ELSEVIER

Journal of Chromatography A, 846 (1999) 39–48

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatographic study of the enzymatic degradation of endomorphins, with identification by electrospray ionization mass spectrometry

Antal Péter[a,*], Géza Tóth[b], Csaba Tömböly[b], Georges Laus[c], Dirk Tourwè[c]

[a]*Department of Inorganic and Analytical Chemistry, Attila József University, Dóm tér 7, H-6720 Szeged, Hungary*
[b]*Institute of Biochemistry, Biological Research Center, Temesvári krt 62, H-6725 Szeged, Hungary*
[c]*Eenheid Organische Chemie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium*

Abstract

The recently discovered native endomorphins play an important role in opioid analgesia, but their metabolic fate in the organism remains relatively little known. This paper describes the application of high-performance liquid chromatography combined with electrospray ionization mass spectrometry to identify the degradation products resulting from the incubation of endomorphins with proteolytic enzymes. The native endomorphin-1, H–Tyr–Pro–Trp–Phe–NH_2 (1), and endomorphin-2, H–Tyr–Pro–Phe–Phe–NH_2 (2), and an analog of endomorphin-2, H–Tyr–Pro–Phe–Phe–OH (3), were synthetized, and the levels of their resistance against carboxypeptidase A, carboxypeptidase Y, aminopeptidase M and proteinase A were determined. The patterns of peptide metabolites identified by this method indicated that carboxypeptidase Y first hydrolyzes the C-terminal amide group to a carboxy group, and then splits the peptides at the Trp^3–Phe^4 or Phe^3–Phe^4 bond. The remaining fragment peptides are stable against the enzymes investigated. Carboxypeptidase A degrades only analog 3 at the Phe^3–Phe^4 bond. Aminopeptidase M cleaves the peptides at the Pro^2–Trp^3 or Pro^2–Phe^3 bond. The C-terminal fragments hydrolyze further, giving amino acids and Phe–NH_2-s while the N-terminal part displays a resistance to further aminopeptidase M digestion. Proteinase A exhibits a similar effect to carboxypeptidase Y: the C-terminal amide group is first converted to a carboxy group, and one amino acid is then split off from the C-terminal side.

Keywords: Electrospray ionization; Endomorphins; Enzymes; Peptides

1. Introduction

Endomorphin-1 (1; H–Tyr–Pro–Trp–Phe–NH_2) and endomorphin-2 (2; H–Tyr–Pro–Phe–Phe–NH_2) have recently been isolated from bovine brain [1] and human brain [2] (Fig. 1). Both peptides are highly potent and selective μ-opioid receptor ago-

H-Tyr-Pro-Trp-Phe-NH_2 **(1)**

H-Tyr-Pro-Phe-Phe-NH_2 **(2)**

H-Tyr-Pro-Phe-Phe-OH **(3)**

Fig. 1. Endomorphin analogs studied: endomorphin-1 (1); endomorphin-2 (2); and an analog of endomorphin-2 (3).

*Corresponding author. Tel.: +36-62-454-000/3656; fax: +36-62-420-505.
E-mail address: apeter@chem.u-szeged.hu (A. Péter)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00146-6

nists. Peptide 1 exerts potent bioactivity both in vitro and in vivo, producing prolonged analgesia in mice after central administration. Stone et al. [3] observed short-lasting antinociceptive effects of 1 and 2. The short duration of action suggests that these peptides are unstable in the spinal cord. Spetea et al. [4] described the binding characteristics of [^{3}H]endomorphin-2 in a rat brain membrane preparation and used peptidase inhibitors in a radioligand binding assay. In the absence of peptidase inhibitors 40% of the radioligand in the incubation mixture was destroyed after incubation for 45 min at 25°C. These different findings highlight the importance of studying the stabilities of 1 and 2 against enzymatic degradation.

The cerebrospinal fluid and central nervous system contain amino-, endo- and carboxypeptidases capable of processing and/or degrading opioid peptides, and altered rates of degradation have been reported in some pathologic conditions [5,6].

This paper describes the application of high-performance liquid chromatography (HPLC) combined with electrospray ionization mass spectrometry (ESI-MS) [6,7] to identify the degradation products of these endomorphins after incubation with neuropeptide-degrading proteases. Peptides 1 and 2 and a modified analog of 2 (3; H–Tyr–Pro–Phe–Phe–OH) were incubated with carboxypeptidase A, carboxypeptidase Y, aminopeptidase M and proteinase A.

Carboxypeptidase A, a metallopeptidase, removes C-terminal aromatic and long side-chain aliphatic residues most rapidly [8]. Native endomorphins, being amidated peptides, are probably stable against carboxypeptidase A digestion, but analog 3 could be a substrate.

Carboxypeptidase Y is a Ser-type enzyme, and accepts a broad spectrum of substances. Ser carboxypeptidase does not require a free C-terminal carboxylate group. It can catalyse the hydrolysis of peptide esters and amides [9]. Additionally, Ser carboxypeptidase acts on peptide amides, releasing either ammonia (amidase activity) or amino acid amides (dipeptidyl amino acid amide hydroxylase activity) [10]. Carboxypeptidase Y exhibits a preference for peptides containing hydrophobic amino acid residues (except Pro).

