Isolation and structural characterization of enkephalins in the brain of the Rhynchobdellid leech *Theromyzon tessulatum*

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Abstract This paper reports the purification of four peptides related to enkephalins from the brain of the leech *Theromyzon tessulatum*. After reverse-phase HPLC purification, the sequence of the enkephalins (YGGFM, YGGFL, FM, FL) was established by a combination of automated Edman degradation, electrospray mass spectrometry measurement, and co-elution experiments in reverse-phase HPLC with synthetic peptides. ELISA titrations performed on each purified peptide indicated that the major amount was borne by the leucine-enkephalin. The ratio of leucine-enkephalin and methionine-enkephalin of 2:1 is in line with previous immunocytochemical data obtained on *T. tessulatum* brains. The presence of enkephalins in *T. tessulatum*, an animal belonging to the oldest group of coelomate metazoans (the Annelida) establishes the very ancient phylogenetic origin of opioids and their conservation in the course of evolution.

Key words: Leech; Enkephalin; Microsequence; Electrospray mass spectrometry; Invertebrate; Phylogeny

1. Introduction

Since their discovery in mammalian brain by Hughes et al. [1], opioids have been presumed to be specific to vertebrates. However, by means of radioimmunoassay and immunocytochemistry, evidence of enkephalin-like substances in all phyla of invertebrates has been demonstrated [2–6]. Finally in 1984, enkephalins (methionine-enkephalin (Met-enk), leucine-enkephalin (Leu-enk)) and the heptapeptide [Met-enk-Arg^6-Phe7], were isolated from the pedal ganglia of the mollusc *Mytilus edulis* [7]. Recently, in crustacea, by a combined approach of mass spectrometry and amino acid sequence analysis, a Leu-enk and a Met-enk has been characterized from thoracic ganglia of the shore crab *Carcinus maenas* L. [8].

In leeches, enkephalin-like immunoreactivities were found in two species, the gnatobdellid *Hirudo medicinalis* [9–13] and the rhynchobdellid *Theromyzon tessulatum* [14–16]. These immunoreactivities were detected in brain [15,16], the ventral nerve cord [9–14] and the peripheral nervous system [13,14]. Since these previous studies in immunocytochemistry, no further peptides related to the opioid family have been isolated in leeches.

We now report the isolation and the characterization of enkephalins in leeches, animals belonging to the oldest group of coelomate metazoans (the Annelida).

2. Materials and methods

2.1. Animals

Mature specimens of the rhynchobdellid leech *T. tessulatum*, reared under laboratory conditions as described by Malecha et al. [17], were used in this study.

2.2. Antisera

Polyclonal antisera anti-methionine-enkephalin (a-Met-enk) and anti-leucine-enkephalin (a-Leu-enk) raised in rabbits, kindly provided by Dr G. Tramu (Laboratoire de Neurocytochimie fonctionnelle, Université de Bordeaux I, Talence, France), were used in immunobinding assays. Their specificities were previously described elsewhere [18]. In short, the a-Leu-enk did not cross-react with Met-enk, and the a-Met-enk showed a cross-reactivity of 0.4% with Leu-enk.

2.3. Immunoassays

Enzyme-linked immunosorbent assays (ELISA) and dot immunobinding assays (DIA) were conducted according to Salzet et al. [19,20] with a-Met-enk and a-Leu-enk employed at a dilution of 1:1000. As a control, preadsorption of anti-enkephalins was carried out using homologous peptides. Prior to ELISA and DIA, a-Met-enk or a-Leu-enk, at their working dilution, were respectively incubated overnight at 4°C with Met-enk and with Leu-enk (Sigma) (100 μg of synthetic peptide per ml of undiluted antiserum).

2.4. Purification

A five-step procedure was employed (Table 1).

