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Isolation and structural characterization of a novel peptide related to γ-melanocyte stimulating hormone from the brain of the leech *Theromyzon tessulatum*

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Abstract

This paper reports the purification of a novel pro-opiomelanocortin derivative peptide (a γ-melanocyte stimulating hormone-like (γ-MSH-like) molecule) from the brain of the leech *Theromyzon tessulatum*. After reverse-phase HPLC purification, the sequence of the γ-MSH-like peptide (YVMGHFRWDKFamide) was established by a combination of automated Edman degradation, electrospray mass spectrometry measurement, enzymatic treatment and co-elution experiments in reverse-phase HPLC with synthetic peptides.

Key words: Leech; Neuropeptide; γ-Melanocyte stimulating hormone-like peptide; Invertebrate; Evolution

1. Introduction

Processing of pro-opiomelanocortin (POMC) yields a number of bioactive peptides including adrenocorticotropin, lipotropins, β-endorphin and melanotropins (α-, β- and γ-MSH) [1]. These peptides have been isolated throughout vertebrates [2–4]. The POMC cDNa and gene have been characterized in a variety of mammals (bovine, murine and rat), including human [5–9].

In contrast, no peptide belonging to the POMC family has so far been isolated in an invertebrate. However, it has been shown in the molluscs *Aplysia californica* and *Lymnaea stagnalis* that the deduced peptide sequence from the isolated FMRF gene [10,11] contains, besides a series of FMRFamides and FLRFamides, other sequences exhibiting ca. 30% sequence homologies to MSH, corticotropin releasing factor (CRF) and corticotropin-like intermediary pituitary peptide (CLIP). Comparable results were obtained in the insect *Drosophila* where the FMRF gene exhibits ca. 30% sequence homology with mammalian CRF [12].

In annelids, cells immunoreactive to an antiserum raised against γ-MSH (a-γ-MSH) have been detected in the brain of the leech *Theromyzon tessulatum* [13,14]. A category of these immunoreactive cells, the β giant cells, also immunoreacted positively with a polyclonal antiserum directed against angiotensin II (a-AII) and with a monoclonal antibody (Tt159) specific for epitopes contained in *T. tessulatum* supraesophageal ganglia extracts [14]. The three epitopes recognized by a-γ-MSH, a-AII and Tt159 in the β giant cells are different [14] and borne on the same multipeptidic protein of ca. 19 kDa [15]. One of the three epitopes present in this protein, the angiotensin II-like one, has recently been isolated [15]. We now report the full characterization in an invertebrate of a peptide of the melanotropin family: the γ-MSH-like peptide of *T. tessulatum*.

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. [16], were used in this study.

2.2. Antiserum

A rabbit polyclonal antiserum specifically directed against the carboxy-terminus of mammalian γ-MSH (a-γ-MSH) and kindly provided by Dr. G. Tramu (Laboratoire de Neurocytochimie fonctionnelle, Université de Bordeaux I, Talence, France), was used for immunoassays.

2.3. Immunoassays

Enzyme-linked immunosorbent assays (ELISA) and dot immunobinding assays (DIA) were conducted according to Salzet et al. [17,18] with a γ-MSH employed at a dilution of 1:1,000. As a control, preadsorption of γ-MSH was carried out using an homologous peptide. Prior to ELISA and DIA, a-γ-MSH, at its working dilution, was incubated overnight at 4°C with synthetic γ-MSH (Sigma) (100 μg/ml undiluted a-γ-MSH).

2.4. Purification

After anaesthesia of the animals in 0.01% chloretone, 4,000 brains were excised, immediately frozen in liquid nitrogen and stored at −70°C until use. Batches of 400 brains were homogenized and extracted with 400 μl 1 M acetic acid at 4°C. After centrifugation at 12,000 rpm for

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Abbreviations: DIA, dot immunobinding assay; ESMS, electrospray mass spectrometry; HPLC, high performance liquid chromatography; MSH, melanocyte stimulating hormone; R_T, retention time; TFA, trifluoroacetic acid.