Aminopeptidases are present in the brain and peripheral tissues and destroy the activity of opioid peptides [11]. Aminopeptidase M releases an N-terminal amino acid from peptides, amides or arylamides. The amino acid released is preferably Ala, but most amino acids including Pro (slow reaction) may also be involved. When a terminal hydrophobic residue is followed by a Pro residue, the two may be released as an intact dipeptide [12]. Aminopeptidase cleaves opioid peptides into inactive fragments by splitting off Tyr [13]. As concerns dynorphins, two aminopeptidases that could participate in N-terminal amino acid removal were identified in the rat brain: the puromycin-sensitive aminopeptidase and aminopeptidase M [14].

Proteinase A is a nonpepsin-type acid endopeptidase; it cleaves small peptides at only one site and the rates of hydrolysis differ markedly among them. It also cleaves amidated peptides, e.g., Substance P and its analogs degrade rapidly [15].

2. Experimental

2.1. Chemicals and reagents

Peptides 1 and 2 were prepared by solid-phase synthesis on a 4-methylbenzhydrylamine resin by means of Boc chemistry, and 3 was prepared by using Merrifield resin, also with Boc protection (Fig. 1) (nomenclature and abbreviations are in accordance with the IUPAC-IUB Joint Commission of Biochemical Nomenclature (JCBN) recommendations [16]). The synthetic peptides were purified on a Merck–Hitachi HPLC system.

Proteinase A (endopeptidase, from baker's yeast; EC 3.4.23.6), carboxypeptidase A (type II DFP from bovine pancreases, treated with diisopropylfluorophosphate to eliminate trypsin and chymotrypsin activity; EC 3.4.17.1,), carboxypeptidase Y (from baker's yeast; EC 3.4.16.1) and aminopeptidase M (leucine aminopeptidase, type IV-S: from porcine kidney microsomes; EC 3.4.11.2) were obtained from Sigma–Aldrich (Steinheim, Germany). The homogenity of each of these commercially available enzymes was checked by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Different batches of enzymes were checked, and only enzymes giving only one protein band were used in digestion measurements. Tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl), tri-

fluoroacetic acid (TFA) of analytical-reagent grade, acetonitrile and methanol of HPLC grade and other reagents of analytical-reagent grade were purchased from Merck (Darmstadt, Germany). Buffers were prepared with Milli-Q water and were further purified by filtration on a 0.45-μm filter type HV (Millipore, Molsheim, France).

2.2. Apparatus

Analytical and semipreparative chromatography were performed on a chromatographic system consisting of an L-6200A Intelligent pump, an L-3000 multichannel photo detector, a T-6300 column thermostat, and a D-6000 HPLC Manager with interface (Merck–Hitachi, Darmstadt, Germany). The samples were introduced via a Rheodyne Model 7125 valve (Cotati, CA, USA) equipped with a 20-μl loop.

The column used was a Vydac 218TP54 C_{18} (250×4.6 mm I.D.), 5-μm particle size (Separations Group, Hesperia, CA, USA). Gradient elutions were run with a 0.1% aqueous solution of TFA as mobile phase A and 0.1% TFA in acetonitrile as phase B, ranging from 0% to 50% B within 20 min.

2.3. Enzymatic cleavage assay

2.3.1. Preparation of solutions

Solutions of peptides 1–3 were made by dissolving them in 50 m*M* Tris·HCl (pH 7.4) buffer to give a final concentration of 1 m*M*.

Carboxypeptidase A and aminopeptidase M were used in their original concentrations, 18 and 10 mg protein/ml, respectively. Carboxypeptidase Y solution (0.5 mg/ml) was made by dissolving 0.16 mg enzyme in 320 μl 50 m*M* Tris·HCl buffer (pH 7.4). Proteinase A solution (0.5 mg/ml) was made by dissolving 0.2 mg enzyme in 400 μl 50 m*M* Tris·HCl buffer (pH 7.4) containing 10% (v/v) glycerol.

2.3.2. Enzymatic digestion

To 1000 μl 50 m*M* Tris·HCl buffer, 200 μl peptide solution was added, and the reaction mixture was incubated for 30 min at 37°C in a Julabo SW-200 agitating water bath (Julabo Labortechnik, Seelbach, Germany). After preincubation, one of the enzymes was added and the mixture was kept at 37°C. (The volumes of added enzyme solutions were as follows: 10 μl aminopeptidase M, 10 μl carboxypeptidase A, 30 μl carboxypeptidase Y or 30 μl proteinase A.) At designated intervals, 20 μl aliquots were removed and quenched in 20 μl 0.1 *M* HCl. The time interval of sampling was chosen so that a kinetic curve could be constructed. Samples were analyzed by HPLC for the quantitative determination of digestion products. For LC–ESI-MS analysis, the reaction was stopped at a degree of conversion of about 50%, and the whole reaction mixture was used for prepurification on Sep-Pak cartridges.

