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High-performance liquid chromatography–mass spectrometry of an osteocalcin derivative

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Abstract

High-performance liquid chromatography (HPLC) was combined on-line with electrospray ionization mass spectrometry (ESI-MS) for structural analysis of a synthetic osteocalcin derivative and its degradation products. Initial determination of amino acid sequence of the synthetic peptide was performed after tryptic degradation. Hydrolytic degradation of the osteocalcin derivative was studied under different pH conditions: pH 2, pH 7 and pH 10 at 60°C up to 20 h. According the HPLC-ESI-MS results, the chemical stability was dependent on pH. Two major degradation products and a number of other fragments were obtained in acidic solution, whereas the osteocalcin molecule was rather stable in neutral and alkaline conditions. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Electrospray ionization; Osteocalcin; Peptides

1. Introduction

Osteocalcin (OCN), also called bone Gla protein is a vitamin K-dependent bone matrix peptide of 49 amino acid residues [1]. It is produced mainly by bone osteoblasts. The amount of osteocalcin in the circulation reflects the status of bone metabolism [2]. The structure of human osteocalcin is given in Fig. 1. [3]. Osteocalcin peptide contains two to three residues of γ-carboxyglutamic acid (Gla) among its amino acids and Kurihara et al. were the first, who synthesized Gla^{17}-human osteocalcin by using a γ-carboxyglutamic acid derivative [3].

Protein drugs are being used for treating various diseases, for example diabetes (insulin), cystic fibrosis (Dnase), dwarfism (human growth hormone) and anemia (erythropoietins). While proteins have the advantage of being very potent and highly specific, they have the disadvantage of being unstable during production, purification and formulation. To produce a commercial formulation of a protein drug, it is indispensable to be aware of the chemical and physical instability of the protein [4].

The stability of proteins is affected by a number of degradation pathways of which the most prominent occur via oxidation of methionine (Met), deamidation of asparagine (Asn) and cleavages of peptide bond at aspartic acid (Asp) [5]. Osteocalcin contains one Asn and four Asp residues, and there is one -Asp-Pro- bond, which is susceptible to acid-catalyzed cleavage [6].

Mass spectrometry detection is one of the most powerful tools available in chemical analysis due to its ability to provide the molecular mass of analytes in most cases [7]. Electrospray ionization (ESI) is a mild ionization mode, very appropriate for peptide and protein analysis [8]. Because of the ability of ESI-MS to directly analyze compounds from aqueous or aqueous/organic solutions and of the depth of

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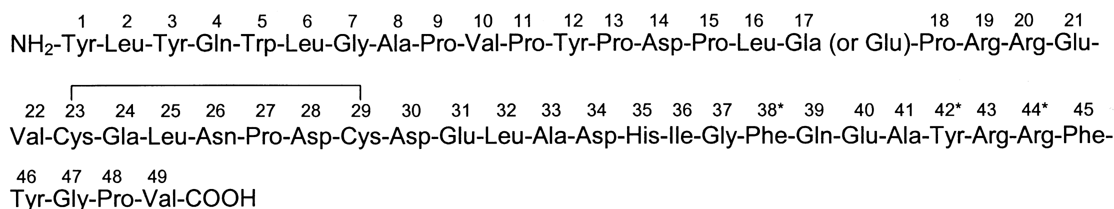
Human osteocalcin:



Synthetic human osteocalcin-derivative:

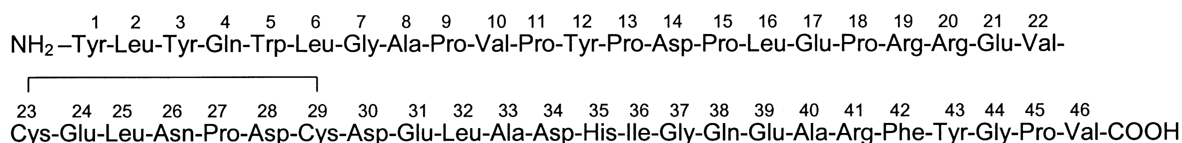


Fig. 1. The primary structure of human osteocalcin 1–49 [3]. The peptide consists of 49 amino acids containing 2 or 3 residues of γ-carboxyglutamic acid (Gla) and a single disulfide bond. Structure of the synthetic human osteocalcin derivative was determined with HPLC-ESI-MS. In the osteocalcin derivative amino acids Phe-38, Tyr-42 and Arg-44 are missing. Instead of Gla residues there are glutamic acid residues.

resulting structural information, it is the most desirable and powerful type of liquid chromatography detection available [8–10].