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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 water acidified with 0.1% trifluoroacetic acid (TFA; Pierce). The eluted fractions were reduced 20-fold in a vacuum centrifuge (Savant) to remove acid and organic solvent. The total amount of γ-MSH-like material was quantified using ELISA. The 50% eluted fraction was made up to 250 μl with acidified water (0.1% TFA) and fractionated 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 effluent was monitored by absorbance at 226 nm and the presence of γ-MSH-like material detected on aliquots of each fraction by DIA. The fractions that contained the immunoreactive material were applied on the same column with a shallower gradient. Elution was performed with a linear gradient of 20–40% acetonitrile in acidified water for 20 min at a flow rate of 1 ml/min. After a 20-fold concentration by freeze drying, fraction aliquots of 0.5 μl were tested by DIA. The γ-MSH-like material was finally purified on an ODS C_{18} reverse-phase column (Ultrasphere, 250 × 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 MSH peptides (α, β, γ) purchased from Sigma and with synthetic carboxy-terminus amidated leech γ-MSH-like peptide purchased from Neosystem.

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

2.5. Structure determination

Prior to microsequencing, the purity of the peptide was controlled by capillary electrophoresis. Separation was performed on an Applied Biosystems model 270A-HT capillary electrophoresis system. Silica capillary (72 cm length) was used. Under these conditions, separation was achieved from anode to cathode in a citrate buffer (20 mM) at pH 2.5. Detection was at 200 nm, temperature 30°C and the volume injected 2 nl.

Automated Edman degradation of the purified peptide and detection of phenylthiohydantoin (PTH-Xaa) derivatives were performed on a pulse liquid automatic sequenator (Applied Biosystems, model 473A). The molecular mass was determined by electrospray mass spectrometry (ESMS) using a VG Biotech Bio-Q mass spectrometer (Manchester) as described elsewhere [18]. The presence of carboxy-terminus amidation was researched by carboxypeptidase A treatment under the conditions described by the manufacturer (Boehringer-Mannheim).

3. Results and discussion

3.1. Isolation of the γ-MSH-like peptide

4,000 brains were subjected to peptide extraction in 1 M acetic acid pH 2. ELISA revealed 20.3 ± 4.8 fmol γ-MSH-like material/brain in the crude extract (prepurified using Sep-Pak C_{18} cartridges). The fraction eluted by 50% acetonitrile was reduced 20-fold by freeze drying and applied to a C_{18} peptide protein column. Eluted fractions tested in DIA revealed an immunoreactive zone at a retention time (R_T) between 27 and 28 min (corresponding to 32–33% of acetonitrile) (Fig. 1). The total amount of γ-MSH-like material determined by ELISA at this step of the purification was 15.7 ± 3.1 fmol/brain (recovery ca. 78% of the starting material). Results obtained after preadsorption of a-γ-MSH by synthetic γ-MSH established the specificity of the immunodetection.

The immunoreactive zone containing the γ-MSH-like material was analyzed on the same column with a more resolutive gradient. A peak immunoreactive to a-γ-MSH, with a R_T between 13 and 13.4 min (corresponding to 26–26.8% acetonitrile), was resolved. At this step of the purification, quantification by ELISA indicated 12.3 ± 4.6 fmol γ-MSH-like material/brain (recovery ca. 61% of the starting material). This peak was then purified to homogeneity on an ODS C_{18} reverse-phase column and gave at a R_T of 38.8 min. The purity of the immunoreactive material was established by capillary electrophoresis (Fig. 2). Quantification by ELISA at this step of the purification indicated 11.6 ± 2.3 fmol γ-MSH-like material/brain (final recovery of ca. 57%).

Co-injection in an ODS C_{18} reverse-phase HPLC column of the purified γ-MSH-like peptide with a synthetic vertebrate melanotropin (α-, β- or γ-MSH) revealed, after elution, two peaks, whatever the vertebrate melanotropin co-injected. This result suggested that the leech γ-MSH-like peptide possesses a structure different from the ones of α-, β- and γ-MSH.

3.2. Characterization of the γ-MSH-like peptide

The amino acid sequence determinated by automated Edman degradation of the *T. tessulatum* γ-MSH-like peptide is: Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Lys-Phe. The two following cycles (12 and 13) did not reveal any further PTH-Xaa signals, which indicated the end of the peptide.