After anaesthesia of the animals in 0.01% chloretone, 1000 brains were excised, immediately frozen in liquid nitrogen and stored at −70°C until use. Batches of 200 brains were homogenized and extracted with 200 μl 1 M acetic acid at 4°C. After centrifugation at 12,000 rpm for 30 min at 4°C, the pellet was re-extracted once. The two supernatants were combined and loaded onto Sep-Pak C_{18} cartridges (500 μl extract/cartridge; Waters). After washing the cartridges with 5 ml 1 M acetic acid, elution was performed with 5 ml 50% acetonitrile in acidified water (0.1% TFA; Pierce). The 50% eluted fraction was reduced 20-fold in a vacuum centrifuge (Savant). The total amount of enkephalin-like material was quantified using ELISA. The 50% eluted fraction was taken up to 250 μl with acidified water (0.1% TFA) and fractionated on a HPGPC column (Ultraspherogel, 7,5 × 300 mm, SEC2000; Beckman). Samples were eluted with 30% acetonitrile in acidified water at a flow rate of 500 μl/min. Fractions immunoreactive in DIA to either a-Met-enk or a-Leu-enk were concentrated before being separated on a C_{18}-peptide protein column (250 × 4.6 mm; Vydac) equilibrated with acidified water (0.1% TFA). Elution was performed with a discontinuous linear gradient of acetonitrile in acidified water over 0–15% for 10 min and over 15–45% for 30 min at a flow rate of 1 ml/min. The column

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Abbreviations: a-Leu-enk, anti-leucine-enkephalin; a-Met-enk, anti-methionine-enkephalin; DIA, dot immunobinding assay; ELISA enzyme-linked immunosorbent assays; ESMS, electrospray mass spectrometry; HPLC, high performance liquid chromatography; HPGPC, high performance gel permeation chromatography; Leu-enk, leucine-enkephalin; γ-MSH, γ-melanocyte stimulating hormone; Met-enk, methionine-enkephalin; POMC, pro-opiomelanocortin; Proenk, pro-enkephalin; Prodyn, prodynorphin; R_T, retention time; TFA, trifluoroacetic acid.

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effluent was monitored by absorbance at 226 nm and the presence of enkephalin-like material detected on aliquots of each fraction by DIA. The fractions that contained the immunoreactive material were analyzed on the same column with a shallower gradient of acetonitrile from 0–15% in 10 min followed by a gradient of 15–45% in 40 min (increase of 0.75%/min instead of 1%/min for the previous step). After concentration by freeze drying, fraction aliquots of 0.5 μl were tested by DIA. The enkephalin-like material was finally purified on an ODS C_{18} reverse-phase column (Ultrasphere, 250times;2 mm; Beckman) developed with a linear gradient of 0–60% acetonitrile in acidified water for 60 min at a flow rate of 50 μl/min. The column effluent was monitored by absorbance at 226 nm and the immunoreactive material detected as above.

Co-elutions in reverse-phase HPLC were performed with synthetic Met-enk and Leu-enk peptides purchased from Sigma.

All HPLC purifications were performed with a Beckman Gold HPLC system equipped with a photo diode array detector Beckman 168.

2.5. Peptide oxidation

50 μl of a solution of 50 pmol of purified peptide were oxidized in 100 μl of pure H_2O_2 for 1 h. Then, the oxidized peptide was separated by reverse-phase HPLC.

2.6. Structure determination

Automated Edman degradation of each purified peptide and detection of phenylthiohydantoin (PTH-Xaa) derivatives were performed on a pulse liquid automatic sequentator (Applied Biosystems, model 473A).

The molecular mass of each peptides was determined by electrospray mass spectrometry (ESMS). The purified peptide was dissolved in water/methanol (50/50, v/v) containing 1% acetic acid and analyzed on a VG Biotech BioQ mass spectrometer (Manchester). This instrument consists of an electrostatic ion-spray source operating at atmospheric pressure, followed by a quadrupole mass analyzer with a mass range of 1–4000. The extraction cone voltage value was 55 V. Scanning was performed from m/z = 500–1500 for 10 s, with the resolution adjusted so that the m/z = 998 peak from horse myoglobin was 1.5–1.7 Da wide at its base. The data system was operated as a multi-channel analyzer and several scans were summed to obtain the final spectrum. Each molecular species produced a series of multiply charged protonated molecular ions from which the molecular mass was determined by simple calculation. Calibration was performed using the multiply charged ions from a separate introduction of horse heart myoglobin (16,951.4 Da). Molecular masses are given as average values based on the atomic weights of the elements (C = 12.011, H = 1.00794, N = 14.0067, O = 15.9994 and S = 32.06); only average masses were measured.