By correlating the information from collision induced dissociation (CID) mass spectra of a peptide, its amino acid sequence can be identified. Fragmentation of a peptide sequence in CID is well known and fragments produced from a peptide by cleavage can be labeled using the system of Biemann [11]. The most common fragment ions produced in low energy CID are carboxy terminal y-series and amino terminal b-series [11].

In this study the aim was to develop a method for analyzing degradation products of peptides using a synthetic osteocalcin derivative (OCN-D) as a model compound. The amino acid sequence of OCN-D was determined, in order to be clear on the accurate amino acid sequence of the peptide, using HPLC-ESI-MS and an amino acid sequence analyzer. In order to get degradation products for identification with HPLC-ESI-MS the osteocalcin derivative was incubated in hardened conditions. The method provided useful information on sensitive sites of OCN, which is useful in developing more stable peptide drug formulations.

2. Experimental

2.1. Chemicals

About 90% pure synthetic human osteocalcin derivative was obtained from Erilab (Kuopio, Finland). It had been synthesized using the solid-phase method and its structure was uncertain. According the HPLC-ESI-MS-MS spectra quoted in the Results below, the product differs from native OCN: Phe-38, Tyr-42 and Arg-44 are missing (Fig. 1) and the amino acid sequence contains glutamic acid residues instead of γ-carboxyglutamic acid residues.

Dithiothreitol was obtained from Boehringer Mannheim (Germany), urea was from Riedel-de Haen (Seelze, Germany), ammoniumbicarbonate and trifluoroacetic acid were from Fluka (Buchs, Switzerland), trypsin (TPCK treated) was from Sigma (St. Louis, MO, USA), acetonitrile was from Rathburn (Walkerburn, UK) and formic acid was from Merck (Darmstadt, Germany). Universal buffers: pH 2 citrate buffer, pH 7 phosphate buffer and pH 10 borate buffer were obtained from FF-Chemicals (Yli-Ii, Finland). Water was deionized using a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Methods

2.2.1. Amino acid sequence of the synthetic osteocalcin derivative

Trypsin digestion: In order to analyze the molecular mass of the OCN-D molecule, it was dissolved (100 μg/ml) in 10% acetonitrile (0. 1% trifluoroacetic acid). The digestion was carried out in two steps; first a reduction using urea, ammoniumbicarbonate and dithiothreitol, and then the trypsin incubation overnight. The sample was prepared as follows: 100 μl of the dissolved OCN-D was taken and 50 μl of a solution containing 6–8 M urea and 0.4 M ammoniumbicarbonate, pH 8 and 5 μl of 45 mM dithiothreitol was added and stirred carefully. The solution was incubated at 50–55°C for 15 min, and Milli-Q water (140 μl) was added and stirred. Then 0.5 μg of trypsin in water was added. The enzyme-protein ratio was 1:20, and digestion was carried out overnight at 37°C.

Sequence analysis: Fractions for sequence analysis were collected from flow of HPLC column (see below), splitted between ion source and manual fraction collection. The collected fractions were pooled and dried. A peptide was dissolved in 0.1% trifluoroacetic acid (about 5 μg/60 μl) and 30 μl was taken for the sequence analysis. An Applied Biosystems 477A Pulsed Liquid Phase Sequencer 120A Analyzer (Foster City, CA, USA) was used.

HPLC-electrospray ionization mass spectrometry: Positive ion mass spectra were acquired with on-line 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) and the spray needle potential was set to 3.5 kV. The stainless steel inlet capillary was heated to 225°C. The tube lens offset was set to 5 V. The parameters used in the instrument were optimized using autotune-method. Tuning was done using MRFA (=Met-Arg-Phe-Ala) standard (4 μg/ml).

A peptide was dissolved in water-acetonitrile (90:10)-trifluoroacetic acid (0.1%) solution (100 μg/ml) and 20 μl was injected to reversed-phase HPLC (Syncropak RP-8, 50×2.1 mm, SynChrom I, Lafayette, IN, USA). The HPLC gradient was 15% to 70% acetonitrile (containing 0.1% TFA) in 30 min. The full scan mass spectra from m/z 410 to m/z 2000 were measured using 500 ms for collection of the ions in the trap; 2 scans were summed. CID-MS-MS spectra of the peptide were obtained using the dependent scan mode of the instrument. Collision energy was 35%. The tryptic digests of osteocalcin derivative (20 μl, concentration 33 μg/ml) were separated using same Syncropak column. The full scan mass spectra from m/z 410 to m/z 2000 were measured using 500 ms for collection of the ions in the trap; 2 scans were summed. CID spectra of the peptides were obtained using the dependent scan mode of the instrument. Fraction collection was carried out using reversed phase HPLC column (Purospher C18e, 125x3 mm, Merck).

