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In vitro metabolism of LVV-hemorphin-7 in human plasma studied by reversed-phase high-performance liquid chromatography and micro-electrospray mass spectrometry

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Abstract

In these experiments we have studied the in vitro metabolism of LVV-hemorphin-7 in human plasma by using reversed-phase high-performance liquid chromatography (RP-HPLC) in combination with micro-electrospray mass spectrometry (micro-ES-MS). Tandem mass spectrometry (MS–MS) was performed in order to verify the structure of the peptide fragments found. Incubations were performed with and without different protease inhibitors. Results showed that LVV-hemorphin-7 was metabolized from the N-terminal end of the peptide, probably by an amastatin-sensitive exopeptidase.

Keywords: Hemorphins; Peptides

1. Introduction

Hemorphins are endogenous peptides that belong to the family of "non-classical" opioid peptides generated by hydrolysis of proteins originally derived from non-neuronal tissue (Table 1). The first report on these novel opioid peptides was in 1986, when Brantl et al. [1] were able to isolate the tetrapeptide Tyr–Pro–Trp–Thr after treatment of bovine blood with a mixture of gastrointestinal enzymes. This tetrapeptide was named hemorphin-4 and its opioid activity was confirmed by use of the electrically stimulated myenteric plexus–longitudinal muscle preparation of the guinea-pig ileum (GPI) as well as by radioreceptor assay [1,2]. Furthermore, hemorphin-4 has been shown to induce release of endogenous opioids (β-endorphin and dynorphin) from pituitary tissue in vitro [3]. In recent studies, hemorphins have been isolated from human pituitary [4], plasma [5] and cerebrospinal fluid [6]. In addition to their opioid effects, the hemorphins have been shown to have an inhibitory action on angiotensin converting enzyme activity, suggesting a possible involvement in blood pressure regulation [7,8]. All hemorphins, regardless of their source, are found in the same region of the β-chain of hemoglobin. For example, the decapeptide LVV-hemorphin-7 is identical with residues 32–41 of the β-chain of human hemoglobin (Fig. 1). This part of the β-chain in the hemoglobin molecule has been suggested to be a "strategic zone" that may have a biological function [9]. To exert their biological effects in vivo, the hemorphins must be formed from the inactive state inside the hemoglobin structure. It has been suggested that proteolytic enzymes, present in the circulatory system, could be involved in a sub-

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Table 1
Amino acid sequences of human hemorphins

Peptide[a]	Fragment[b]	Sequence
		1 2 3 4 5 6 7 8 9 10
Hemorphin-4	4–7	Tyr–Pro–Trp–Thr
Hemorphin-6	4–9	Tyr–Pro–Trp–Thr–Gln–Arg
Hemorphin-7	4–10	Tyr–Pro–Trp–Thr–Gln–Arg–Phe
LVV-hemorphin-4	1–7	Leu–Val–Val–Tyr–Pro–Trp–Thr
LVV-hemorphin-6	1–9	Leu–Val–Val–Tyr–Pro–Trp–Thr–Gln–Arg
LVV-hemorphin-7	1–10	Leu–Val–Val–Tyr–Pro–Trp–Thr–Gln–Arg–Phe

[a] Nomenclature according to references [1] and [4].
[b] Actual fragment number in sequence of LVV-homorphin-7, fragment 1–10.

sequent degradation of the hemorphins to smaller fragments [5]. Thus, Piot et al. [9] suggested that LVV-hemorphin-7 or VV-hemorphin-7, both initially produced by pepsin-like enzymes, could be metabolized into more or less active forms, such as hemorphins-4, -5 and -6 [9].

Peptides and their fragments are commonly analyzed by immunological methods, such as radioimmunoassays (RIA) in combination with high-performance liquid chromatography (HPLC). The RIA methods provide high sensitivity (attomole to femtomole range) but lack complete specificity due to cross-reactivity of the antibody with substances other than the specific peptide of interest. The antibody used in a RIA is often directed to the C- or N-terminus of a peptide and may therefore react with an entire family of peptides. In contrast to the RIA, mass spectrometry provides a high level of molecular specificity. This technique combined with HPLC