3. Results

3.1. Isolation of enkephalin-like peptides

1000 brains of *T. tessulatum* were subjected to peptide extraction in 1 M acetic acid, pH 2. ELISA of crude extract revealed ca. 7 pmol of Leu-enk/brain and ca. 4 pmol of Met-enk/brain. The crude extract was prepurified using Sep-Pak C_{18} cartridges. The fraction eluted by 50% acetonitrile containing the enkephalin-like material was reduced 20-fold by freeze-drying and applied to a HPGPC column. The eluted fractions obtained were tested in DIA and revealed a single immunoreactive zone of both a-Met-enk and a-Leu-enk corresponding to peptides with molecular masses of ~0.2–1 kDa (Fig. 1). An amount of 5.35 ± 0.35 pmol of Leu-enk/brain (recovery of ca. 84%) and of 3.05 ± 0.55 pmol of Met-enk/brain (recovery of ca. 82%) was obtained at this step 2 of the purification. Results obtained after preadsorption of the antisera by synthetic enkephalins established the specificity of the immunodetection. The immunoreactive fractions were then concentrated 20-fold and applied to a reverse-phase HPLC.

In the first step of reverse-phase HPLC on a Vydac C_{18} column, Met-enk-like substances eluted in several fractions with R_T ranging from 13–14 min and 21–22 min and Leu-enk-like substances between 13–14 min and 23–24 min (Fig. 2). Under these conditions vertebrate Met-enk eluted at a R_T of 21.8 min and Leu-enk at a R_T of 23.3 min. In the second step of reverse-phase HPLC, conducted on each immunoreactive fraction, Met-enk-like material eluted as two sharp peaks at a R_T of, respectively, 13.25 and 23.4 min and Leu-enk-like material at a R_T of, respectively, 14.5 min and 25.4 min. The final step of purification was performed on an ODS C_{18} reverse-phase column which gave four homogeneous peaks, designated P1–P4, at R_T, respectively, of 19.87, 20.37 min, 24.6 min and 26.22 min.

3.2. Characterization of the enkephalin-like peptides

A total of four immunoreactive peptides, designated P1–P4, were isolated in this study.

Given that peptides P3 and P4 co-eluted in reverse-phase HPLC with, respectively, Met-enk and Leu-enk, experiments were conducted in order to check that P3 and P4 corresponded effectively to these two pentapeptides. As Met-enk contained a methionine, the oxidized form of Met-enk [M(o)-enk], is more hydrophilic than Met-enk and thus elutes earlier than Met-enk. P1–P4 were oxidized. Separation of P1, P2, P3 and P4 on a Vydac column showed that P1 and P3 gave two peaks shifted to an earlier time (17.35 min vs. 19.87 min for P1 and 22. 4 min vs. 24.6 for P3) whereas P2 and P4 did not shift. These results suggested the existence of a methionine amino acid residue only in P1 and P3. In order to confirm such a hypothesis, each purified peptide was subjected to automated Edman degradation followed by ESMS. The amino acid sequences of P1–P4 determined by automated Edman degradation are Phe-Met for P1, Phe-Leu for P2, Tyr-Gly-Gly-Phe-Met for P3 and Tyr-Gly-Gly-Phe-Leu for P4 (Table 2). The sequences obtained were

Table 1
Purification of enkephalins in the brain of the leech *Theromyzon tessulatum*

Step	Column material	Elution conditions (acetonitrile)	Yield (%)
1 Solid phase extraction	Sep-Pak C_{18} cartridges	50%	100
2 HPGPC	Ultraspherogel, 7.5 × 300 nm	30%	83
3 Reverse-phase HPLC	Vydac C_{18}, 4.6 × 250 nm	0–15% (10 min) 15–45% (30 min)	75
4 Reverse-phase HPLC	Vydac C_{18}, 4.6 × 250 nm	0–15% (10 min) 15–45% (40 min)	70
5 Final purification	Ultrasphere C_{18}, 2 × 250 nm	0–60% (60 min)	62