2.2.2. Degradation product analysis

Degradation assay: Solid osteocalcin derivative (430 μg) was dissolved in 1 ml of three different buffers: pH 2 citrate buffer (citric acid·1H_2O 6.5 g/l, sodium hydroxide, hydrogen chloride, sodium azide as a preservative 20 mg/ml), pH 7 phosphate buffer (potassium dihydrogenphosphate 3.5 g/l, disodium hydrogenphosphate·2H_2O 7.2 g/l, sodium azide as a preservative 20 mg/l) and pH 10 borate buffer (potassium chloride 4.0 g/l, boric acid 3.3 g/l, sodium hydroxide). Incubation was carried out at 60°C and six samples were collected (0, 4, 8, 12, 16 and 20 h). Samples were stored in a freezer (−20°C).

For the analysis the degradation samples were thawed and diluted to 215 μg/ml, and 20 μl was injected to reversed phase HPLC (Syncropak RP-8, 50×2.1 mm, SynChrom). The degradation products were analyzed with the HPLC-ESI-MS instrument described with following modifications: Samples were injected using LaChrom Autosampler L-7200 Merck-Hitachi (Hitachi, Tokyo, Japan) and spray needle potential was set to 4 kV. The degradation products were separated using a gradient in water from 2% to 52% acetonitrile in 30 min. The full scan mass spectra from m/z 410 to m/z 2000 were measured using 200 ms for collection of the ions in the trap; 2–4 scans were summed. CID-MS-MS spectra of degradation products were obtained using

the dependent scan mode of the instrument. Collision energy was 40%.

3. Results and discussion

3.1. Amino acid sequence of the synthetic peptide

Peptide sequencing by tandem mass spectrometry is used in peptide characterization. Mass spectrometry obtains structural information on peptides by CID, in which the precursor ions are subjected to collisions and the structures of the resulting ions are characterized. The use of CID is well established [12–18].

In order to determine the molecular mass of the whole OCN-D molecule, it was dissolved in the water-acetonitrile (90:10)-trifluoroacetic acid (0.1%) solution was analyzed with on-line HPLC-ESI-MS. There was one major peak in HPLC-ESI-MS base peak ion chromatogram at 13.12 min (Fig. 2A). The ESI-MS spectrum (Fig. 2B) shows $(M+3H)^{3+}$ at m/z 1777.9 and $(M+4H)^{4+}$ at m/z 1333.6. Using deconvolution the average molecular weight was determined to be 5330.6. Expected average mass for the human OCN peptide (including three Glu residues) is 5799.5. The molecular mass observed now was 468.9 units different from this, indicating a lack of amino acid residues vs the human OCN. To get further information on the structure the tryptic digestion was carried out. The human OCN is known to form three fragments, when treated with trypsin [19]: 1–19, 20–43 and 45–49. In our study the tryptic digestion fragments were separated and detected using HPLC-ESI-MS method (Fig. 3A). Peak I corresponds to ion $(M+1H)^{1+}$ at m/z 582.3 (expected 582.3) and it was identified as sequence FYGPV (Fig. 3B, Table 1). The peak III shows ion $(M+2H)^{2+}$ at m/z 1138.3 (Fig. 3D), which was identified as fragment 1–19: YLYQWLGAPVPYPDPLEPR (Table 1). Comparing our result and an earlier published result about the tryptic digestion of osteocalcin [19], we concluded that the peak II is the middle sequence of the peptide (sequence 20–43). The HPLC-ESI-mass spectrum gave ions $(M+2H)^{2+}$ at m/z 1256.1 and $(M+3H)^{3+}$ at m/z 837.9 (Fig. 3C). The expected average molecular mass for