1- Val-His-Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala-Val-Thr-Ala-Leu-Trp-
16- Gly-Lys-Val-Asn-Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-Arg-
31- Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-Phe-Glu-Ser-Phe-
46- Gly-Asp-Leu-Ser-Thr-Pro-Asp-Ala-Val-met-Gly-Asn-Pro-Lys-Val-
62- Lys-Ala-His-Gly-Lys-Lys-Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-
76- Ala-His-Leu-Asp-Asn-Leu-Lys-Gly-Thr-Phe-Ala-Thr-Leu-Ser-Glu-
91- Leu-His-Cys-Asp-Lys-Leu-His-Val-Asp-Pro-Glu-Asn-Phe-Arg-Leu-
106- Leu-Gly-Asn-Val-Leu-Val-Cys-Val-Leu-Ala-His-His-Phe-Gly-Lys-
121- Glu-Phe-Thr-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-
136- Gly-Val Ala-Asn-Ala-Leu-Ala-His-Lys-Tyr-His

Fig. 1. The entire amino acid sequence of the β-chain of human hemoglobin. The LVV-hemorphin-7 sequence is underlined.

[10] or capillary electrophoresis [11] appeared to be powerful for the analysis of selected peptide structures.

Recently, a micro-electrospray (micro-ES) mass spectrometry (MS) analysis system was developed [12] that utilizes flow-injection techniques at a low (nl/min) flow-rate to achieve ultra-high sensitivity. This system is specially suited for analysis of sample volumes of 0.1–10 μl [13,14]. The sensitivity of the micro-electrospray has been shown to be in the attomole per microliter range for peptides and in the femtomole per microliter range for proteins [12,15]. The sensitivity and specificity has been further enhanced by coupling the micro-ES source with a tandem quadrupole mass spectrometer. In the tandem MS analysis (MS–MS), the first mass analyzer (quadrupole) is set to transmit only a specific analyte ion into the system. This provides the first level of specificity of the analysis. The selected ion is subsequently subjected to energetic collision with an inert gas in the second quadrupole region of the instrument. These collisions yield characteristic, structurally informative, fragment ions, which are subsequently selected for transmission by the third quadrupole region to the MS detector. This second selection again adds specificity to the analysis and furthermore greatly reduces the contribution of chemical background noise to the signal.

In the present study we investigated the in vitro stability of the synthesized peptide LVV-hemorphin-7 in human plasma. Incubations were performed with and without different protease inhibitors. The peptide fragments formed were separated by HPLC and analysed by mass spectrometry.

2. Experimental

2.1. Peptides and chemicals

Synthetic LVV-hemorphin-7 (LVVYPWTQRF) was synthesized by Dr. G. Lindeberg (Department of Medical Immunology, Uppsala University, Uppsala, Sweden). Amastatin, phosphoramidon and captopril were purchased from Sigma (St. Louis, MO, USA). Acetonitrile was of HPLC grade. All aqueous HPLC eluents were filtered on Millipore (Bedford, MA, USA) 0.22 μm filters and were degassed with helium prior to use. All other common chemicals and solvents were of analytical grade from various commercial sources.

2.2. Equipment

A Pharmacia LKB Biotechnology system (Uppsala, Sweden) was used for the RP-HPLC separation. Mass spectrometry was performed on a Finnigan TSQ70 quadrupole mass spectrometer, upgraded with TSQ700 software and a 20-kV dynode (Finnigan MAT, San Jose, CA, USA).

2.3. Sample preparation

Synthetic LVV-hemorphin-7 was incubated with human plasma at a concentration of 255 μM (in Eppendorf tubes at 37°C, total volume 150 μl). Separate incubations were performed in the presence of the different protease inhibitors, amastatin (20 μM), phosphoramidon (20 μM) or captopril (20 μM). Aliquots (20 μl) were collected at time zero, 30 min and after 1, 3, 7 and 24 h. The incubation was terminated by adding 1 ml of ice-cold methanol–0.1 *M* HCl, followed by centrifugation at 3000 *g* for 5 min. The supernatant was evaporated in a Speed Vac centrifuge (Savant, Hicksville, NY, USA) prior to separation on RP-HPLC.

2.4. Reversed-phase high-performance liquid chromatography

The RP-HPLC system was equipped with a Nova-Pak C_{18} column (300×3.9 mm, 4 μm particle size, 60 Å pore diameter) from Waters (Milford, MA, USA). Degradation products were resolved on the column using a linear gradient of acetonitrile (ACN), containing 0.04% trifluoroacetic acid (TFA). The gradient applied was 15–60% ACN in 0.04% TFA over 40 min. Prior to injection, the dried sample was dissolved in 200 μl of 15% ACN–0.04% TFA, and filtered through a sterile Millex-HV 0.45 μm filter (Millipore). Fractions (0.5 ml) were collected at a flow-rate of 1.0 ml/min and evaporated in the Speed Vac centrifuge prior to MS analysis.