2.3.3. Sample handling and data processing

For ESI-MS and HPLC–ESI-MS analysis the digested samples were prepurified on C_{18} cartridges. For this purpose, the digested sample was taken up in an 0.1% aqueous solution of TFA and adsorbed on prewetted Sep-Pak C_{18} cartridges (Millipore, Bedford, USA) [17]. The cartridge was washed with 0.1% TFA and peptides were eluted with 0.1% TFA–acetonitrile (20:80, v/v).

Blanks were run by incubating the enzyme and the enzyme-free reaction mixture for several hours at 37°C, and the products formed were taken into account.

The results were calculated by taking into account the amount of starting peptide consumed and the amounts of degradation fractions produced, and are given in area% calculated from the HPLC measurements.

2.4. Mass spectrometry

The MS measurements were carried out on a Quattro II apparatus (Micromass UK, Altrincham, UK) with ESI and coupled to an HPLC system. The HPLC system consisted of a low-pressure gradient pump type 325, a UV detector type 332 and an Autosampler Type 465, all from Kontron (Milan, Italy). The column used was a Vydac 218TP54 C_{18} (250×4.6 mm I.D.), 5-μm particle size. The effluent of the HPLC column was split by an LC Packings split system with a split ratio of 1 to 10. For loop injection analysis, a Harvard Model 22 syringe infusion pump (South Natich, MA, USA) and a Rheodyne 7125 injector with a 50-μl loop were used. The flow-rate of the syringe pump was kept at 30 μl/min.

The mass spectrometer was operated under the

following conditions: capillary voltage 3.5 kV; cone voltage 50 V; scan duration 0.5 s (data type: compressed centroid); source temperature 60°C; mass range 110 to 1100. Data were acquired in positive-ion mode and the spectra were recorded with a Mass Lynx Application Software System (Micromass UK, Wythenshawe, UK). Peptides were identified via their molecular ions. Identification of the fragments by the direct application of MS to the digestion mixture was not possible. The on-line LC–ESI-MS was applied for identification of the peptide fragments. An example of the identification of the fragments of 1 after digestion with carboxypeptidase Y and HPLC fractionation is shown in Fig. 2.

3. Results and discussion

Four different enzymes were chosen to investigate their effects on the stabilities of endomorphins. Carboxypeptidase Y can catalyze the hydrolysis of amidated peptides and may release ammonia in consequence of its amidase activity [9,10]. The accommodation of an amino acid amide is probably aided by a beneficial hydrogen-bond between the C-terminal NH_2 group of the substrate and the deprotonated form of Glu-145 of the enzyme [10]. Endomorphins 1 and 2 were good substrates of enzymatic digestion with carboxypeptidase Y.

The results of HPLC and MS analyses of enzymatic digestion with carboxypeptidases are presented in Table 1 and Figs. 3 and 4. Fig. 3 relates to the separation of the degradation fragments and the starting peptides. The sequence of elution of the fragments could be rationalized in terms of their hydrophobicities. Fragments containing Trp in position 3 always had higher retention times than the corresponding fragments containing Phe in position 3. As concerns the separation of fragments containing a C-terminal amide or hydroxy group, the former are more hydrophilic and have shorter retention times.

For 1 and 2, three major peaks appeared in the chromatogram after digestion with carboxypeptidase Y (Fig. 3). The fragments observed indicate that the digestion of 1 and 2 with carboxypeptidase Y took place in two steps. In the first step, the C-terminal amide group underwent hydrolysis to the carboxy group; then, in the second step, cleavage of the Trp^3–Phe^4 or Phe^3–Phe^4 bond occurred:

H-Tyr-Pro-Trp-Phe-NH_2	H-Tyr-Pro-Phe-Phe-NH_2	(1)
↓	↓	
H-Tyr-Pro-Trp-Phe-OH	H-Tyr-Pro-Phe-Phe-OH	(2)
↓	↓	
H-Tyr-Pro-Trp-OH + Phe	H-Tyr-Pro-Phe-OH + Phe	(3)

The total degradation of 1 requires more than 20 min, whereas 2 was no longer present after 5 min. The remaining fragment peptides (H–Tyr–Pro–Trp–OH or H–Tyr–Pro–Phe–OH) are stable: even after hydrolysis for 20 h, no sign of new products could be observed in the chromatogram. Results concerning the stabilities of the peptides against carboxypeptidase Y digestion are presented in Table 1. The half-lives ($t_{1/2}$) for 1 and 2 were calculated from the rate constants (k), as $0.693/k$, using the kinetic curves of consumption of the peptides, and were about 3.0 and 1.0 min, respectively.

Endomorphins 1 and 2, being amidated peptides, exhibited high stability against enzymatic digestion with carboxypeptidase A: no sign of degradation could be observed after digestion for 24 h at 37°C. Analog 3 degrades rapidly in the presence of carboxypeptidase A, with a half-life of less than 5 s, and gives two major products: H–Tyr–Pro–Phe–OH and Phe (Table 1). This behavior is in agreement with literature data; carboxypeptidase A truncates peptides from the C-terminal side, releasing one amino acid [8].

