; received in revised form 18 April 2002; accepted 18 April 2002

Abstract

Two mass spectrometry methods, high-performance liquid chromatography combined on-line with electrospray ionization mass spectrometry (HPLC–ESI-MS) and electron-capture (EC) dissociation tandem mass spectrometry (MS–MS), were applied for structural analysis of bovine and human osteocalcins. Osteocalcin contains γ-carboxyglutamic acid (Gla) residues, which bind metal ions, among its amino acids. Ethylenediaminetetraacetic acid (EDTA) was added to all samples in order to chelate bound metal ions. After elimination of interfering metal ions MS spectra became uncomplicated to interpret. EDTA is incompatible with ESI and it was removed from samples using either on-line HPLC or micropurification method. The number of Gla residues varies in osteocalcin. These subforms, which contain different amounts of Gla residues, were separated using the HPLC–ESI-MS method. In order to determine locations of Gla residues in human osteocalcin, which contained two Gla residues, dissociation MS–MS method was successfully applied.

Keywords: Electron-capture dissociation; Mass spectrometry; Carboxyglutamic acid; Osteocalcin

1. Introduction

Osteocalcin (OCN), also called bone Gla protein, is a bone matrix protein of 49 amino acid residues in humans [1]. OCN was independently discovered by two separate research groups of Hauschka et al. [2] and Price et al. [3]. OCN is presently known as the most abundant non-collagenous bone protein [4,5]. OCN is secreted by osteoblasts and is a specific biochemical marker for bone formation [6]. A special feature of OCN is its γ-carboxyglutamic acid (Gla) residues, which are located at positions 17, 21 and 24 [7]. The Gla residues increase the affinity of OCN to calcium [8]. In the presence of calcium the polypeptide backbone of OCN adopts a α-helical conformation [9]. It is demonstrated that the Gla residue at position 17 is essential for calcium-dependent conformational transition of OCN [10]. The space between the Gla residues in three dimensional

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00451-X

structure correspond to the interatomic distance of calcium atoms in hydroxyapatite lattice and it is demonstrated that this interaction accounts for tight adsorption of OCN onto hydroxyapatite surface and OCN's affinity for bone [8,9].

Mass spectrometry plays an important role in the characterization of peptides and proteins [11]. Detection of ever more unstable modifications, such as glycosylation, carboxylation, oxidation, and phosphorylation, to biomolecules is possible using methods as electrospray ionization mass spectrometry (ESI-MS) [12]. However, further microcharacterization of these unstable species with such energetic methods can be difficult as a result of ejection of the modification before backbone bond cleavage [12]. Formation of γ-carboxyglutamic acid residue from glutamic acid is an example of labile post-translational modifications. Several mass spectrometric methods have been introduced to analyze Gla residues: gas chromatography–mass spectrometry (GC–MS) [13–15], matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) [1,16,17]. However, these methods either need reasonably complicated sample preparation methods or spectra give inadequate data, when further analyzing methods are needed to identify the sample. Electron-capture (EC) dissociation is a new tandem MS (MS–MS) method [18,19]. EC dissociation cleaves peptide backbones primarily at the C_α–N bond rather than at the amide linkage as with collisionally activated dissociation (CAD). The potential of EC dissociation for analysis and sequencing of post-translationally modified peptides and proteins has been demonstrated for O-glycosylated peptides [20], sulfapeptides [12,21], phosphorylated peptides [22], phosphorylated proteins [23] and γ-carboxylated peptides [12]. As a gentle fragmentation method EC dissociation does not induce loss of labile conjugated groups in peptides in MS–MS experiments. A technical description for EC dissociation will not be reviewed here, as this has been covered in detail recently [24].

