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ORIGINAL ARTICLE

Chromacin-like peptide in leeches

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Abstract

We demonstrate the presence in leech hemolymph of high levels of a peptide recognized by antiserum directed against bovine chromacin. The purification of the chromacin-like peptide was carried out by an acidic extraction, followed by solid phase and high pressure gel permeation chromatography and reversed-phase HPLC purification. Its sequence (GDFELPSIADPQATFESQRGPSAQQVDK) was established by a combination of techniques, including automated Edman degradation, MALDI-TOF measurement and DOT immunobinding assays with anti-chromogranin A. Mass spectrometry measurement revealed a m/z 3177Da, revealing the fact that the molecule is phosphorylated. ELISA titrations performed at each step of the purification revealed a major increase in the level of the peptide (ca. 125 nmol/μl of coelomic fluid) 15 min after LPS exposure. The increase in chromacin-like peptide levels is both time and concentration dependent. The level of this peptide decreased significantly 4 hours after LPS exposure. This report is the first discovery of a chromogranin derived like peptide in invertebrates.

The abbreviations used are:

a-CGA	anti-chromogranin A;
a-CGB	anti-chromogranin B,
DIA	dot immunobinding assay;
ELISA	enzyme-linked immunosorbent assays;
LPS	lipopolysaccharides;
MALDI-TOF	matrix assisted laser desorption/ionisation time of flight
HPLC	high performance liquid chromatography;
HPGPC	high performance gel permeation chromatography; R_T

Introduction

In mammals, opioid peptides have been implicated in neural, neuroimmune and autoimmunoregulatory signaling [1, 12, 15]. These phenomena have been supported by studies documenting the presence of stereospecific opioid receptors on specific leukocytes, *i.e.*, granulocytes, and nerve cells [14, 15]. Additionally, these cells express the actual signaling molecules used for this chemical signaling, including the expression of mRNA [15]. On a functional level, with regard to immunocytes, opioid peptides have the ability to induce chemotaxis, as well as initiate the release of cytokines [13–15].

In invertebrates, these same signaling peptides induce chemotaxis and the release of mammalian-like cytokines [15]. Furthermore, invertebrate immunocytes contain both δ_1 and δ_2 opioid receptors, which also occur on human granulocytes, that appear to mediate these actions [13–15]. Recently, we isolated from leeches a proenkephalin precursor [10]. The structure of the leech proenkephalin material demonstrates considerable amino acid sequence similarity with amphibian proenkephalin (26.2%) [10]. The amount of leucine-enkephalin is identical to that found in vertebrates. Sequence comparison and a specific antiserum raised against bovine proenkephalin A (209–237), enkelytin, an antibacterial peptide [18], demonstrates this molecule is present with high sequence identity to that found in human [17, 19].

The presence of the extended form of enkelytin (and peptide B) in invertebrate proenkephalin, with a nearly perfect sequence match to that found in bovine chromaffin cells (98%) [2], further supports the hypothesis that these molecules first evolved in simpler animals [18]. Indeed, peptide B, with its high antibacterial activity, further associates opioid peptides with immune related activities. We surmise that immune or neural signaling may lead to enhanced proenkephalin proteolytic processing, freeing both opioid peptides and enkelytin/peptide B [18]. In this scenario, the opioid peptides would stimulate immunocyte chemotaxis and phagocytosis, as well as the secretion of cytokines [15]. During this process, the simultaneously liberated enkelytin/peptide B would attack bacteria immediately, allowing time for the immunocyte stimulating capabilities of the opioid peptides to manifest themselves. This hypothesis is further supported by the presence of specific met-enkephalin receptors on these cells [15]. Thus, it appears that many of the mammalian molecular and cellular survival strategies first appeared in organisms that evolved at least 500 million years ago. They may have evolved first to supplement immune actions, i.e., enkelytin/peptide B, by covering the latency period before total or partial immune activation occurs [6].

In order to establish this hypothesis, we isolate from leech coelomocytes several antibacterial peptides induced in the first 15 min after lipopolysaccharide (LPS) injection. Near the enkelytin, we characterized another mammalian chromaffin cell peptide that we also found in human immunocytes [19], a chromogranin-like derived peptide sharing 68% sequence homology with chromacin, an antimicrobial peptide coming from chromogranin A processing [16]. The increase of chromacin-like peptide levels is both time and concentration dependent.