Aminopeptidases release an N-terminal amino acid from the peptide or peptide amide [11]. The present endomorphins were split between Pro^2–Trp^3 or Pro^2–Phe^3 (Table 2). This unusual finding can be explained in terms of the Pro content of the molecules. Neubert et al. [12] showed that β-casomorphins, which are Pro-rich peptides, are very resistant to proteases. For their degradation study, they used dipeptidyl peptidase IV, which belongs in the aminopeptidase family, and splits off dipeptides from the N-terminal end of the peptides. The literature data indicate that, when an N-terminal hydrophobic residue is followed by a Pro residue, the two amino acids may be released as an intact dipeptide by aminopeptidase M, as was observed in our case [18].

The total degradation of 1 requires more than 1 h,

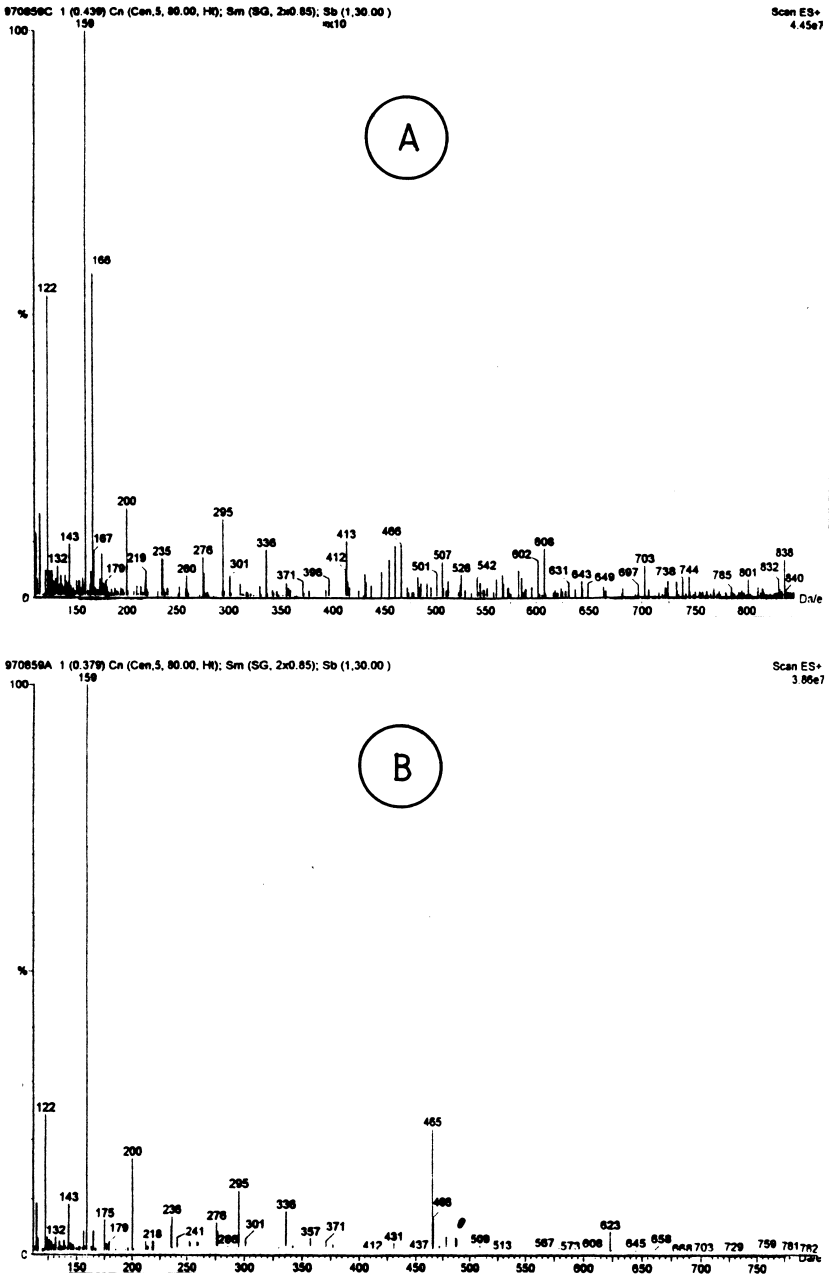

Fig. 2. Mass spectra of fragments of 1 after enzymatic digestion with carboxypeptidase Y and HPLC fractionation. Composition of digested mixture: 1, 166 μM; carboxypeptidase Y, 12.5 μg protein/ml. Incubation time, 15 min. (A) Phe, $m/z=166$; (B) H–Tyr–Pro–Trp–OH, $m/z=465$; (C) H–Tyr–Pro–Trp–Phe–OH, $m/z=612$.

while 2 had disappeared from the reaction mixture after a digestion period of 3 h. The peptide fragments formed displayed different stabilities. The resulting C-terminal dipeptide fragments, H–Trp–Phe–NH_2 or H–Phe–Phe–NH_2, were unstable and hydrolyzed further. They were split into the corresponding