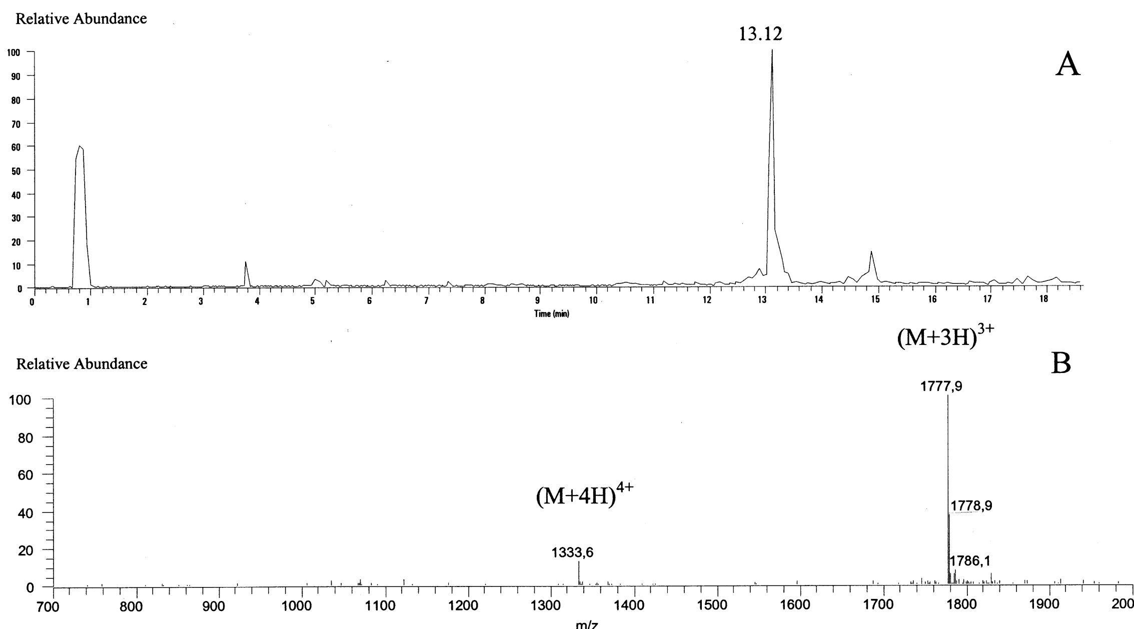


Fig. 2. HPLC-ESI-MS base peak ion chromatogram of the synthetic osteocalcin derivative (A). Peak at 13.12 shows ions $(M+3H)^{3+}$ at m/z 1777.9 and $(M+4H)^{4+}$ at m/z 1333.6 (B). Observed average molecular mass was 5330.6.