The molecular mass of the γ-MSH-like peptide measured by ESMS (m/z = 1484.2) differed by 1 Da from the monoisotopic molecular mass (1485.7 Da) calculated from the amino acid sequence determined by Edman degradation. This result predicted that the leech γ-MSH-like peptide possesses an amidated carboxy-terminus, the calculated monoisotopic molecular mass of γ-MSHamide-like peptide being 1484.7 Da. In order to determine whether the carboxy-terminus of the peptide is blocked by amidation, treatment of the purified γ-MSH-like peptide with carboxypeptidase A was performed, but it did not affect the retention in reversed-phase HPLC. Moreover, co-injection in reverse-phase HPLC of both the puri-

Table 1
A comparison of the sequences of *T. tessulatum* γ-MSH-like peptide and γ_1-MSH of different species of vertebrates

Species	Name	Structure (1 5 10)	References
Mammal			
Human	γ_1-MSH	YVMGHFRWDRF (NH_2)	[20]
Amphibians			
Xenopus laevis	γ_1-MSH	YVMTHFRWNKF (NH_2)	[4]
Rana ridibunda	γ_1-MSH	KYVMSHFRWDKF (NH_2)	[2]
Leech			
T. tessulatum	γ-MSH-like	YVMGHFRWDKF (NH_2)	

Amino acids underlined represent those differing from the leech γ-MSH-like sequence

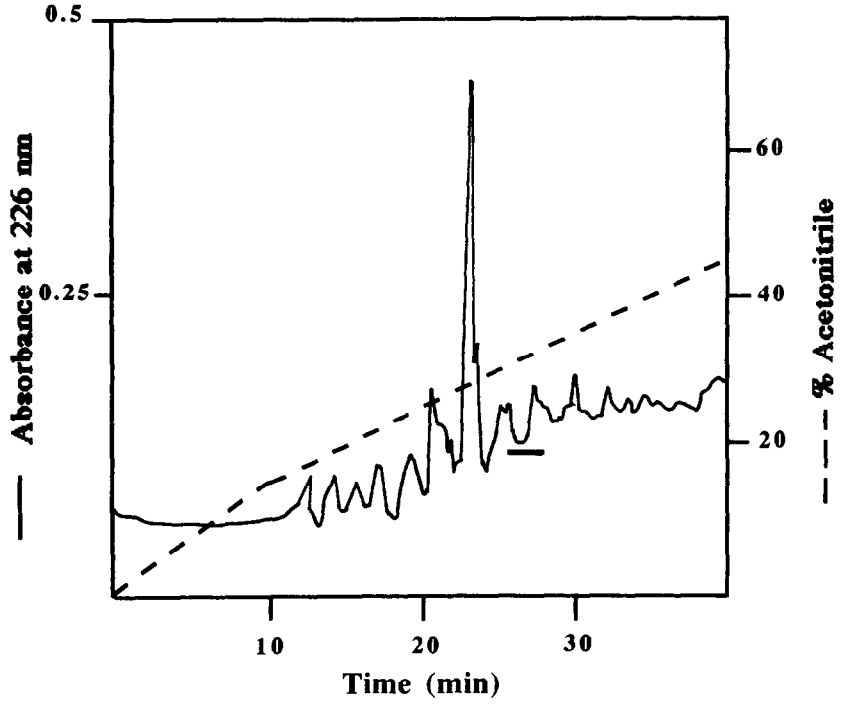

Fig. 1. Separation of a brain extract of *T. tessulatum* by reverse-phase HPLC. The solid bar indicates the material immunoreactive to a-γ-MSH.

fied and the synthetic carboxy-terminus amidated γ-MSH-like peptides indicated that a single peak was eluted at 38.2 min. These results were consistent with the following structure for the *T. tessulatum* γ-MSH-like peptide: Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Lys-Pheamide.

The characterization of a γ-MSH-like peptide in the leech *T. tessulatum* constitutes the first report on the presence of a peptide of the melanotropin family in the brain of an invertebrate. Indeed, so far in invertebrates, only a partial purification of an α-MSH-like peptide in the insect *Locusta migratoria* [19] has been reported.