Materials and methods

Animals

Rhynchobdellid leeches of the species *T. tessulatum* in stage 3 of their life cycle were used for these experiments. This stage corresponds to the gametogenesis phase which is characterized by an uptake of water, followed by water retention. The animals were reared under laboratory conditions as described in detail by Malecha *et al.* [5]. Hemolymph was collected by aspiration with a tuberculin syringe at room temperature at different times after lipopolysaccharide (LPS) or vehicle injection. Immunocytes were separated from the liquid hemolymph by centrifugation and washed in their respective saline. 1μM per animal of LPS was injected directly into the body of the animals and their hemolymph collected at 5 min intervals up to 4 hours. 100μM phosphoramidon and 100μM captopril were added in the collected samples before the biochemical purification. Surgical cuts in the integument of both animals were performed with a thin razor, and then the hemolymph was collected as above.

Antisera

Polyclonal antisera used in the ELISA, RIA and cytochemical procedures were kindly provided by Dr M.H. Metz-Boutigue (INSERM U338, Strasbourg, France). These rabbit antibodies recognize synthetic peptides corresponding to bovine enkelytin (PEA 220–237), and the chromogranin A derived peptide, the chromacin [17–19].

Immunoassays

Purification of leech antibacterial peptides was monitored by dot immunobinding (DIA) and enzyme linked immunosorbent assays (ELISA) using a panel of specific antisera [8, 9, 11]. For DIA, 1μl from each fraction collected during the purification steps was spotted onto a nitrocellulose membrane (0.45μm pore size, Schleicher and Schuell). Membranes were baked (30 min;110°C), blocked with 5% skim milk (in 50 mM PBS;150 mM NaCl, pH 7.4 including 0.05% Tween 20) to reduce background (1 hr under gentle agitation; room temperature) then incubated overnight at 4°C with the different primary antisera [diluted 1:1000