In this study the aim was to develop a MS method to analyze OCN and its Gla residues. First a high-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI-MS) method was successfully developed to separate subforms of OCN containing different amounts of Gla residues. Further microcharacterization of OCN was impossible with this HPLC–ESI-MS method: CO_2 of Gla was ejected by ESI-MS–MS during CAD. The ejection of CO_2 occurred before backbone bond cleavage. Secondly an EC dissociation MS–MS method was applied in order to locate Gla residues in OCN peptide. The ECD-MS–MS method has been demonstrated to be suitable for analysis of small 28 residue peptides containing Gla residues [12], however suitability for larger peptides containing Gla residues has not been demonstrated earlier. Here we report application of EC dissociation MS–MS for determination of γ-carboxylation sites in OCN. Using these two methods it is possible to fully characterize OCN and its subforms, which contain different amounts of Gla residues. These methods provide a useful tool to determine OCN's exact function in bone and to evaluate its importance as a biochemical marker of bone function. These methods are also useful in developing more stable peptide drug formulations because it is possible to get more information on labile post-translational modifications of ever more larger proteins.

2. Experimental

2.1. Chemicals

Bovine osteocalcin (bOCN) was isolated from bovine bone powder using a previously described method [25] and further purified by a method described by Kaartinen et al. [26]. The amino acid sequence (Fig. 1) contains two to three residues of Gla at positions 17, 21 and 24, hydroxyproline (Hyp) at position 9 and disulfide bond between cysteine residues (Cys-23 and Cys-29) [9]. Samples were diluted with water to a final concentration. (Glu-17, Gla-21, Gla-24)–Osteocalcin human 1–49 (hOCN) was obtained from Peninsula Labs. (Belmont, CA, USA). The amino acid sequence contains two Gla residues at positions 21 and 24 and disulfide bond between cysteine residues (Fig. 1). Solid protein was dissolved in concentration 50 μg/ml in phosphate buffer pH 7 obtained from FF-Chemicals (Yli-Ii, Finland) [27], divided into aliquots and stored in a freezer (−20 °C).

Ethylenediaminetetraacetic acid (EDTA) Titriplex III and formic acid were obtained from Merck

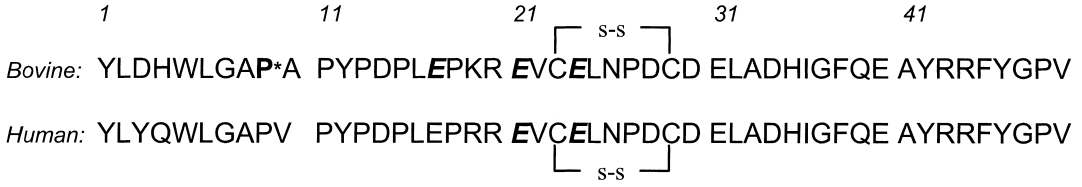

Fig. 1. The primary structure of bovine osteocalcin 1–49 and of human osteocalcin 1–49 (Glu-17, Gla-21, 24). *P**=hydroxyproline, *E*=γ-carboxyglutamic acid.

(Darmstadt, Germany), dithiothreitol was from Boehringer–Mannheim (Germany), urea was from Riedel-de Haen (Seelze, Germany), ammonium hydrogencarbonate was from Fluka (Buchs, Switzerland), acetonitrile from Rathburn (Walkerburn, UK) and Poros R2 from PerSeptive Biosystems (Cambridge, MA, USA). Water was deionized using a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Methods

2.2.1. Chelation of metal ions with EDTA

bOCN was analyzed both without any additional EDTA and with EDTA. EDTA was dissolved in water and added to samples in order to get a final concentration from 5 to 10 m*M*.

2.2.2. Determination of Gla residues

In order to determine the Gla residues the disulfide bond was reduced and the Cys residues were alkylated using the following procedure: 30 μl bOCN (20 μg/ml) was taken and 3.5 μl EDTA (100 m*M*), 25 μl NH_4HCO_3 (0.1 *M*) and 6 μl dithiothreitol (100 m*M* DTT in 0.1 *M* NH_4HCO_3) was added and stirred carefully. The solution was incubated at 56 °C for 15 min. After incubation 4 μl of 0.5 *M* iodoacetamide in 0.1 *M* NH_4HCO_3 was added and the solution was protected from light and incubated at room temperature for 15 min.