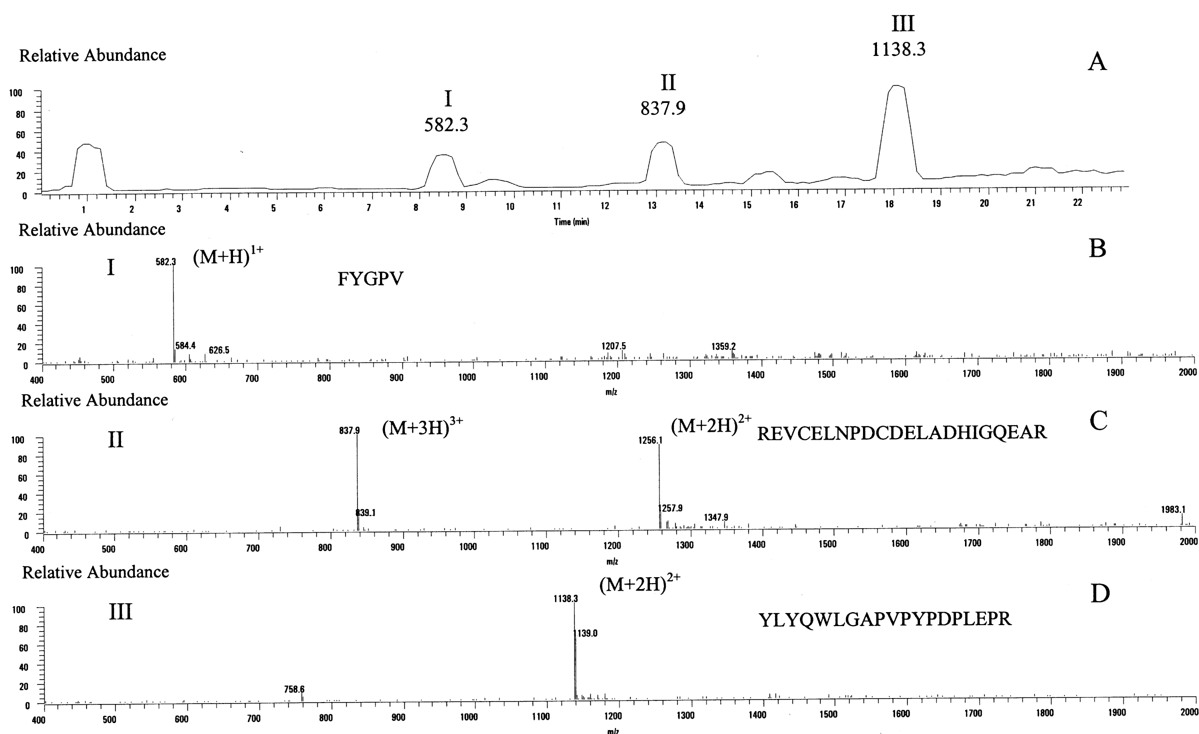


Fig. 3. HPLC-ESI-MS base peak ion chromatogram of trypsin digested osteocalcin derivative: three fragments were separated (A). Peak I shows ion $(M+H)^{1+}$ at m/z 582.3, which corresponds to sequence FYGPV (B). Peak II shows ions $(M+2H)^{2+}$ and $(M+3H)^{3+}$ at m/z 1256.1 and at m/z 837.9, respectively. Obtained sequence was REVCELNPDCDELAD HIGQEAR. Peak III shows ion $(M+2H)^{2+}$ at m/z 1138.3. Sequence is 1–19: YLYQWLGAPVPYPDPLEPR.

fragment 20–43 is 2823.1 and observed was 2510.7. The difference between the expected and observed was 312.4 units. This difference can be explained by lack of amino acids Phe-38 and Tyr-42 (expected mass 310.4) (Table 1). The peptide II was collected using HPLC-ESI-MS and analyzed with amino acid sequence analyzer. According to the results, the two above mentioned amino acid residues were missing. The amino acid sequence obtained is in Fig. 4. Molecular masses of peptides I, II (Phe and Tyr excluded) and III were summed in order to get molecular mass for the whole peptide and obtained mass was 5330.6, which was exactly the same as recorded for the osteocalcin derivative. Accordingly it was concluded that Arg-44 was also missing. A HPLC-ESI-MS-MS spectrum from parent ion (M+

Table 1
Obtained sequences from trypsin digestion of the osteocalcin derivative

Peak	Sequence expected	Ions observed	Molecular mass observed (M_r^{obs})	Molecular mass expected (M_r^{ex})	ΔMW= ($M_r^{ex}-M_r^{obs}$)	Comment	Corrected sequence
I	45–49	(M+H)1+ 582.3	581.3	581.7	0.4		42–46=FYGPV
II	20–43	(M+2H)2+ 1256.1 (M+3H)3+ 837.9	2510.7	2823.1	312.4	Two amino acids Phe-38 and Tyr-42 are missing	20–41= REVCELNPDCDELADHIGQEAR
III	1–19	(M+2H)2+ 1138.3	2274.6	2274.6	–		1–19= YLYQWLGAPVPYPDPLEPR

[a] The expected sequences are according to Rosenqvist et al. [19]. Used molecular masses are average.

Fig. 4. MS-MS spectrum of ion $(M+3H)^{3+}$ at m/z 837.9. Fragment ions (a-, b- and y-series) are labeled and located in the sequence 20–41 REVCELNPDCDELADHIG QEAR

$3H)^{3+}$ at m/z 1777.9 (Fig. 2B) is given in Fig. 5: b- and y-series fragment ions are located in the obtained sequence of 46 amino acid residues. The amino acid sequence of the synthetic human osteocalcin derivative 1–46 is in Fig. 1. The expected average mass of this peptide containing one disulfide bond is 5330.9, the observed average mass 5330.6, i.e. only three decimals difference between observed and expected molecular masses.

