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Characterization of a novel *Sepia officinalis* neuropeptide using MALDI-TOF MS and post-source decay analysis

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Abstract

A novel neuropeptide acting as a myosuppressor on esophagus, funnel and mantle muscular fibers has been isolated from the stellar ganglia of the mollusk cephalopod *Sepia officinalis* by means of HPLC analysis. Fractions were monitored using a myotropic bioassay. After three separation steps, MALDI-TOF spectrum revealed one main peak at m/z 756.6. The partial N-terminal and C-terminal digestions by exopeptidases followed by MALDI-TOF analysis allowed the determination of the nature of the two C-terminal and N-terminal amino acids. Post Source Decay fragmentation of the molecular ion accurately determined the following primary sequence: Val-Tyr-Ser-Ala-Pro-Tyr-Gly-OH. The mapping of this heptapeptide performed in ESI-MS revealed that its distribution is restricted to the stellar ganglia, the giant fibers III, and the nervous bundle containing the giant fibers II and the palleal nerve. The neuropeptide was not detected in the hemolymph suggesting a release by nerve endings next to the targets. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: MALDI-PSD-TOF MS; ESI-MS; HPLC; neuropeptide; Invertebrate; Mollusk; *Sepia officinalis;* stellar ganglia; esophagus

1. Introduction

During the last 15 years, the use of in vitro myotropic bioassays has allowed the isolation in invertebrates of numerous bioactive peptides. These molecules, which were purified on the basis of their ability to modulate the contraction of various organs are often involved in a large variety of physiological mechanisms such as pheromone production, water-hydromineral balance, secretion and reproduction [10,1]. Thus, reproducible myotropic bioassays provide an important means for testing novel regulatory molecules. In particular, purification of bioactive compounds involved in the control of egg-laying in the cephalopod *Sepia officinalis* has been successfully monitored using a myotropic bioassay based on the recording of oviduct contractions [2,3,4,13,14,15]. In order to eliminate myotropic factors not specifically involved in the regulation of reproduction processes, a second bioassay was carried out using muscular mantle fibers and esophagus as controls for the HPLC fractions tested. By virtue of one of these specificity controls, it was found that one of the fractions was able to lower the tonus of the contraction of the funnel and mantle fibers and to diminish the amplitude of the contractions of the esophagus without affecting the contractile activity of the *Sepia* oviduct.

The present report describes the isolation of a neuropeptide monitored by means of a myotropic bioassay. The purification has been performed from a pool of stellar ganglia which contain the giant nerve fibers responsible for the coordination of the different muscles involved in the locomotion *of Sepia officinalis* [12]. The strategy which has been used to elucidate the primary sequence of this myotropine is based on exopeptidase digestions followed by MALDI-TOF analysis. The partial sequence obtained has been completed with MALDI-PSD-TOF MS analysis.

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2. Methods

2.1. Animals

All mature cuttlefish were trapped in the Bay of Seine from January to June. They were maintained in 1000 liter outflow tanks at the Marine Station of Luc sur Mer (University of Caen, France).

2.2. Peptide synthesis

Peptide VYSAPYG was synthetized (0.1 mmol scale) by the solid phase methodology on a 433A APPLIED BIOSYSTEMS peptide synthetizer, using the standard Fmoc procedure as previously described in [7]. The synthetic peptide was purified by reverse phase HPLC on a 1 × 25-cm Vydac C_{18} column (TOUZART & MATIGNON, Courtaboeuf, France) using a linear gradient (10–45% over 45 min) of acetonitrile-TFA (99.9: 0.1, v/v). Analytical HPLC, on a 0.45 × 25-cm VYDAC C_{18} column, revealed that the purity was greater than 99%.

2.3. Biological activities

The muscles were dissected from freshly collected anaesthetized animals and suspended on a displacement tranducer (PHYMEP, BIONIC INSTRUMENTS) connected to a computer controlling the recorder and the DATAC (Dispositif d'Acquisition et de Traitement Automatique de la Contraction). The muscle chamber (2.5 ml) was perfused at a flow rate of 0.5 ml.min^{-1} with synthetic filtered sea water (INSTANT OCEAN) containing 1 mM glucose and maintained at 15°C in a cold room. The HPLC fractions and the synthetic peptide were injected into the perfusing flow using a three-way valve in order to avoid mechanical and thermal stress. The injections were traced into the muscle chamber by addition of phenol red. The muscle containing chambers were washed for at least two hours before the application of synthetic peptides or HPLC fractions. In all cases, the experiments were performed when the activity had returned to basal level.