2.2.3. HPLC–ESI-MS

2.2.3.1. HPLC–ESI-MS Method A

Positive ion mass spectra were acquired with online HPLC–ESI-MS. The measurements were carried out using a LCQ quadrupole ion trap mass spectrometer equipped with an ESI ion source (Finnigan MAT, San Jose, CA, USA), a Rheos 4000 HPLC pump (Flux Instruments, Danderyd, Sweden) and a Rheodyne 7725 injector with a 20 μl loop (Cotati, CA, USA). The spray was stabilized using nitrogen sheath gas flow (value 80–85). The parameters used in the instrument were optimized using the auto-tune method. Tuning was done using a MRFA (=Met–Arg–Phe–Ala) standard. The column was a reversed-phase HPLC column (Syncropak RP-8, 50×2.1 mm, SynChrom I, Lafayette, IN, USA). The gradient was 2 to 70% acetonitrile (containing 50 m*M* HCOOH) in 40 min, flow 200 μl/min. Samples were injected using LaChrom Autosampler L-7200 from Merck–Hitachi (Hitachi, Tokyo, Japan), injection volume 20 μl. The full scan mass spectra from m/z 410 to m/z 2000 were measured.

2.2.3.2. HPLC–ESI-MS Method B

Positive ion mass spectra were acquired with online micro (μ) HPLC–ESI-MS. The HPLC–ESI-MS instrument described in Method A was used with the following modifications: The flow from the HPLC pump was split with a flow controller (Acurate, LC Packings, Netherlands). The column was a Vydac C_8, 150×0.3 mm (5 μm, 300 Å) (LC Packings). The gradient was 7 to 70% acetonitrile (containing 50 m*M* HCOOH) in 30 min, flow 4 μl/min. The original ESI orifice was replaced by a PicoTip adapter with fused-silica needles (New Objective, Cambridge, MA, USA).

2.2.4. EC dissociation MS–MS

In order to chelate metal ions EDTA was added to the hOCN sample. EDTA has to be removed before MS analysis and the following procedure was used: Micro column of Poros R2 was prepared in a gel-loader tip using Poros R2 suspension in 1:1 methanol and water. The hOCN solution containing 10 m*M* EDTA was loaded into the column, followed by a

washing with 5% formic acid. Peptides were eluted using 50% methanol in 5% formic acid and loaded into the MS sample capillary.

A 4.7 Tesla Ultima (IonSpec, Irvine, CA, USA) Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer was used to perform electron-capture dissociation. External accumulation in the hexapole of the ESI-source (Analytica of Branford, MA, USA, modified with a heated metal capillary), followed by gated trapping was used before the desired charge states were selected by applying a pre-programmed waveform. Isolated cations were irradiated by electrons from a heated tungsten filament for 12 s. Due to the electron thermal energy and the non-zero potential in the center of the cell, +1.25 V bias of the filament center was used to ensure <0.2 eV electron energy in the region occupied by the parent ions.

3. Results and discussion

3.1. Chelation of metal ions with EDTA

bOCN was analyzed without any additional EDTA (Fig. 2A) using the HPLC–ESI-MS Method A. The HPLC–ESI-MS base peak ion chromatogram shows several peaks at retention time 17–18 min. The molecular ions $(M+3H)^{3+}$ at m/z 1950.7, $(M+4H)^{4+}$ at m/z 1463.7, $(M+5H)^{5+}$ at m/z 1171.2 and $(M+6H)^{6+}$ at m/z 976.0 give an average mass of 5850.2 (Fig. 2A, Table 1). The expected average molecular mass (M_r^{Ex}) for bOCN, including three Gla and one Hyp residues and a single disulfide bond, is 5850.3. The molecular ions $(M+3H)^{3+}$ at m/z 1936.4, $(M+4H)^{4+}$ at m/z 1452.6, $(M+5H)^{5+}$ at m/z 1162.3 and $(M+6H)^{6+}$ at m/z 968.8 give an average mass of 5806.5 (Fig. 2A, Table 1). M_r^{Ex} for bOCN, including two Gla and one Hyp residues and a single disulfide bond, is 5806.3. These molecular ions correspond to the expected molecular masses, but the HPLC–ESI-MS spectra show also additional molecular ions, which differ from the expected ions. These molecular ions give average molecular masses of, for example, 5873.8, 5903.4 and 5906.9 (Table 1). The difference between the expected and the observed is 23.5, 53.1 or 56.6 units. These differences can be explained by the affinity of metal ions, for example, sodium (atomic mass 22.99), magnesium (atomic mass 24.31), chromium (atomic mass 52.00) or iron (atomic mass 55.85) to the OCN molecule. After this observation OCN was analyzed with the addition of EDTA (Fig. 2B). The HPLC–ESI-MS base peak ion chromatogram shows one major peak. This peak corresponds both to molecular ions from bOCN including three Gla residues (tri-Gla bOCN) and to molecular ions from bOCN including two Gla residues (di-Gla bOCN). The two forms of bOCN co-elute and are therefore found in the same peak. Any interfering molecular ions, which include metal ions, are not observed because of the addition of EDTA.