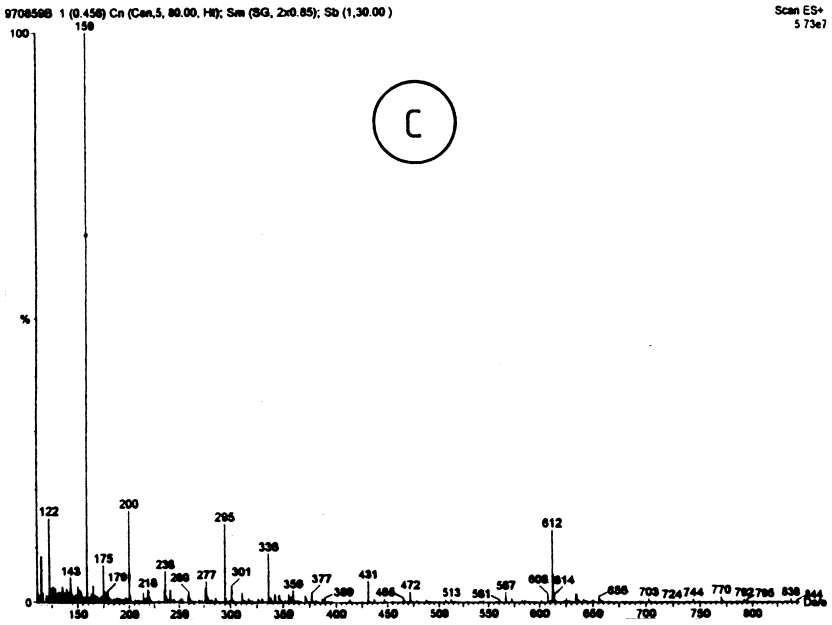

Fig. 2. (*continued*)

Table 1
Main degradation products of 1 and 2 on digestion with carboxypeptidase Y, and of analog 3 with carboxypeptidase A

Endomorphin	t_r [a]	m/z [b]	$M_{r(calc)}$ [c]	Sequence assignment	Sequence no.	$t_{1/2}$ [d] (min)
1	9.8	166	165.1	Phe	4	
	15.2	465	464.3	H–Tyr–Pro–Trp–OH	1–3	
	17.9	612	611.4	H–Tyr–Pro–Trp–Phe–OH	1–4	
	17.1	611	610.4	H–Tyr–Pro–Trp–Phe–NH_2 (intact peptide)	1–4	3.1
2	9.8	166	165.1	Phe	4	
	14.9	426	425.3	H–Tyr–Pro–Phe–OH	1–3	
	17.6	573	572.4	H–Tyr–Pro–Phe–Phe–OH	1–4	
	16.6	572	571.4	H–Tyr–Pro–Phe–Phe–NH_2 (intact peptide)	1–4	1.0
3	9.8	166	165.1	Phe	4	
	14.9	426	425.3	H–Tyr–Pro–Phe–OH	1–3	
	17.6	573	572.4	H–Tyr–Pro–Phe–Phe–OH (intact peptide)	1–4	<0.1

[a] HPLC retention time in min. HPLC conditions: column, Vydac 218TP54 C_{18}; gradient elution, 50% B in 20 min, see Experimental; detection, 210 nm; flow-rate, 0.8 ml/min.

[b] m/z Values for the ion $[M+H]^+$ in the mass spectra.

[c] Monoisotopic masses.

[d] Half-lives of peptides in the presence of carboxypeptidase Y and carboxypeptidase A.