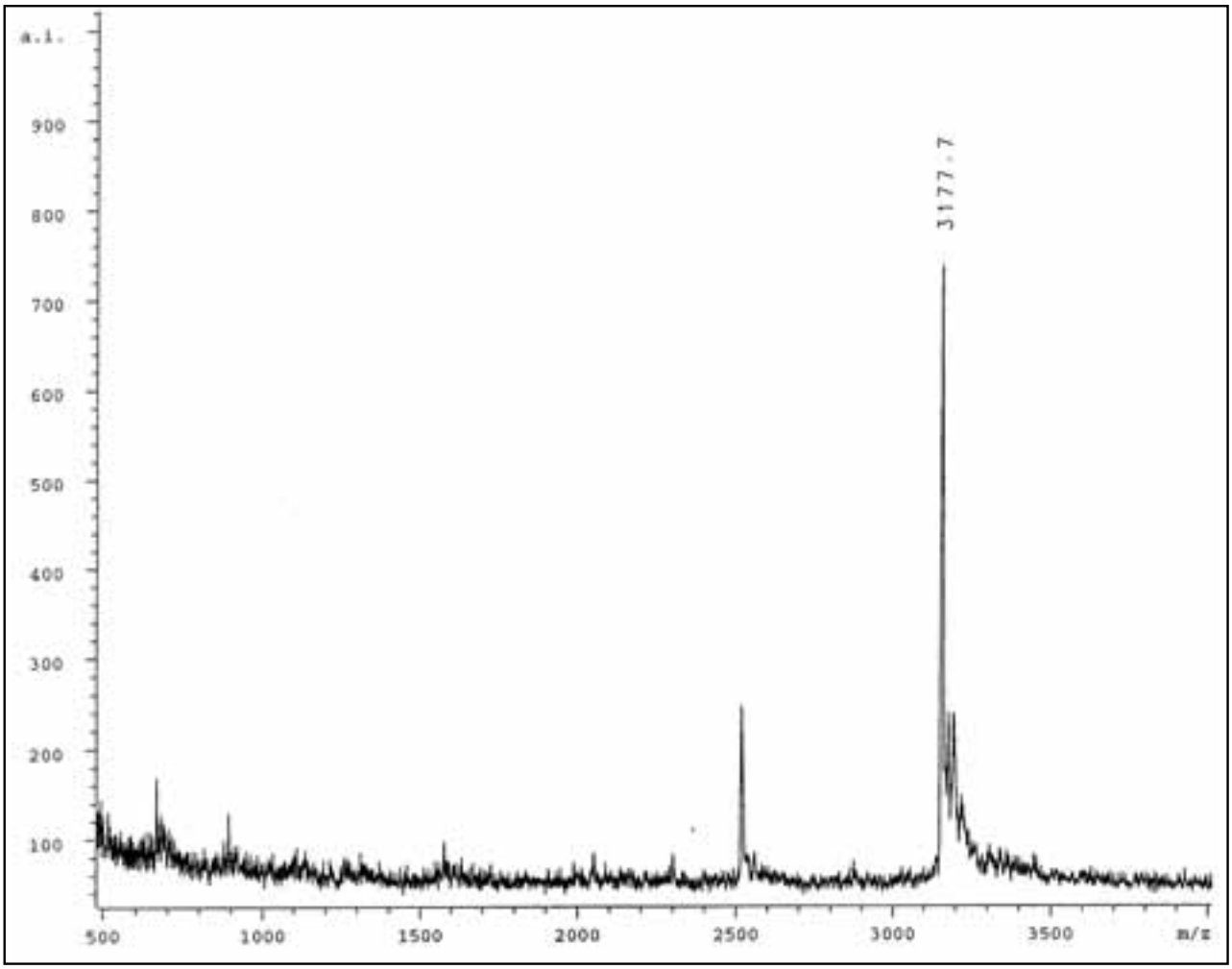

Figure 1. Maldi-MS measurement of the purified peptide. A peak at a MH+ of 3177.7 is observed, as well as salt adducts ($[M+Na]^+$, $[M+K]^+$, $[M-H^{+2\ Na}]^+$). The other peak at 2521 Da corresponds to a contaminant which is in low level because it was not detected during Edman degradation.

in PBS/0.05% Tween 20 (buffer A)]. Membranes were washed (4x5 min with buffer A); incubated for 1 hr with peroxidase-conjugated goat anti-rabbit IgG (1:1000 in buffer A); washed again and developed in DAB (45 mg of 3-3'-diaminobenzidine tetra-hydrochloride and 20 μl of hydrogen peroxide in 100 ml of buffer A). To further confirm that the isolated material was authentic, and not an epitope, prior to ELISA and DIA, the diluted antisera were incubated overnight at 4 °C with their homologous antigens (Sigma or Neosystem) (100 μg of synthetic peptide per ml of undiluted antiserum).

Purification

Batches of 5 ml of hemolymph collected from LPS exposed and control leeches were added to 5 ml of 1 M acetic acid at 4 °C. After centrifugation (12,000 rpm for 30 min at 4 °C), the pellet was discarded and the supernatant loaded onto Sep-Pak C_{18} cartridges (500 μl extract/cartridge; Waters). After washing the cartridges with 5 ml 1 M acetic acid, an 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 immunoreactive material was checked before being quantified by ELISA. The 50% eluted fraction was taken up in 250 μl of acidified water (0.1% TFA) and fractionated on a HPGPC column (Ultraspherogel, 7,5 x 300 mm, SEC2000, Beckman). These samples were then eluted with 30% acetonitrile at a flow rate of 500 μl/min.

Fractions immunoreactive in DIA were concentrated before being separated on a C_8–RP100 Lichrosphere column (125 mm x 4.6 mm, Waters; equilibrated with acidified water, 0.1% TFA). Elution was performed with a linear gradient (1%/min) of acetonitrile in acidified water at a flow rate of 1 ml/min. The column effluent was monitored by absorbance at 226 nm and the presence of the immunoreactive material was determined on aliquots of each fraction by DIA. The fractions that contained the immunoreactive material were analyzed on a narrowbore column (250 x 2 mm, Backman) with a shallower gradient of acetonitrile (0.5% /min) at a flow rate of 200 μl/min. After concentrating the material by freeze drying, fraction aliquots of 0.5 μl were tested by DIA and antibacterial activity. The purity of the CGA-like derived peptide was finally checked by capillary zonal electrophoresis. Samples (2 nl) were injected under vacuum into a PACE 5000 capillary electrophoresis system (Beckman) equipped with a fused silica capillary (72 cm length). Separation from anode to cathode was carried out in 20 mM citrate buffer (pH

2.5) with a voltage of 20 kV, at 30 °C. The capillary effluent was monitored by absorption at 200 nm.

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

Structure determination

Automated Edman degradation of the purified peptide and the detection of the phenylthiohydantoin (PTH-Xaa) derivatives were performed on a pulse liquid automatic sequencer (Applied Biosystem, 473A). The purified peptide (1 μl) was deposited on a thin layer of α-cyano-4-hydroxycinnamic acid crystals made by the fast evaporation of a saturated solution in acetone. The droplet was allowed to dry under a gentle vacuum before introduction into the mass spectrometer. MALDI-MS measurement was performed in a Bruker BIFLEX™ (Bruker, Bremen, Germany) operating in the positive linear mode. Ions were formed by a pulse ultraviolet laser beam (nitrogen laser, $\lambda = 337$ nm). Mass spectra were obtained by averaging 50–100 laser shots. External mass calibration was provided by the $[M + H]^+$ ion of angiotensin II (1047.20 Da), ACTH (18–39) (2466.73 Da), and bovine insulin (5734.56 Da) and/or using matrix peaks $[M + H]^+$: 190.05; $[2M + H]^+$: 379.09 Da).