Metal ions interfere with the analysis, but they have a remarkable effect on the stability of OCN. A sample containing bOCN 1 μg/ml in 0.1 m*M* EDTA was stored at 23 °C for 24 h. MS spectra of degradation fragments showed that only 12.5% of the original peptide was left. Major degradation products were fragments 15–49 (56.5%) and 1–14 (7%). Moreover, dimers formed via the re-organization of disulfide bonds were found in the spectra (24%). For that reason EDTA was added only to those samples, which were going to be analyzed immediately.

The majority of the components of the peptide mixture were successfully separated by HPLC. However, those OCN subforms, which contain either two or three Gla residues and thus differ one Gla from each other, did co-elute. These forms were separated in the mass spectra according to their masses. Buffer salts, for example EDTA, which was added to OCN samples, may interfere with the electrospray. For that reason it is a convenient way to use on-line LC in order to remove salts.

3.2. Determination of Gla residues

Further analyzing of bOCN was carried out using HPLC–ESI-MS Method B. EDTA was added to all samples. According to our previous observations (Fig. 2B) the degree of carboxylation varies: from mass spectra we obtain molecular masses for both three and two Gla residues including forms of bOCN. In the HPLC the forms co-elute, but they can be separated in the mass spectra according to their masses. CAD-MS–MS spectra were obtained from molecular ions $(M+5H)^{5+}$ at m/z 1171.0 (tri-Gla