Experimental details are indicated in section 2

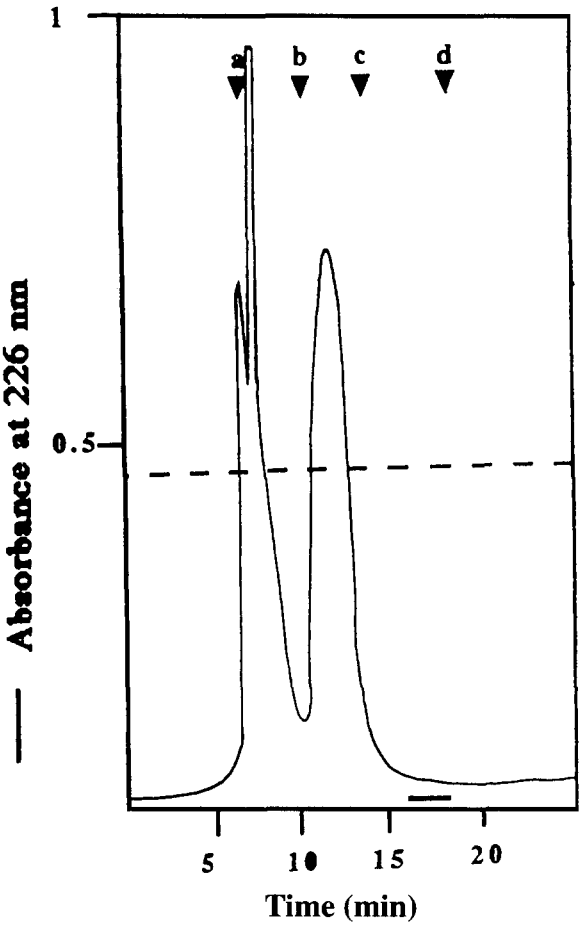

Fig. 1. HPGPC elution profile of an acidic extract of 1000 brains from *T. tessulatum*. Arrowheads indicate the elution position of standards (a, hirudin (7 kDa); b, adrenocorticotropic hormone (4.5 kDa); c, angiotensin II (1.0 kDa); d, tryptophan (0.20 kDa)). The black bar indicates the zone immunoreactive to enkephalin antisera. The dotted lines indicate the isocratic gradient (30% acetonitrile).

confirmed by mass spectrometry analyses. The molecular masses of P1–P4 measured by ESMS yielded a m/z of 164.8 ± 0.2 Da for P1, 278.15 ± 0.3 Da for P2, 573.4 ± 0.33 Da for P3 and 555.5 ± 0.6 Da for P4. These different m/z are in good agreement with the calculated mono-isotopic mass of FM (165.04 Da), FL (278.36 Da), YGGFM (573.2 Da) and YGGFL (555.2 Da). Moreover, co-injection in reverse-phase HPLC of either P3 and synthetic Met-enk or of P4 and synthetic Leu-enk indicated that in both cases a single peak was eluted.

These results established the presence of a methionine-enkephalin (P3), leucine-enkephalin (P4) and two dipeptides FM (P1) and FL (P2) in the brain of the leech *T. tessulatum*. The amount of these peptide estimated at the final step of purification is ca. 0.70 for P1, ca. 0.45 for P2, ca. 1.5 for P3 and ca 3.5 pmol/brain for P4 (Table 3).

4. Discussion

After reverse-phase HPLC purification, the sequence of the enkephalin-like peptides was established by a combination of automated Edman degradation, electrospray mass spectrometry measurement, and co-elution experiments in reverse-phase HPLC with synthetic peptides. Our data show that Leu-enk and Met-enk from the leech *T. tessulatum* are structurally identical to those of vertebrates. Similar results were previously obtained by Leung and Stefano [7] in the mollusc *Mytilus edulis* and by Luschen et al. [8] in the crustacean *Carcinus maenas* L. However, by the isolation of these enkephalins in leeches, we establish the existence of opioids in the oldest group of coelomate metazoans, the Annelida. We could postulate that enkephalins have a very ancient phylogenetic origin and are well conserved during evolution. These results lead to two central questions: what was the ancestral opioid gene? What are the opioid precursors in leeches?

4.1. Phylogeny of enkephalins in leeches

In vertebrates, opioids are derived from three different large precursor molecules, i.e. the proenkephalin (Proenk), the