2.4. HPLC preparation

One hundred stellar ganglia were crushed in cold 0.1 N acetic acid and centrifuged at 30000× g. The supernatants were evaporated in a Speed Vac concentrator and prepurified on Chromafix C_{18} cartridges (MACHEREY-NAGEL). Dry eluates were resuspended in aqueous 0.1% TFA and fractionated by HPLC onto a gel-permeation **(**gp) protein pak I-125 (WATERS), a C_{18} column (MACHEREY-NAGEL, 250 × 4.6, 7 μm) with a 45-min acetonitrile linear gradient of 1.33% per minute in 0.1% TFA, and a C_{18} column (MACHEREY-NAGEL, 250 × 4.6, 7 μm) with a 90-min acetonitrile linear gradient of 1.33% per minute in 25 mM ammonium acetate. HPLC analysis was performed with the software LC-STAR VARIAN connected to a VARIAN 9012 solvent delivery system and a VARIAN 9050 wave length UV-VIS detector set at 214 nm

2.5. Mass spectrometry analysis

Positive ion MALDI mass spectra were recorded using a TOFSPEC E (MICROMASS Manchester, UK) mass spectrometer equipped with a nitrogen laser (337 nm), using the reflectron-mode with an acceleration voltage of 20 kV. External calibration was provided with corticotropin-like intermediate lobe peptide (CLIP; $ACTH_{18-39}$), substance P and leucine-enkephalin

For PSD analysis, the PSD spectra were recorded at 26 kV acceleration voltage. The reflectron voltage was reduced in steps to obtain a MALDI-PSD spectrum. The spectrometer was calibrated with CLIP.

A part of the pure HPLC fraction was resuspended in 1:1 acetonitrile/0.1% TFA solution. One microliter was mixed with an equal volume of the standard matrix solution of α-cyano-4-hydroxycinnamic acid (10 mg.ml^{-1} in acetonitrile/methanol (50:50, v/v)). One μl of this mixture was deposited onto the target and dried under vacuum.

2.6. Enzyme hydrolyses

Another part of the pure HPLC fraction was resuspended in 5 μl of carboxypeptidase buffer (PERKIN-ELMER KIT). Aliquots of 0.5 μl of this mixture were mixed with an equal volume of carboxypeptidase Y directly on the MALDI target. Kinetic digestion was performed at room temperature and stopped by addition of the acidic matrix.

For aminopeptidase (SIGMA) hydrolysis, a sodium citrate buffer was used and the reaction was carried out directly on the MALDI target. Successive incubations performed at room temperature were stopped by addition of the acidic matrix.

The mass differences between monoprotonated forms $[M+H]^+$ of the partially digested peptides allowed identification of the amino-acids released by the digestions, and thus determination of partial N- and C-terminal sequences of the native peptide.

2.7. Mapping of VYSAPYG in organs and nerves by electrospray ionization mass spectrometry (ESI-MS)

Electrospray mass spectra were acquired using a quadrupole mass spectrometer (NERMAG R 10–10 U) equipped with an analytical Branford atmospheric pressure electrospray source (Quad Service, Poissy, France). The rpHPLC fractions were diluted in H_2O: MeOH (50:50; v/v) and introduced into the ion source by infusion at a flow-rate of 1.5 μl.min^{-1} with a Harvard Apparatus syringe pump. The measured source temperature was 80°C. Full-scan spectra were acquired over the range m/z 100 to 1500 at a scan rate of 9 s. The analysis of the experimental sample was fol-