The sequence of *T. tessulatum* γ-MSH-like peptide is close to the γ_1-MSH peptides isolated throughout the animal kingdom. A sequence alignment of the different γ_1-MSH peptides with *T. tessulatum* γ-MSH-like peptide (Table 1) indicated that the γ-MSH-like peptide differs from human γ_1-MSH [20] by only one amino acid residue at position 10 (Lys residue in *T. tessulatum* γ-MSH-like peptide and Arg in human γ_1-MSH) and from amphibians (*Rana ridibunda* and *Xenopus laevis*) γ_1-MSH by two residues. In *Rana ridibunda* [2], the γ_1-MSH has a NH_2-terminus extended by a Lys residue and a Gly at position 4. In *Xenopus laevis* [4], the γ_1-MSH has two amino acid residues different from the *T. tessulatum* γ-MSH-like peptide (Thr-4 and Asn-9 for the γ_1-MSH, a Gly-4 and Asp-9 for the γ-MSH-like peptide). The data deduced from molecular biology experiments (Table 2) have confirmed biochemical results obtained in human [20] and amphibians [21]. Moreover, in rat (Table 2), at the level of the POMC cDNA, the nucleic acid sequence coding for the γ_1-MSH has been found, although a γ_1-MSH peptide has not been biochemically isolated [23]. This result is explained by the fact that in rat, at the level of the POMC, the γ_1-MSH is not flanked by pairs of dibasic amino acid residues which represent potential sites of proteolytic cleavage by processing enzymes. On the other hand, in fishes (salmon and trout), a γ_1-MSH would not exist as the isolation of POMC cDNA revealed (Table 2) a gap in the nucleic acid sequence coding for the γ_1-MSH [24,25].

It thus seems that although it does not exist in fishes, the γ-MSH has been well conserved in the course of evolution. Nevertheless, the structure of POMC, the precursor of the γ-MSH, seems to have changed in the course of evolution. In invertebrates, the existence of POMC has yet to be demonstrated. In the leech *T. tessulatum*, the γ-MSH-like peptide would be localized on a

Table 2
Alignment of the γ_1-MSH derived-peptides deduced from POMC cDNA isolated from different species of vertebrates

Species	Sequence	References
Human	N P K R Y V M G H F R W D R F G R R N S	[21]
Rat	N P K R Y V M G H F R W D R F G P R N S	[23]
Xenopus	S I R K Y V M T H F R W N K F G R R N S	[22]
Salmon, trout	S --------- gap ---------- >	[24,25]

The dibasic amino acid residues underlined represent potential sites of proteolytic cleavage by processing enzymes; gap, no amino acid residues corresponding to a γ-MSH were found in sequences deduced from cDNA sequences coding for POMC.

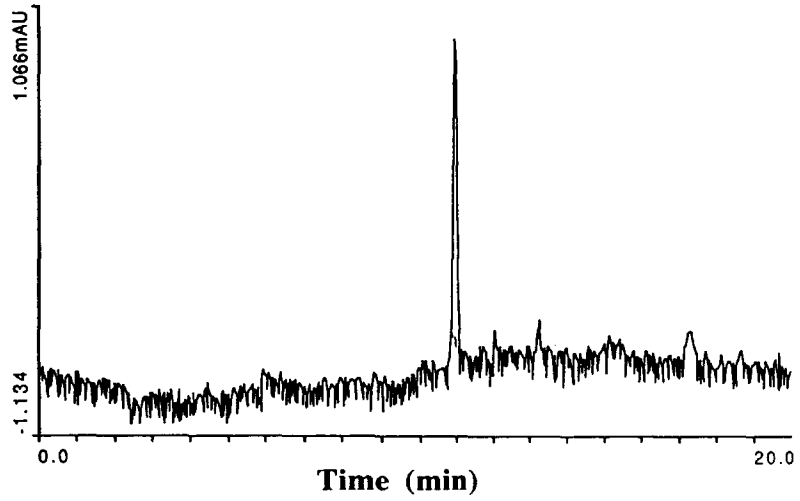

Fig. 2. Capillary electrophoresis of purified *T. tessulatum* γ-MSH-like peptide. Analyses were performed on a 72 cm capillary in 20 mM citrate buffer at pH 2.5.

precursor which bears other peptides [15]. One of these peptides, the angiotensin IIamide, has recently been isolated in leeches (unpublished data). These results favor the idea that the POMC-like protein in leeches would be different to the one isolated in vertebrates.

3.3. Physiological function

The physiological function of the leech cerebral γ-MSH-like peptide remains to be determined. However, an involvement of this peptide in the regulation of the color change can be suggested. Indeed, control by the central nervous system of this phenomenon in leeches has been established [26–29]. Moreover, in vertebrates the overall role of γ-MSH peptides in the regulation of pigmentation, although small, exists [30–32]. It has been suggested that selected γ-MSH peptides could modulate pigmentation by modifying the cellular response to other melanotropins [33]. As the structure of the γ-MSH-like peptide is close to that of vertebrate γ_1-MSH, we suspect that this peptide has conserved its biological function in the course of evolution.

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