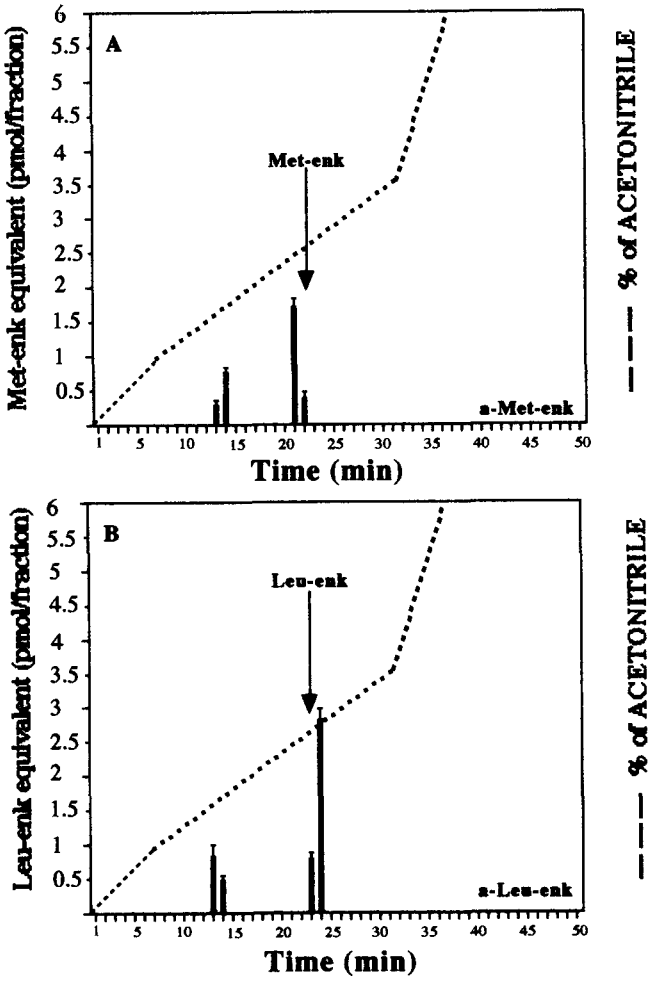

Fig. 2. Reverse-phase HPLC separation of the zone immunoreactive to the enkephalin antisera previously detected after separation of an acidic extract of *T. tessulatum* brains in HPGPC. (A) The filled bars indicate the material immunoreactive to the anti-Met-enkephalin; (B) the filled bars indicate the material immunoreactive to the anti-Leu-enkephalin. Each zone containing the immunoreactive material was detected by ELISA. Values given for each assayed fraction correspond to aliquots of 1 μl. Arrows indicate the eluted position of synthetic Met-enkephalin (Met-enk) and synthetic Leu-enkephalin (Leu-enk). The acetonitrile/water gradient is shown by a dashed line (see section 2 for conditions).

Table 2
Automated Edman degradation of enkephalins (P1–P4)

Cycle no.	Amino acid sequences							
	P1		P2		P3		P4	
	PTH-AA	Yield (pmol)	PTH-AA	Yield (pmol)	PTH-AA	Yield (pmol)	PTH-AA	Yield (pmol)
1	F	43	F	30	Y	1556	Y	2683
2	M	21	L	27	G	1763	G	3770
3					G	1868	G	4144
4					F	1811	F	2020
5					M	985	L	1982

prodynorphin (Prodyn) and the POMC [21]. Cleavage of these precursors gives, respectively, the enkephalins, the dynorphins and the endorphins [21]. The mammalian Proenk contains four copies of Met-enk, one of Leu-enk and two Met-enk extended (Met-enk-RF (YGGFMRF) and Met-enk-RGL (YGGFMRGL)) [22]. Such an organisation has also been found with little variation in other vertebrates phyla (for a review see [23]). In invertebrates, the existence of an opioid precursor(s) is still unknown. Additionally, it is unknown whether invertebrates possess opioid peptides other than enkephalins [24].

In the leech *T. tessulatum*, immunocytochemical studies performed at the level of the brain revealed that Leu-enk and Met-enk are expressed in different neurons [14]. These results suggest: (i) a genic organisation different from that found in mammals, with the presence of two separated genes, one coding for the Met-enk and the other one for Leu-enk; or (ii) if we consider the hypothesis of the existence of an ancestral Proenk in invertebrates, these two pentapeptides could result from a post-translational processing mechanism where the Leu-enk is not liberated from cells expressing Met-enk and vice versa.

The first hypothesis is sustained by results obtained in the agnata *Ichthyomyzon castanaus* and *Petromyzon marinus* [25]: the authors demonstrated Met-enk but not Leu-enk in pars intermedia. Theses results were interpreted by the authors as (i) the result of a unique post-translational processing mechanisms where the Leu-enk sequence is not liberated from the Proenk in any of these fish; or (ii) the Proenk is relatively simple and contains only Met-enk sequences but no copy of the Leu-enk sequence [25]. Furthermore data obtained in the anuran *Xenopus laevis* have revealed that a relatively simple organization of the Proenk provides the more likely explanation. In this species, the Proenk gene was isolated [26] and the single copy of the Leu-enk sequence found to be replaced by a Met-enk sequence [26]. Moreover, Leu-enk as well as the Prodyn end-products (α-neo-endorphin and dynorphin A (1–8)) have been detected in *X. laevis* brain [27]. However, dynorphin (1–17) and dynorphin B were not immunologically detected [27]. These data are in favor of the conversion of some *Xenopus* Prodyn products into Leu-enk and establish the existence of two different genes coding for enkephalins in amphibians [28].