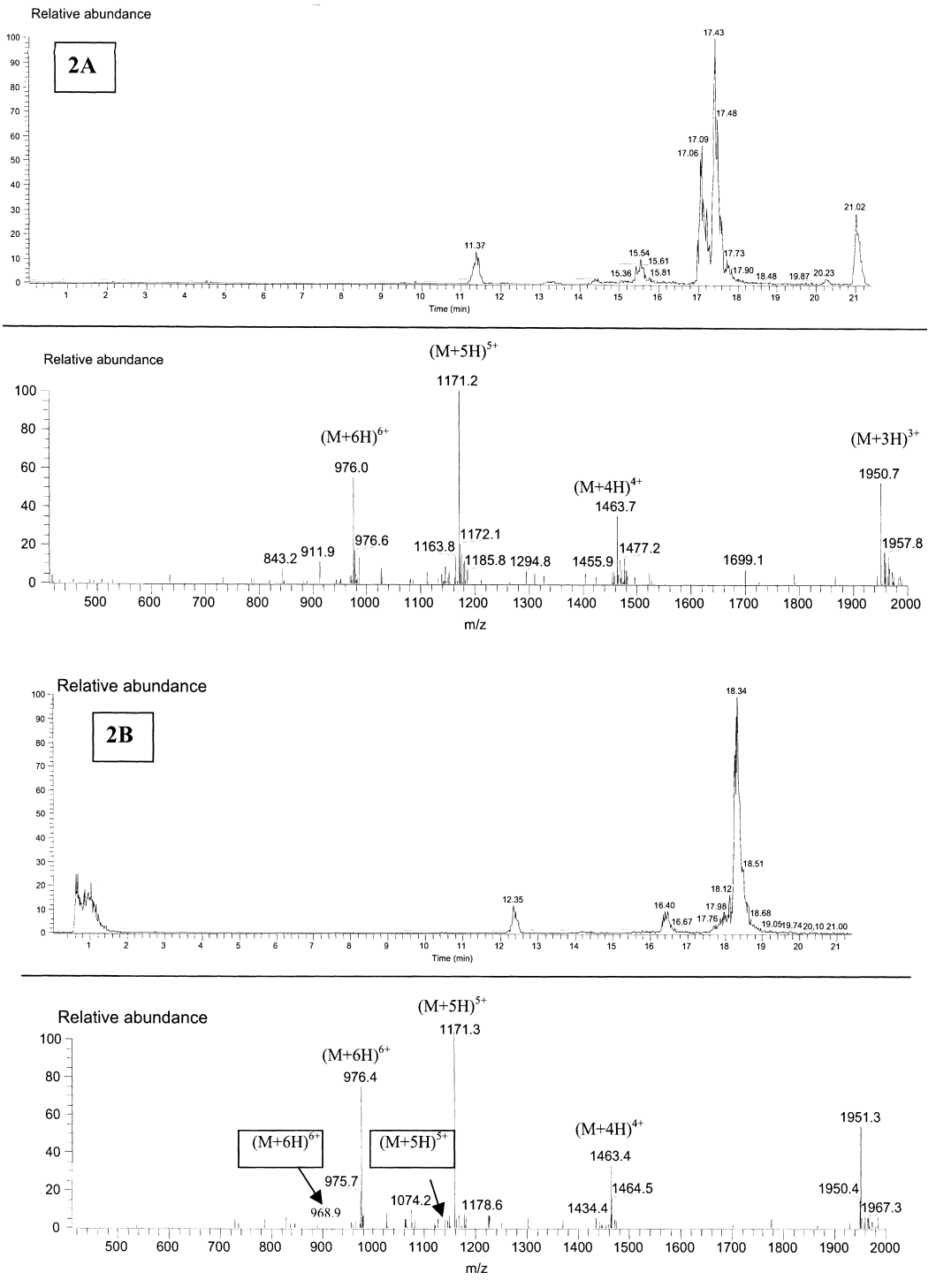

Fig. 2. HPLC–ESI-MS base peak ion chromatogram of bovine osteocalcin (20 μg/ml, 20 μl) without EDTA (A) and with 10 m*M* EDTA (B). Analysis conditions are described in Method A. $(M+5H)^{5+}$ and $(M+6H)^{6+}$ (text in box) are molecular ions from osteocalcin, which has two Gla residues, other molecular ions are from osteocalcin, which has three Gla-residues.

Table 1
Obtained and expected molecular masses of the bovine osteocalcin 1–49[a]

Molecular mass expected (M_r^{Ex})	Gla residues	Molecular mass observed (M_r^{Obs})	Ions observed	ΔM_r ($M_r^{Ex}-M_r^{Obs}$)	Comment
5850.3	3	5850.2	$(M+3H)^{3+}$ 1950.7 $(M+4H)^{4+}$ 1463.7 $(M+5H)^{5+}$ 1171.2 $(M+6H)^{6+}$ 976.0	0.1	
5806.3	2	5806.5	$(M+3H)^{3+}$ 1936.4 $(M+4H)^{4+}$ 1452.6 $(M+5H)^{5+}$ 1162.3 $(M+6H)^{6+}$ 968.8	0.2	
5850.3	3	5873.8	$(M+3H)^{3+}$ 1958.8 $(M+4H)^{4+}$ 1469.4 $(M+5H)^{5+}$ 1175.8 $(M+6H)^{6+}$ 980.0	23.5	Metal ion
5850.3	3	5903.4	$(M+3H)^{3+}$ 1969.3 $(M+4H)^{4+}$ 1477.1 $(M+5H)^{5+}$ 1181.5 $(M+6H)^{6+}$ 984.6	53.1	Metal ion
5850.3	3	5906.9	$(M+3H)^{3+}$ 1970.2 $(M+4H)^{4+}$ 1478.4 $(M+5H)^{5+}$ 1182.4 $(M+6H)^{6+}$ 984.9	56.6	Metal ion