3.2. Degradation product analysis

In order to develop a method for detection and identification of degradation products, a stability

Fig. 5. HPLC-ESI-MS-MS spectrum of ion $(M+3H)^{3+}$ at m/z 1777.9. The corrected sequence of osteocalcin derivative 1–46 is YLYQWLGAPVPYPDPLEPRREVCELNPDCDELADHIGQEARFYGPV. Obtained b- and y-series fragment ions are located in the sequence. Product ions are labeled according to Biemann [11].

pH 10 ⇓ pH 10 ⇓ pH 10 ⇓ pH 10 ⇓

YLYQWLGAPVPYPD PLEPRREV CELNPDC D ELAD HIG QEARFYGPV

⇑ pH 2, pH 7 ⇑ pH 2 ⇑ pH 2 ⇑ pH 2, pH 7

Fig. 6. Obtained cleavage sites from the degradation assay in the osteocalcin derivative peptide. The peptide was incubated in pH 2, pH 7 and pH 10.

experiment was carried out in three different pH conditions at 60°C. According to earlier studies it was most obvious that in an acidic condition cleavage between Asp-14 and Pro-15 would occur [6]. There are four Asp residues altogether, as other possible cleavage sites in the molecule.

Effects of the incubations were followed for 20 h. Obtained cleavage sites are in Fig. 6. Major degra-

Fig. 7. HPLC-ESI-MS base peak ion chromatogram of incubated (pH 2, 12 h, 60°C) osteocalcin derivative (A). Peak A shows major degradation products 1–14 and 15–46, which coelute and found in same peak. Peak B is the original peptide. Molecular ion from sequence 1–14 is $(M+H)^{1+}$ at m/z 1682.6 (text in box) and ions from sequence 15–46 are $(M+2H)^{2+}$ at m/z 1834.0, $(M+3H)^{3+}$ at m/z 1223.0, $(M+4H)^{4+}$ at m/z 917.6 and $(M+5H)^{5+}$ at m/z 734.4 (bold font) (B). CID-MS-MS spectra are showed in C and D spectra. Below spectra is sequence 1–46 cut into two pieces: sequence 1–14 and sequence 15–46. Obtained fragment ions are labeled and located in the fragments.

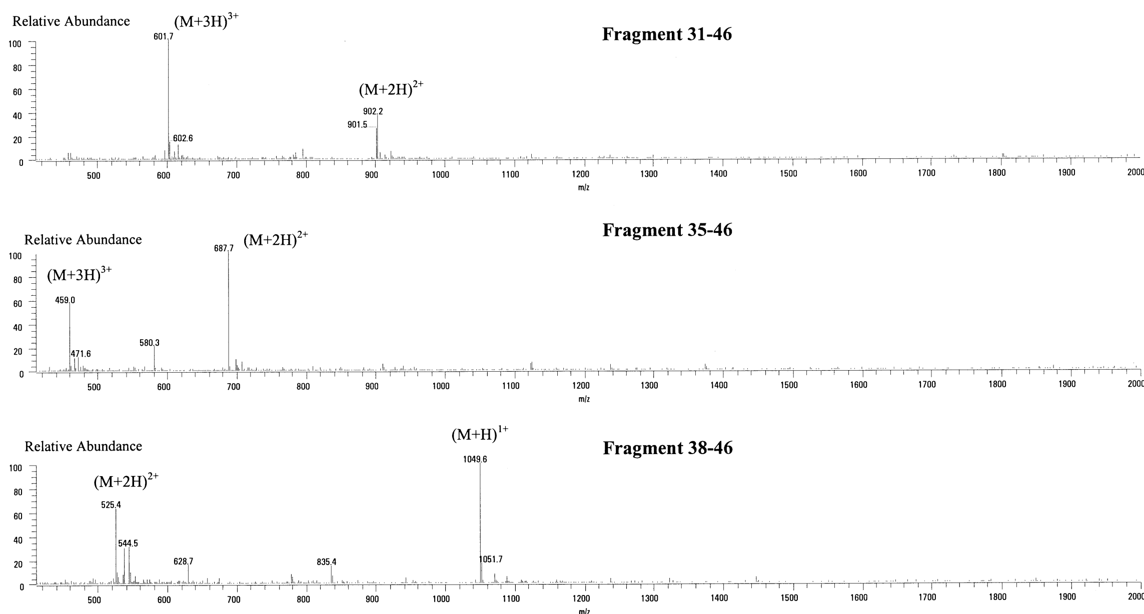


Fig. 8. MS spectra of degradation fragments 31–46, 35–46 and 38–46. Molecular ions from fragment 31–46 are $(M+2H)^{2+}$ at m/z 902.2 and $(M+3H)^{3+}$ at m/z 601.7. Ions from fragment 35–46 are $(M+2H)^{2+}$ at m/z 687.7 and $(M+3H)^{3+}$ at m/z 459.0 and ions from fragment 38–46 are $(M+H)^{1+}$ at m/z 1049.6 and $(M+2H)^{2+}$ at m/z 525.4.