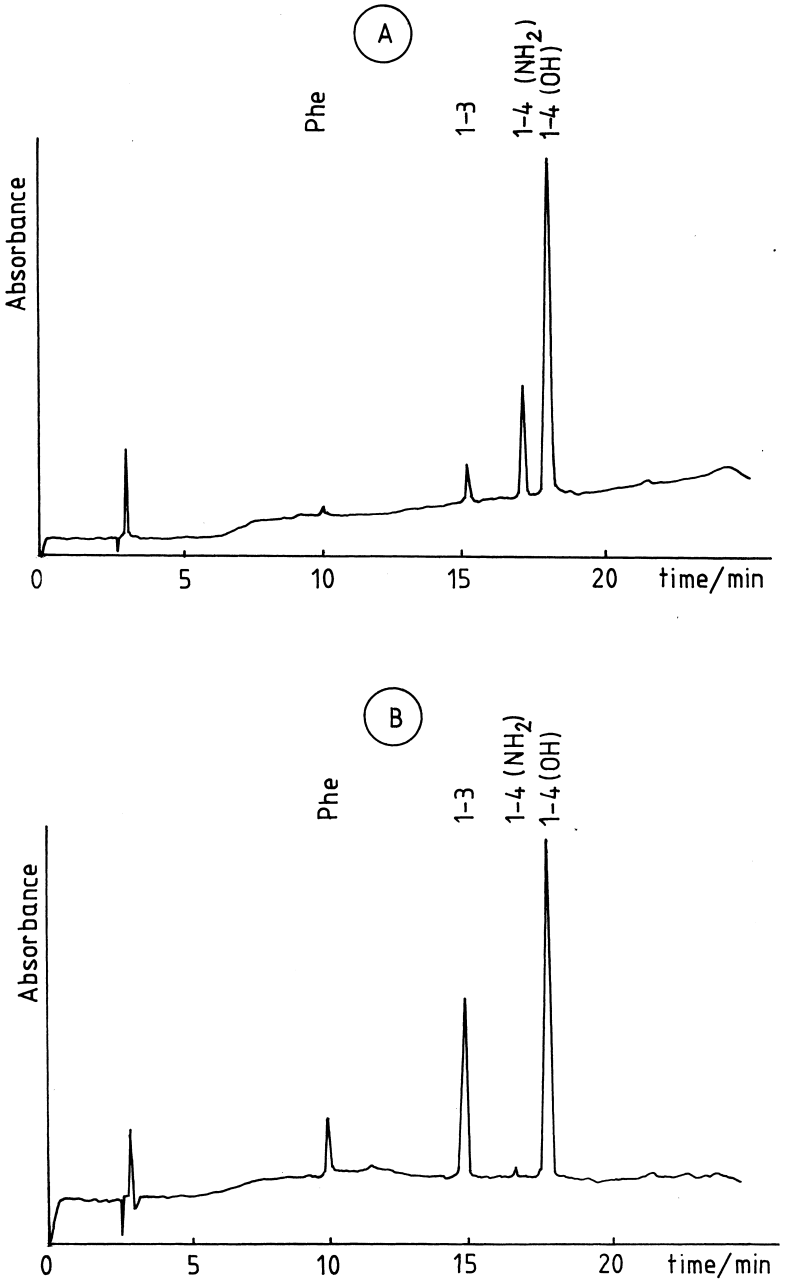

Fig. 3. HPLC separation of mixtures of products of degraded endomorphins after digestion with carboxypeptidase Y. (A) 1; (B) 2. Composition of digestion mixture: 1, 166 μM; 2, 166 μM; Carboxypeptidase Y, 12.5 μg/ml. Incubation time, 15 min; conditions of analysis: column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 210 nm; gradient elution, 50% B in 20 min, see Experimental. Peaks: see Table 1.