2.5. Mass spectrometry analysis

A Finnigan TSQ 70 mass spectrometer was equipped with a micro-ES source [modification (see ref. [12]) from Vestec Products, PerSeptive Biosystems, Boston, MA]. Microspray needles were constructed from fused-silica capillaries with an I.D. of 50 μm and an O.D. of 220 μm. The opposite end of the needle was attached to a stainless-steel zero dead volume fitting with a 0.02″ through-hole and the high voltage connection was made through this union. Standard ES operating conditions were as follows: needle voltage, 3.5 kV; nozzle voltage, 205 V; repeller voltage, 9–10 V; block temperature, 230–240°C. The drying gas was ultra-pure, dry N_2 at approximately 1 atm. Samples were dissolved in 50 μl of 50% methanol in 0.25% acetic acid and analyzed by continuous infusion at a flow-rate of 820 nl/min.

3. Results and discussion

Following incubation of LVV-hemorphin-7 with human plasma, several distinct fragments were released from the parent peptide. Fig. 2a–c shows the HPLC chromatograms of the separations of LVV-hemorphin-7 and the produced metabolites after 0, 3 and 7 h of incubation. Two C-terminal fragments (2–10 and 6–10) were detected after 3 h, as well as the substrate peptide LVV-hemorphin-7. The substrate peptide showed a relatively high stability in plasma and approximately 30% of LVV-hemorphin-7 remained unconverted after 3 h. Incubation for 7 h completely metabolized LVV-hemorphin-7 and fragment 2–10 (VV-hemorphin-7) and two additional C-terminal fragments were identified (7–10 and 9–10).