Peptide Synthesis

Chromacin-like peptide was synthesized according to classical Fmoc chemistry on $_p$-alkoxybenzyl alcohol resin on a 25-μmol scale with a ABI 432 A. Conventional side chain-protecting groups were used 2,3, 5, 7, 8-Pentamethylchroman 6-sulfonyl (Arg), triphenylmethyl (Cys, Asn and Glu), *t*-butoxycarbonyl (Lys) and *t*-butyl (Ser and Tye). Briefly, a standard Fmoc deprotection was used in conjunction with benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate /*N*-hydroybenzotriazole /diisopropyethylamine. Coupling reactions were allowed to proceed for 15min. After two dimethylformamide washings, a second coupling with the same excess of reagents was routinely performed. At the end of the synthesis, the resin was washed with dichloromethane and ether and dried under nitrogen. The final trifluoroacetic acid cleavage was performed in the same reaction vessel with 5 ml of reacting buffer (100μl trisopropylsilane, 100μl ethanedithiol and 1.8 ml trifluoroacetic acid) during 150 min. At the end, the peptide was drained in a 40-ml polypropylene centrifuge tube previously filled with 25 ml of cold ether. The peptide was then centrifuged, and the pellet was washed twice with ether. After the last centrifugation, the pellet containing the reduced peptide was taken up in 0.1 M ammonium acetate buffer (pH 8.5) at a concentration of 35 mg/liter and was allowed to refold by air oxidation for 17 h at room temperature under constant stirring. The refolded peptide was purified by semi preparative reversed-phase chromatography (Aquapore RP300 column, 250 x 7.0 mm) with a linear gradient of 1% /min in acidified water (0.1%) at a flow rate of 1 ml/min.

Results

In vehicle treated leeches, the hemolymph was examined for the presence of the antibacterial peptides. In control animals, 5 ml of hemolymph subjected to an acetic acid extraction followed by ELISA titrations. ELISA revealed immunopositive materials to anti-CGA peptides. An amount of 8.63 ± 3.21 nM CGA-like peptide/µl of hemolymph was found (*Table 1*). In animals exposed to LPS (100 µM) the level of this material significantly increased in a time and concentration dependent manner (Tables 1, 2). For example, in the leech following the acid extraction and ELISA at 15 min 125 nmol/μl of CGA-like material was found in the hemolymph. To further substantiate the material as CGA-like peptide, the positive material was reduced 20-fold by freeze-drying and subjected to DIA with anti-CGA. This material was then applied to a HPGPC column. Again, the eluted fractions were tested in DIA. A single immunoreactive zone corresponding to peptides with a molecular mass of ~2.5–5 kDa was found. It contained 98 nM of CGA-like material/μl of hemolymph (recovery of 72.6%). The positive immunoreactive fractions were then concentrated and applied to a reversed-phase HPLC. In reversed-phase HPLC, the anti-CGA immunoreactive material (86 nmol/μl hemolymph) eluted from the column at an RT of 30.4 min (corresponding to 30.4% ACN, recovery 63.7%). After the second reversed-phase HPLC run, the CGA-

Table 1: Time course chromacin-like peptide amount titration by competitive ELISA after LPS injection (100 µM)

	Leech (nmol/µl)
Control	5.7 ± 2.7
5 min	32 ± 4.6
15 min	125 ± 6.7
30 min	145 ± 5.6
1 h	138 ± 7.4
2 h	121 ± 5.4
3 h	93 ± 2.7
4 h	85 ± 2.1

Table 2: Chromacin-like peptide amount titration after 15 min injection in function of the dose of LPS injected. (SD = 5)

Concentration LPS	**Leech** (nmol/µl of hemolymph)
100 nM	89 ± 9.6
1 µM	135 ± 10.6
10 µM	189 ± 12.6

Table 3: comparison between leech CGA derived peptide and CGA derived peptides isolated from vertebrates. In bold: Amino acid residue different in all sequence

Leech	DPQATFESQRGPSAQQ
Bovine CGA (193-207)	PQA**KE**DSE-GPS**QG**P
Human CGA (193-207)	PQA**EG**DSE-G**L**S**QGL**

like material eluted as a sharp peak at 13.32 min (corresponding to 35% ACN). An identity determination with capillary zonal electrophoresis confirmed that the isolated peptide was pure. The final amount of CGA-like material present was 74 nM per μl of hemolymph (recovery 54.8%; *Table 1*).