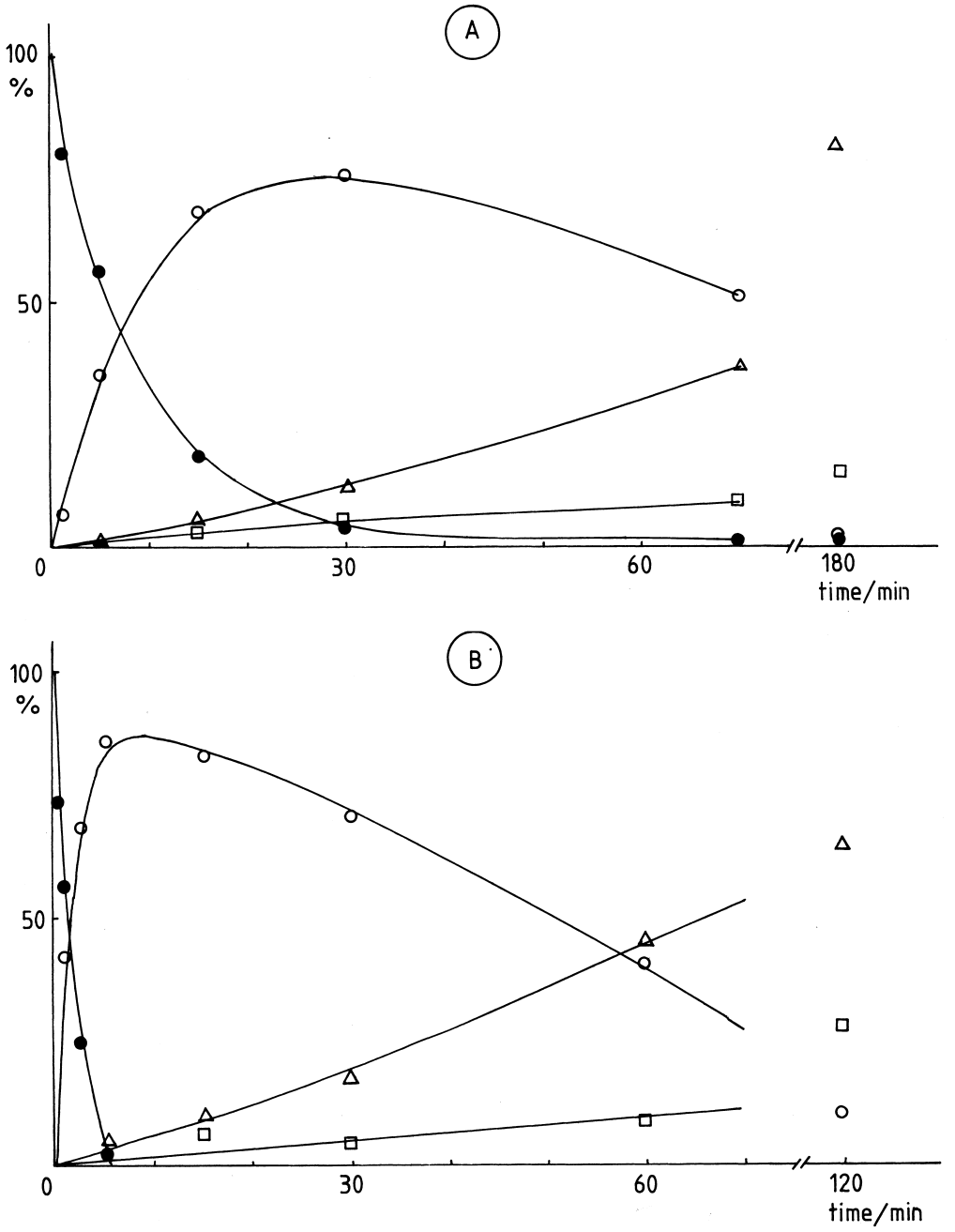

Fig. 4. Rates of change of peak intensities of the starting peptides and digestion products of endomorphins on incubation with carboxypeptidase Y. (A) 1; (B) 2. Composition of digestion mixture: 1, 166 μM; 2, 166 μM; Carboxypeptidase Y, 12.5 μg/ml; Conditions of analysis: column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 210 nm; gradient elution, 50% B in 20 min, see Experimental. ● (A,B) starting peptide; ○ (A) H–Tyr–Pro–Trp–Phe–OH; (B) H–Tyr–Pro–Phe–Phe–OH; △ (A) H–Tyr–Pro–Trp–OH; (B) H–Tyr–Pro–Phe–OH; □ (A,B) Phe.

Table 2
Main degradation products of 1 and 2 on digestion with aminopeptidase M

Endomorphin	t_r [a]	m/z [b]	$M_{r(calc)}$ [c]	Sequence assignment	Sequence no.	$t_{1/2}$ [d] (h)
1	9.0	165	164.1	Phe–NH_2	4	
	10.0	279	278.2	H–Tyr–Pro–OH	1–2	
	11.9	205	204.1	Trp	3	
	13.7	351	350.2	H–Trp–Phe–NH_2	3–4	
	17.1	611	610.4	H–Tyr–Pro–Trp–Phe–NH_2 (intact peptide)	1–4	0.17
2	9.0	165	164.1	Phe–NH_2	4	
	9.8	166	165.1	Phe	3	
	10.0	279	278.2	H–Tyr–Pro–OH	1–2	
	13.4	312	311.2	H–Phe–Phe–NH_2	3–4	
	16.6	572	571.4	H–Tyr–Pro–Phe–Phe–NH_2 (intact peptide)	1–4	0.25

[a] HPLC retention time in min. HPLC conditions: column, Vydac 218TP54 C_{18}; gradient elution, 50% B in 20 min, see Experimental; detection, 210 nm; flow-rate, 0.8 ml/min.
[b] m/z Values for the ion $[M+H]^+$ in the mass spectra.
[c] Monoisotopic masses.
[d] Half-lives of peptides in the presence of aminopeptidase M.

amino acids (Trp or Phe) and amino acid amide (Phe–NH_2) within 1 h of the start of digestion. The N-terminal part of the hydrolysis product (H–Tyr–Pro–OH) was relatively stable; Tyr appeared in the chromatogram after 5 h. The half-lives calculated from k were 0.17 h for 1 and 0.25 h for 2.

Proteinase A degrades native endomorphins in two steps, similarly to carboxypeptidase Y (Table 3). In the first step, the C-terminal amide group is converted to a carboxy group, and the C-terminal amino acid is then released. This degradation mechanism is supported by the products of digestion of 3, which

Table 3
Main degradation products of 1, 2 and 3 on digestion with proteinase A

Endomorphin	t_r [a]	m/z [b]	$M_{r(calc)}$ [c]	Sequence assignment	Sequence no.	$t_{1/2}$ [d] (h)
1	9.8	166	165.1	Phe	4	
	15.2	465	464.3	H–Tyr–Pro–Trp–OH	1–3	
	17.9	612	611.4	H–Tyr–Pro–Trp–Phe–OH	1–4	
	17.1	611	610.4	H–Tyr–Pro–Trp–Phe–NH_2 (intact peptide)	1–4	3.5
2	9.8	166	165.1	Phe	4	
	14.9	426	425.3	H–Tyr–Pro–Phe–OH	1–3	
	17.6	573	572.4	H–Tyr–Pro–Phe–Phe–OH	1–4	
	16.6	572	571.4	H–Tyr–Pro–Phe–Phe–NH_2 (intact peptide)	1–4	1.7
3	9.8	166	165.1	Phe	4	
	14.9	426	425.3	H–Tyr–Pro–Phe–OH	1–3	
	17.6	573	572.4	H–Tyr–Pro–Phe–Phe–OH (intact peptide)	1–4	1.4

[a] HPLC retention time in min. HPLC conditions: column, Vydac 218TP54 C_{18}; gradient elution, 50% B in 20 min, see Experimental; detection, 210 nm; flow-rate, 0.8 ml/min.
[b] m/z Values for the ion $[M+H]^+$ in the mass spectra.
[c] Monoisotopic masses.
[d] Half-lives of peptides in the presence of proteinase A.

contains a C-terminal carboxy group and degrades directly to H–Tyr–Pro–Phe–OH and Phe. The remaining peptide fragments (H–Tyr–Pro–Trp–OH or H–Tyr–Pro–Phe–OH) are relatively stable. No sign of degradation products could be observed in the chromatogram after digestion for 8–10 h. The literature data indicate that proteinase A can catalyze the hydrolysis of amidated peptides [15]. Substance P (Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2) and various of its analogs degrade rapidly in the presence of proteinase A. Results concerning the stabilities of the peptides against enzymatic degradation are shown in Table 3. The total degradation of 1 requires more than 8 h, whereas 2 and 3 were no longer present after 6 h. The half-lives ($t_{1/2}$) for 1, 2 and 3 were calculated from the rate constants (k) and were found to be 3.5, 1.7 and 1.4 h, respectively.