[a] Gla-residues=γ-carboxyglutamic acid residues. Used molecular masses are average.

bOCN) and $(M+5H)^{5+}$ at m/z 1162.2 (di-Gla bOCN) (Fig. 3). The two spectra obtained were almost identical: one shows molecular ion $(M+5H)^{5+}$ at m/z 1144.3 and the other shows $(M+5H)^{5+}$ at m/z 1144.6. In order to eliminate the disulfide bond the bOCN was reduced and Cys residues were carbamidomethylated (CAM). In the mass spectra of modified bOCN (mod-bOCN) there are molecular ions $(M+3H)^{3+}$ at m/z 1989.3, $(M+4H)^{4+}$ at m/z 1492.1, $(M+5H)^{5+}$ at m/z 1194.2 and $(M+6H)^{6+}$ at m/z 995.2 and the observed average molecular mass is 5965.1. The M_r^{Ex} of tri-Gla mod-bOCN is 5966.3. The obtained mass for di-Gla mod-bOCN was 5922.4 and the M_r^{Ex} 5921.3. CAD-MS–MS spectra from $(M+5H)^{5+}$ at m/z 1194.2 and $(M+5H)^{5+}$ at m/z 1186.0 were almost identical again (Fig. 4): one shows $(M+5H)^{5+}$ at m/z 1167.7 and the other shows $(M+5H)^{5+}$ at m/z 1167.8. The explanation for the peaks 1144.3 and 1144.6 (unmodified bOCN) and 1167.7 and 1167.8 (CAM-modified bOCN) in MS–MS spectra is: Gla contains a labile carboxy group, which is ejected during CAD. Molecular masses obtained from the molecular ions in MS–MS spectra are 5716.5 (1144.3^{5+}) and 5718.0 (1144.6^{5+}) and 5833.5 (1167.7^{5+}) and 5834.0 (1167.8^{5+}). When these masses are compared to di-Gla and tri-Gla bOCN (both unmodified and CAM-modified), we can see a difference of approximately 132.6 or 88.2 units. The number 132.6 correlates to loss of three carboxy groups and 88.2 correlates to loss of two carboxy groups. Multiple MS (MS^n) can be applied for further characterization of the peptide, but obtained daughter ions show only Glu residues since the γ-carboxy groups are ejected during the first CAD.

3.3. EC dissociation MS–MS

Fourier transform (FT) EC dissociation MS–MS mass spectra (Fig. 5) show molecular ions and fragment ions of Glu-17, Gla-21, Gla-24–hOCN 1–49. Totally, 40 scans were integrated. The spectrum