Characterization of the CGA-like peptide

A fraction aliquot of the immunoreactive purified material (20 pmol) was sequenced by Edman degradation (sequencing yield of 96%). Twenty amino acid residues were obtained. The invertebrate chromogranin A-like peptide possessed the following sequence GDFELPSIADPQATFESQRGPSAQQVDK that exhibited a 64.7% sequence identity with chromogranin A's derived peptides (*Table 3*), confirming the identity with antibacterial peptide isolated from bovine chromogranin A, the chromacin [16]. The molecular mass measured by MALDI-MS (m/z: 3177.44 Da; Fig. 1), is in agreement with the calculated isotopic mass (3017Da; average molecular mass of 3019.23) obtained from the amino acid sequence of a bi-phosphorylated peptide after Edman degradation like bovine chromacin [16].

Chromacin-like peptide physiological effects

A seen in *Table 1*, after LPS injection hemolymph levels of leech chromacin increased with time, peaking at 30 min, as we previously demonstrate with enkelytin/peptide B [18] To determine if the LPS induced response is specific, the animals were surgically cut. In animals receiving a surgical cut to the integument as opposed to scraping (gently), chromacin-like substance levels peaked at 15–30 min post injury (121 nM /μl of leech hemolymph) as also noted in the LPS experiments (*Table 1*). Because bovine chromacin is known to possess an antimicrobial activity, we test purified and synthesized leech chromacin according to the method we used for enkelytin/peptide B [18]. However, no antimicrobial activity was found for the leech chromacin suggesting a signaling role of this peptide like human antimicrobial peptides [6].

Discussion

We previously reported the immunohistochemical localization of chromogranin B like protein in leeches for the first time in invertebrates [7]. Here, we demonstrate in leeches a high amount of a peptide related to chromogranin A derived peptide, a chromacin-like substance. This chromacin-like peptide is released in the first 5 min of an injury (LPS, bacteria), before metabolized by endopeptidases such like angiotensin-converting enzyme and neutral endopeptidase [3, 4]. This peptide with others like the antimicrobial peptide, enkelytin/peptide B might be implicated in the stimulation of leech immune response as previously demonstrated [18]. These data allowed us to suggest that the precursors *i.e.* proenkephalin and chromogranins are present in epidermal cells of the tegument and immunocytes [18]. These neuropeptide precursors are already processed by prohormone convertases and are able to release immuno-modulators such like enkelytin/peptide B or chromacin-like peptide after aggressions.

In addition, we recently cloned cDNA fragment of prohormone convertases related to the SPC2 and SPC3 in leeches. Using an RT-PCR strategy based on the HGTRCAGE sequence conserved in all convertases in the animal kingdom and the sequence ALALEAN (near the serine residue of the active site), a 600 bp cDNA fragment covering the active site related to the SPC3 has been cloned (Vieau, unpublished data). Its deduced amino acid residue sequence

(hgtrcageevaaiannnkcgvgvaykakiggirmldgdvtdle aqslsfhiqhvdifsaswgptddgktldgpgtlaallmgiqqgrng sgtifvwasgnggrhldncncdgytnsmytlsissvtsaneqpe ylekcsstlattyssgedrnfqivttdlhnectdthtgtsasapma aaiialalqin)

presented 67% sequence homology with Aplysia SPC3. For the SPC2, a fragment of 180 bp situated at the C-terminal region of the enzyme has been obtained. The deduced sequence

(ylehvqafitlratrrgdvtihlvspmnttsmllgkrvdnnsksg ftkwpfmtthtw)

presents 64% sequence homology with Drosophila SPC2. These preliminary results reflect those prohormone enzymes able to process neuropeptide precursors are also present in leeches.

Taken together, we surmise that neuropeptide peptides like opioid or chromogranin derived peptide are able to stimulate immunocyte chemotaxis while simultaneously liberating neuropeptide antimicrobial derived molecules, attacking bacteria, and allowing time for the immunocyte stimulating capabilities of the signaling peptides to emerge. We also demonstrate that opioids and antibacterial peptides present in tegument cells and immunocytes are required after either surgical cuts or LPS injections. This reflects a high, early detection/surveillance system which would continuously monitor its status in regard to microbial penetration and growth. Hence, the association of antibacterial peptides and signaling peptides exists because both molecules evolved to fight the presence of microbes. In conclusion, in the first minutes of injury, neurosignaling peptides and antibacterial peptides participate in conjunction in order to block bacteria attack and stimulate chemotaxis and the cellular immune response.

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