It is difficult to explain this unusual effect of proteinase A, which belongs among the endopeptidases. On the one hand it catalyzed the conversion of the C-terminal amide group to a carboxy group, while on the other hand it behaved as an exopeptidase, splitting off one C-terminal amino acid. This behavior could not stem from impurities in the enzyme, because SDS-PAGE demonstrated its good homogenity.

4. Conclusions

HPLC combined with ESI-MS proved a useful and rapid tool for investigation of the enzymatic digestion of endomorphins. The qualitative picture of the degradation products provided by MS furnishes sufficient information to establish the possible pathways of degradation. The semiquantitative picture afforded by HPLC measurements yields useful information allowing comparisons of the stabilities of these peptides under different conditions.

In the digestion of native endomorphins, 1 and 2 with carboxypeptidase Y and proteinase A, the C-terminal amide was first converted to a C-terminal carboxy group, and the hydrolysis then led to splitting off the Trp^3–Phe^4 and Phe^3–Phe^4 bonds. The resulting tripeptides were stable against further hydrolysis with these enzymes. Carboxypeptidase A proved effective only against the peptide with a C-terminal carboxylic acid, 3. Aminopeptidase M cleaved the Pro^2–Trp^3 and Pro^2–Phe^3 linkages. The remaining N-terminal dipeptide fragment, H–Tyr–Pro–OH, was relatively stable; Tyr appeared after digestion for 5 h. The C-terminal parts, H–Trp–Phe–NH_2 or H–Phe–Phe–NH_2, hydrolyzed quickly; these dipeptides had disappeared from the reaction mixture after 1 h.

Acknowledgements

This work was supported by Hungarian grants OTKA T 029460, T 030086, FKFP 0087/97 and Flemish–Hungarian Intergovernmental Cooperation in S and T grant B-3/96.

References

[1] J.E. Zadina, L. Hackler, L.-J. Gee, A.J. Kastin, Nature 386 (1997) 499.
[2] L. Hackler, J.E. Zadina, L.-J. Gee, A.J. Kastin, Peptides 18 (1997) 1635.
[3] L.S. Stone, C.A. Fairbanks, T.M. Laughlin, H.D. Nguyen, T.M. Busky, M.W. Wessendorf, G.L. Wilcox, Neuroreport 8 (1997) 3131.
[4] M. Spetea, K. Monory, Cs. Tömböly, G. Tóth, E. Tzavara, S. Benyhe, J. Hanoune, A. Borsodi, Biochem. Biophys. Res. Commun. 250 (1998) 720.
[5] F. Benter, E.M. Hirsch, A.J. Tuchman, P.E. Ward, Biochem. Pharm. 40 (1990) 465.
[6] R. Mentlein, R. Lucius, Brain Res. Protocols 1 (1997) 237.
[7] L. Prokai, H.-S. Kim, A. Zharikova, J. Roboz, L. Ma, L. Deng, W.J. Simonsick Jr., J. Chromatogr. A 800 (1998) 59.
[8] R.P. Ambler, Methods Enzymol. 25 (1972) 262.
[9] P.F. Berne, S. Blanquet, J.-M. Schmitter, J. Am. Chem. Soc. 114 (1992) 2603.
[10] S.J. Remington, K. Breddam, Methods Enzymol. 244 (1994) 231.
[11] A.K. Dua, C. Pinsky, F.S. LaBella, Life Sci. 37 (1985) 985.
[12] K. Neubert, B. Hartrodt, I. Born, A. Barth, H.-L. Reuthrich, G. Grecksch, U. Schrader, C. Liebmann, in: F. Nyberg, V. Brantl (Eds.), β-Casomorphins and Related Peptides, Fyris-Trych, Uppsala, 1990, p. 15.
[13] B.F. Roques, F. Noble, V. Dange, M.-C. Fournie-Zaluski, A. Bleaumont, Pharmacol. Rev. 45 (1993) 87.
[14] A. Safavi, L.B. Hersh, J. Neurochem. 65 (1995) 389.
[15] K. Takahashi, Methods Enzymol. 248 (1995) 146.
[16] IUPAC-IUB JCBN recommendations, J. Biol. Chem. 264 (1989) 668.
[17] A. Péter, S. Devadder, G. Laus, D. Tourwè, J. Chromatogr. A 729 (1996) 137.
[18] A. Bairoch, Nucleic Acids Res. 24 (1996) 221.