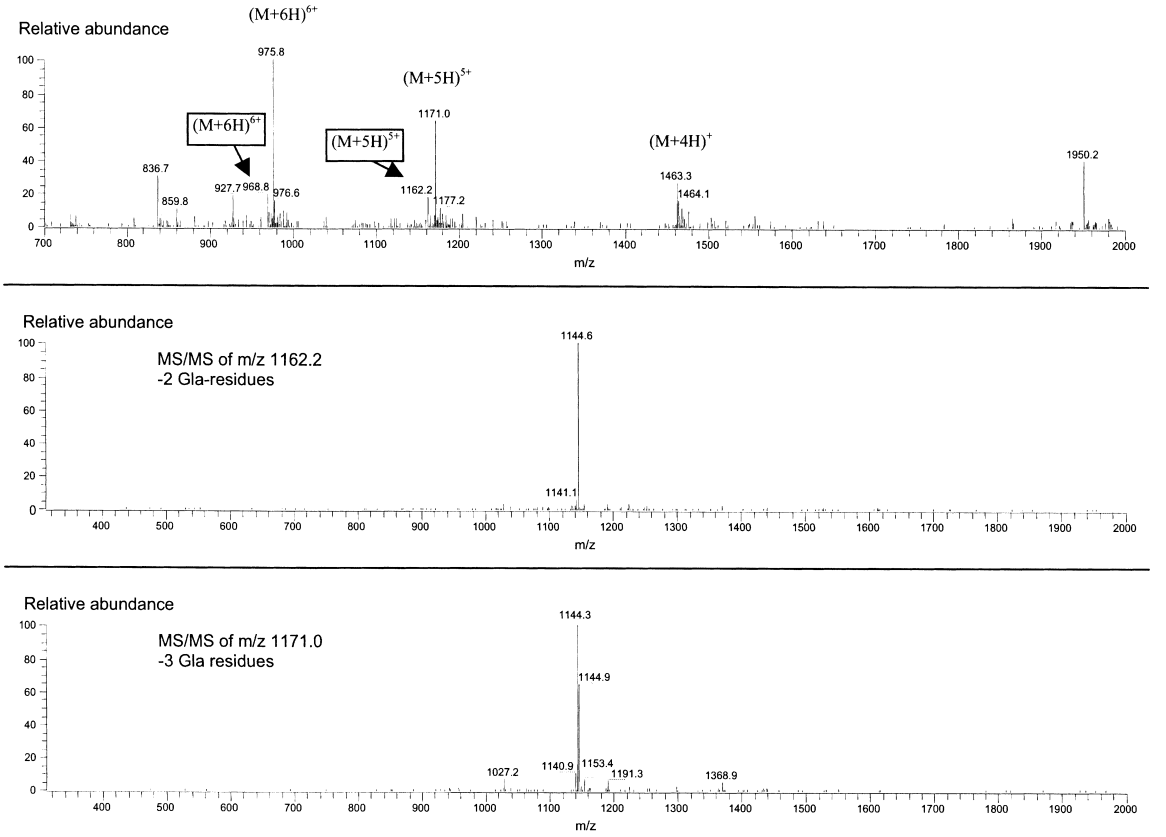

Fig. 3. HPLC–ESI-MS base peak ion chromatogram of bovine osteocalcin (8.7 μg/ml, 5 m*M* EDTA). Analysis conditions are described in HPLC–ESI-MS Method B. $(M+6H)^{6+}$ and $(M+5H)^{5+}$ (text in box) are molecular ions from osteocalcin with two Gla residues, other molecular ions are from osteocalcin with three Gla residues.

in Fig. 5 contains preferentially c and z ions characteristic for EC dissociation; some amount of b, y fragmentation is also present, originating from collisional excitation during the charge-state isolation. No backbone fragmentation is observed from the region protected by the internal disulfide bond (residues 23–29). Outside the ring, all inter-residue bonds were cleaved except those on the N-side of the Pro residue that are EC dissociation-immune [18]. No losses are observed from the even-electron c-ions; minor CO_2 losses were detected from the radical z-fragments, consistent with their lower stability [20]. Since the ring contained only one glutamic acid residue, and the total number of γ-carboxylated residues was known from the molecular mass shift, the observed cleavages allowed for unequivocal assignment of the modification to Glu-21 and Glu-24. The signal for the uncarboxylated form is less than 5% and for that reason Glu-17 and Glu-31 were not to be found modified. Therefore, a single EC dissociation spectrum was sufficient for complete characterization of the γ-carboxylation pattern of hOCN. At this moment it is not possible to connect on-line HPLC to EC dissociation MS–MS equipment and thus the results are not in chromatographic scale. Further study is needed to develop an on-line LC–EC dissociation MS–MS method.