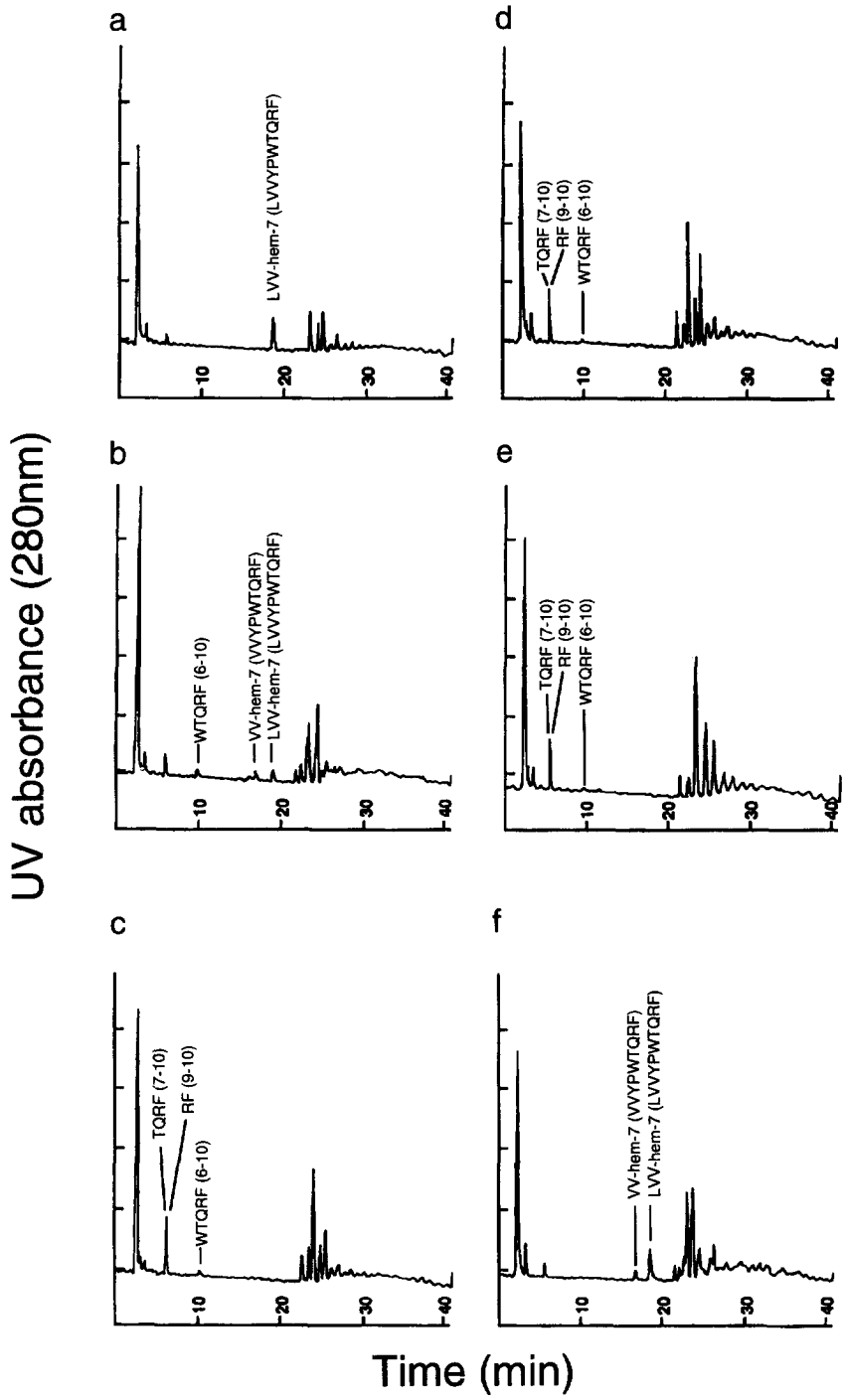

Fig. 2. HPLC separations of LVV-hemorphin-7 and its degradation products after 0, 3 and 7 h of incubation in plasma (a–c) and after 7 h of incubation in plasma containing captopril (20 μM) (d), phosphoramidon (20 μM) (e) or amastatin (20 μM) (f). LVV-hemorphin and its degradation products were resolved on a Nova-Pak C_{18} 300×3.9 mm column with a linear gradient of acetonitrile, containing 0.04% TFA. The gradient applied was 15–60% ACN in 0.04% TFA over 40 min. The identity of the peptide fragments was confirmed by mass spectometry. Peaks eluting between 20–30 min are probably larger peptides/proteins originating from the plasma.

In experiments where various protease inhibitors were included in the incubation mixture, it was found that captopril and phosphoramidon had little or no effect on the conversion rate, whereas amastatin appeared to be a strong inhibitor (Fig. 2d–f). It was thus found that the substrate peptide LVV-hemorphin-7 displayed high stability in human plasma in the presence of the aminopeptidase inhibitor, amastatin. After 7 h of incubation with amastatin included, LVV-hemorphin-7 was only partially hydrolysed to the C-terminal fragment 2–10. Approximately 90% of LVV-hemorphin-7 remained unconverted (Fig. 2a compared with Fig. 2f).

In the present study, MS–MS analysis of the collected HPLC fractions identified LVV-hemorphin-7 and several fragments. LVV-hemorphin-7 was recorded as a doubly charged ion (m/z 655.3), as can be seen in Fig. 3a, which shows the tandem MS spectrum of this peptide. The peptide was fragmented to form mainly singly or doubly charged Y ions and product ions of the A type (nomenclature according to Roepstorff and Fohlman [16]. Also seen were product ions of the A type. Hemorphin-fragment 2–10 (m/z 598.7) produced a similar type of fragmentation, where Y and A ions were detected (Fig. 3b). Micro-ES ionization of the shorter C-terminal fragment 6–10 was recorded as both the singly (m/z 737.8) and the doubly charged state (m/z