dation products in acidic conditions (pH 2) were fragments 1–14 and 15–46 (Fig. 7), formed via cleavage between Asp-14 and Pro-15. Fragments 1–14 and 15–46 coelute and they are found in the same peak A (Fig. 7A). The relative content (% area vs. peak B) of this product increased at the following rate: at 0 h a small amount, at 4 h about 45%, at 8 h about 88%, at 12 h about 85%, and at 16 and 20 h about 125%. Fragment 1–14 shows ions $(M+H)^{1+}$ at m/z 1682.6 and $(M+2H)^{2+}$ at m/z 841.5 [observed average molecular mass (M_r) was 1681.6 and expected M_r 1681.9). The structure of fragment 1–14 was verified by CID-MS-MS of the singly protonated molecule (Fig. 7D). Fragment 15–46 shows ions $(M+3H)^{3+}$ at m/z 1223.7, $(M+4H)^{4+}$ at m/z 917.6 and $(M+5H)^{5+}$ at m/z 734.2 (observed aver. mw was 3668.1 and expected aver. mw was 3669.1). The structure of fragment 15–46 was identified by CID-MS-MS of the $(M+4H)^{4+}$ protonated molecule (Fig. 7C). Small amounts of degradation product 38–46 were found in all samples. It is formed via cleavage between Gly-37 and Gln-38 and N-terminal Gln forms pyroglutamic acid. Observed ions were $(M+H)^{1+}$ at m/z 1049.6 and $(M+2H)^{2+}$ at m/z 525.4 (observed M_r 1048.5 and expected M_r 1049.1) (Fig. 8). Fragment 31–46 was found (small amounts) in 4, 8, 12, 16 and 20 h samples. Fragment is formed via cleavage between Asp-30 and Glu-31 and it shows ions $(M+2H)^{2+}$ at m/z 902.2 and $(M+3H)^{3+}$ at m/z 601.7 (observed M_r 1801.8 and expected 1802.0) (Fig. 8). Fragment 35–46 was found (small amounts) in 12 and 20 h samples. Fragment is formed via cleavage between Asp-34 and His-35 and it shows ions $(M+2H)^{2+}$ at m/z 687.7 and $(M+3H)^{3+}$ at m/z 459.0 (observed M_r 1373.4 and expected 1373.5) (Fig. 8). Appearance of fragment 35–46 at long times can be explained by further degradation of fragment 31–46. Fragment 15–34 (in 20 h sample) is formed via cleavage between Asp-14 and Pro-15 (N-terminal side) and between Asp-34 and His-35 (C-terminal side). Ions $(M+2H)^{2+}$ at m/z 1156.5 and $(M+3H)^{3+}$ at m/z 771.5 were seen and observed M_r was 2311.5 (including one disulfide bond) and expected M_r was 2311.5.

In neutral (pH 7) and alkaline (pH 10) conditions osteocalcin derivative peptide was more stable.

Small amounts of fragments 1–14, 15–46 and 38–46 were found in all samples in pH 7 and no new fragments were found. In pH 10 small amounts of the same fragments and two new ones were found: 1–22 and 30–46. Fragment 1–22 is formed via cleavage between Val-22 and Cys-23 (observed M_r 2658.9 and expected M_r 2659.0) and fragment 30–46 is formed via cleavage between Cys-29 and Asp-30 (observed M_r 1917.4 and expected M_r 1917.1).

The mobile phase used in this assay was water-rich. The low concentration of organic phase in the gradient was used to focus the sample in the inlet-end of the HPLC column in order to avoid the peak broadening in the chromatogram. The use of water-rich mobile phase is a problem. Due to the low proportion of organic solvent there is a possibility of loosing water-soluble degradation products, which elute at the first ten minutes in the chromatogram. The low concentration of organic solvent in the mobile phase may also produce a spray of bad quality, which decreases the analytical signal. The use of a higher proportion of organic phase, especially in the beginning, could have been better. Further study is needed to develop a quantitative method for analysis of the degradation products.

4. Conclusion

Structure of the synthetic osteocalcin derivative was determined and degradation products were successfully separated and identified using the developed on-line HPLC-ESI-MS method. According to this study the method is suitable for qualitative analysis of degradation products obtained from peptide drugs.

Acknowledgements

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