Table 3
Amounts of leucine-enkephalin or methionine-enkephalin immunoreactive material after Sep-Pak C_{18} prepurification and of each identified enkephalin-like peptides (P1–P4) isolated at the final step of purification

	Amount (pmol/brain)
Prepurification step	
Leu-enk-like immunoreactivity	6.32 ± 1.25
Met-enk-like immunoreactivity	3.75 ± 0.95
Final purification step	
Peptide	
FM (P1)	0.69 ± 0.36
FL (P2)	0.45 ± 0.28
YGGFM (P3)	1.32 ± 0.45
YGGFL (P4)	3.49 ± 0.55

Values are expressed as a mean ± S.D. (from four determinations).

In invertebrates, detection by immunocytochemistry of the Prodyn end-products left no doubt that Proenk is not the only source of Leu-enk in neural tissue: Leu-enk could also derive from a Prodyn [29]. Results obtained in the crustacean *Carcinus maenas* L. are in line with this hypothesis. The molar ratio of Leu-enk and Met-enk in crustacea is 3:1 [8]. This result proves, in contrast to what is found in mammals, that Leu-enk is the predominant enkephalin peptide in crustacea. ELISA titrations performed in *T. tessulatum* confirm this result. In leeches, enkephalins are present at a ratio of Leu-enk to Met-enk of 2:1. This value is strengthened by immunocytochemical studies revealing a much higher number of cells immunoreactive to a-Leu-enk than to a-Met-enk [14]. The whole of these results are reinforced by the specificity of the antisera used [18].

These data strongly suggest that, in the leech, Met-enk and Leu-enk are coded by two different genes. The existence of a Prodyn-related precursor is strongly suggested by immunocytochemical results [14]. Cells immunoreactive to antibodies specific to vertebrate Prodyn, i.e. anti-dynorphin and anti-α-neo-endorphin, were detected in *T. tessulatum* brain [14]. Moreover, recently a peptide present in vertebrates on an opioid precursor of ca. 60 kDa, the pro-opiomelanocortin (POMC), has been isolated from *T. tessulatum* brains. This peptide is related to γ-MSH: it has ca. 80% sequence homology to vertebrate γ-MSH [30]. However, in leeches no POMC was detected. Western blot analyses performed in *T. tessulatum* have revealed the existence of a multipeptidic precursor of ca. 19 kDa. This protein possesses at least three epitopes recognized by two different polyclonal antisera (an anti-γ-MSH and an anti-angiotensin II) and by a monoclonal antibody (Tt159) specific for epitopes contained in *T. tessulatum* supraesophageal ganglia extracts [31].

These observations in leech allow us to postulate that enkephalins are the most ancient class of opioids and to propose a form of the Proenk gene as the ancestral opioid gene. This ancestral opioid gene would have been subjected to a first duplication, which would have occurred before or in annelids, to give rise to a Proenk and a Prodyn gene. Nevertheless, to

establish the truth of these suggestions in invertebrates, the isolation of peptides related to the Prodyn end-products and a molecular study of opioid precursors in leeches have to be undertaken.

4.2. Enkephalin peptidases

In parallel with the purification of these two pentapeptides, two dipeptides FM and FL were isolated. The presence of the dipeptides FL and FM could be due to proteolytic action of peptidases. Recently, Leung et al. [32] demonstrated Met-enk peptidases in the hemolymph of the mollusc *M. edulis*. As suggested by the authors, the presence of the dipeptide FM could result from the specific action of an angiotensin-converting enzyme (ACE) in *M. edulis* hemolymph. Such a hypothesis could be sustained in the case of the leech *T. tessulatum*. Indeed, an angiotensin II-like molecule close to vertebrate angiotensin II has been isolated in the brain of leech [31]. However, the existence of a neutral endopeptidase or a carboxypeptidase which cleaves Met-enk and/or Leu-enk, can not be excluded. These results suggest the presence of enkephalin peptidases in leech. The validity of these suggestions requires further experimental testing.

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