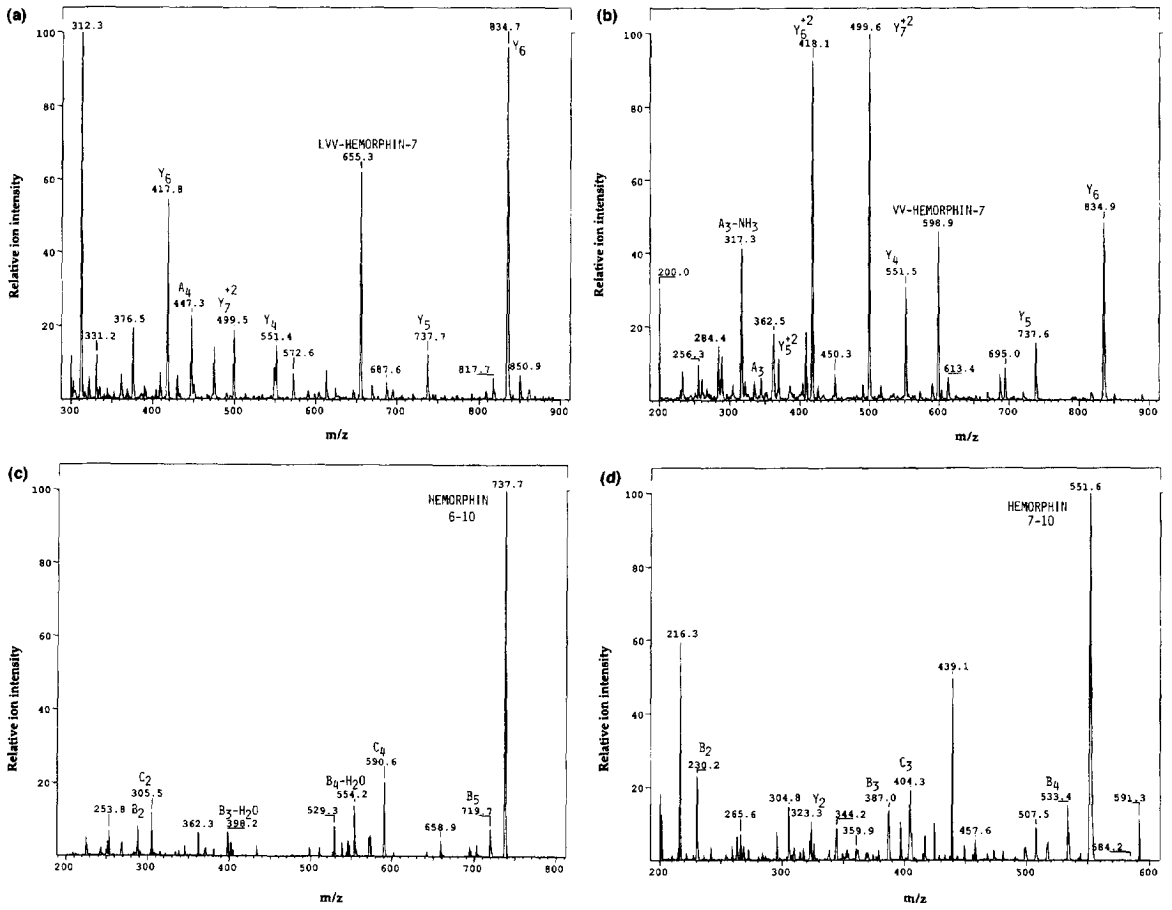

Fig. 3. (a) Tandem mass spectrum of LVV-hemorphin-7. The HPLC fraction was dissolved in 50 μl of methanol–0.25% acetic acid and infused to the micro-ES source. The spectrum shows the doubly charged parent ion, m/z 655.3 and singly and doubly charged Y and A product ions. The spectrum was obtained from an average of thirteen scans. Figures b–d show similar MS–MS spectra for VV-hemorphin-7, hemorphin fragments 6–10 and 7–10.

369.4). Collision-induced dissociation of the singly charged state produced mainly ions of the B and C type. MS–MS analysis of the doubly charged state gave ions of the Y and B type (Fig. 3c). Two hemorphin fragments co-eluted in the HPLC separation and were detected in the same fraction. Both the C-terminal fragments 9–10 (m/z 322.4) and 7–10 (m/z 551.6) were recorded as singly charged ions in the analysis. The MS–MS fragmentation of 7–10 produced Y, B and C ions (Fig. 3d). The shorter 9–10 fragment showed Y and C ions in the mass spectrum (spectrum not shown).

MS–MS capabilities are important in measuring peptides, because of the highly structure-specific nature of this technique, and were used to produce sequence information about the peptides contained in each collected HPLC fraction in these experiments. Protonated peptide ions were selected with the first quadrupole and were transmitted to the collision cell, where the ions were vibrationally excited by multiple low-energy collisions (collision-induced dissociation) with xenon, a neutral gas. The resulting product ions were therefore indicative of the amino acid sequence of the peptide and the predominant fragment ions were type A, B and Y ions. The MS–MS spectrum obtained for LVV-hemorphin-7 in this study produced several fragment (product) ions that could only originate from the parent molecule m/z 655.3, the doubly charged molecular species of LVV-hemorphin-7. Product ions produced from other peptides or proteins were therefore not recorded. Thus, the MS analysis does not only identify the mass-to-charge value of the peptide, but also verifies the primary structure of the peptide by identifying several sequence-specific fragments.

In conclusion, this study has demonstrated that LVV-hemorphin-7 is metabolized in vitro by proteolytic enzymes in the blood. The fragments found were C-terminal fragments 2–10, 6–10, 7–10, 8–10 and 9–10. However, it is also evident from this study that LVV-hemorphin-7 is relatively stable in human plasma. Even after 3 h of incubation, approximately 30% of the peptide remained unconverted. This resistance to proteolysis may be of importance for the biological effects of LVV-hemorphin-7. It was further indicated that the major enzyme responsible for the metabolism of LVV-hemorphin-7 in human plasma is an amastatin-sensitive exopeptidase. Whether this route of degradation also occurs in vivo is not yet clarified but will be the subject for our future research.

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