4. Conclusion

The interfering effect of metal ions to MS spectra was eliminated using an addition of EDTA to the osteocalcin sample. Osteocalcin subforms, which contain different amounts of Gla residues among its amino acids, were separated using the applied on-

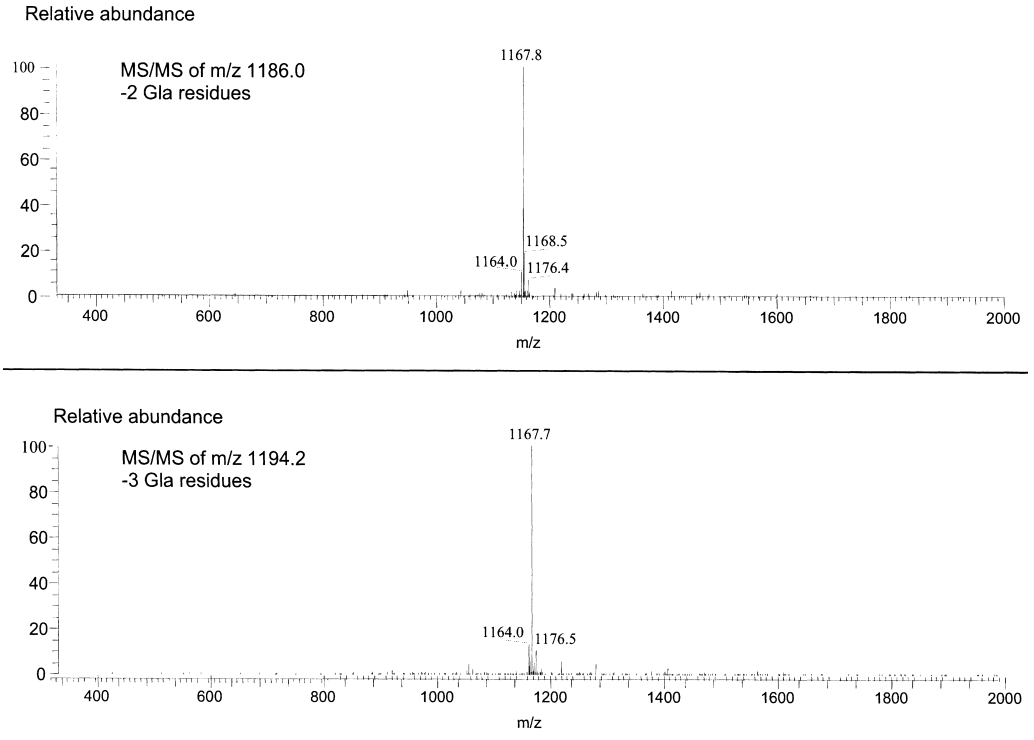

Fig. 4. MS–MS spectra of ion $(M+5H)^{5+}$ at m/z 1186.0 and ion $(M+5H)^{5+}$ at m/z 1194.2.

line HPLC–ESI-MS method. Localization of Gla residues of the peptide was done using the applied EC dissociation MS–MS method. According to this study these MS methods may help to determine the exact function of OCN in bone. When developing more stable peptide drug formulations these MS methods are suitable for qualitative analysis.

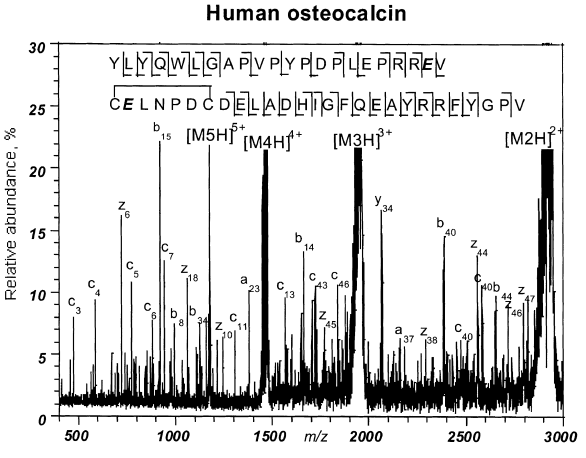

Fig. 5. Electron-capture dissociation MS–MS spectrum (40 scans) of Glu-17, Gla-21, Gla-24–human osteocalcin 1–49.

Acknowledgements

Financial support from the Technology Development Centre of Finland (TEKES, Finland) is gratefully acknowledged. The Odense group is grateful to P.B. Hansen and T. Jensen for technical assistance; the work was supported by the grants STVF-0001242 and SNF-51-00-0358.

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