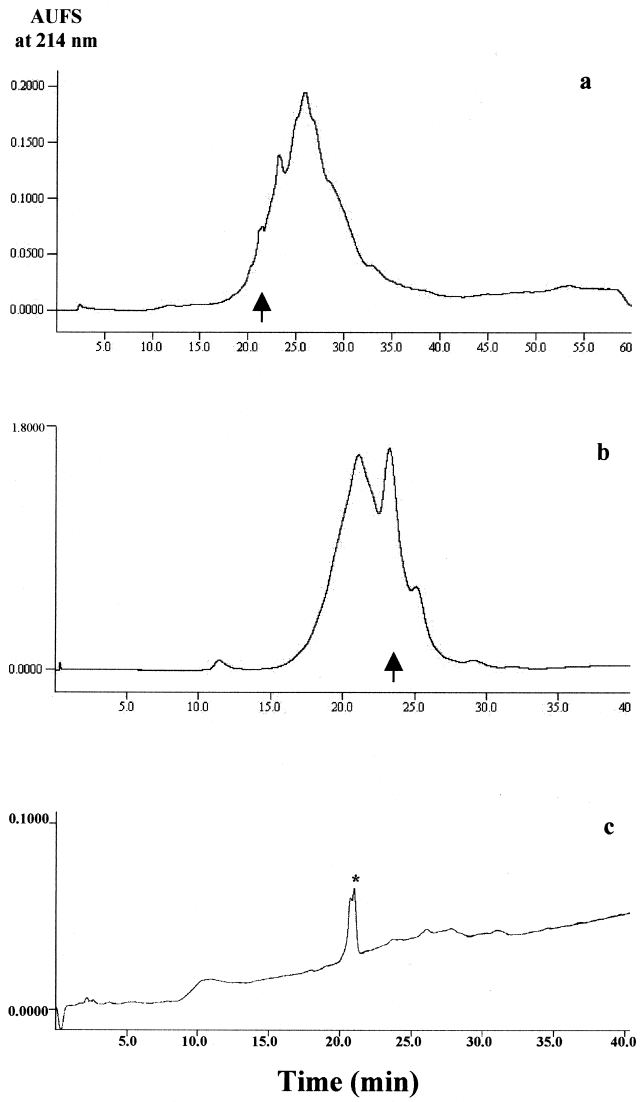

Fig. 1. (a) First separation step. One of the 10 gpHPLC runs of C_{18} Chromafix eluates of 100 stellar ganglia. (b) Second separation step. One of the two C_{18} rpHPLC with a linear acetonitrile gradient of 1.33% per min in 0.1% TFA. (c) Final C_{18} rpHPLC with a linear acetonitrile gradient of 1.33% per min in 25 mM ammonium acetate. The arrows designate the myotropic fractions.

lowed by a calibration with a standard molecular mass containing synthetic VYSAPYG.

3. Results

3.1. HPLC

Chromafix C_{18} eluate of 100 stellar ganglions was fractionated in gpHPLC **(**Fig. 1a). The 1-min fractions were tested on mantle muscle fiber contraction. The fraction eluting at 22 min that contained biological activity was concentrated and fractionated onto a C_{18} rpHPLC column successively in 0.1% TFA **(**Fig. 1b) and in 25 mM ammonium acetate **(**Fig. 1c). The fraction eluting at 21 min that contained biological activity was submitted to MALDI-TOF to check the purity before structural analysis.

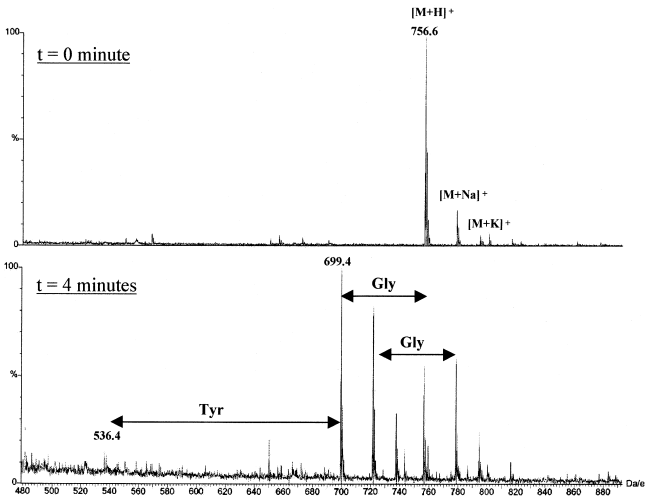

Fig. 2. MALDI-TOF mass spectra after C-terminal digestion by carboxypeptidase Y of the peptide at m/z 756.6. The mass differences allowed identification of the amino acid released by the hydrolysis of the peptide bond. The m/z ions corresponding to $[M+Na]^+$ and $[M+K]^+$ confirmed the nature of the amino acid released based on a typical ion pattern. The digestion demonstrated the following C-terminal sequence: -Tyr-Gly-COOH.

3.2. Structural analysis

MALDI-TOF analysis revealed a main ion at m/z 756.6 $[M+H]^+$ and adduct ions at m/z 778.5 and 794.4 corresponding to $[M+Na]^+$ and $[M+K]^+$ respectively. Kinetic analysis, directly on the MALDI target, successively with a carboxypeptidase and an aminopeptidase, allowed identification of the nature of the C- and N-terminal amino acids. The mass difference between the ions at m/z 756.6, 699.4 and 536.4 led to the following C-terminal amino acid sequence: -Tyr-Gly-COOH (Fig. 2). The mass difference between the ions at m/z 756.6, 657.1 and 493.6 led to the following N-terminal amino acid sequence: H_2N-Val-Tyr- (Fig. 3). The PSD MALDI mass spectrum from the $[M+H]^+$ is represented in Fig. 4. The different peaks corresponding to ions produced by the cleavage of amino acid were presented at m/z 681.9, 518.8, 421.8 and 350.6 (b series), at m/z 654.0, 490.9 and 393.7 (a series), at m/z 355.6, 298.4, 239.5 and 169.3 (internal ions with or without neutral loss) and at m/z 136.3 and 70.4 (immonium ions). The fragmentation interpretation gave the following C-terminal amino acid sequence: -Ala-Pro-Tyr-Gly-COOH.

The association of exopeptidase digestions results and MALDI-PSD TOF MS data led to the following amino acid sequence: Val-Tyr-X-Ala-Pro-Tyr-Gly. The mass difference between the $[M+H]^+$ and the elucidated amino acid sequence (87.4 Da) corresponded to the mass of a serine (87.1). The final amino acid sequence of this novel neuropeptide was Val-Tyr-Ser-Ala-Pro-Tyr-Gly.

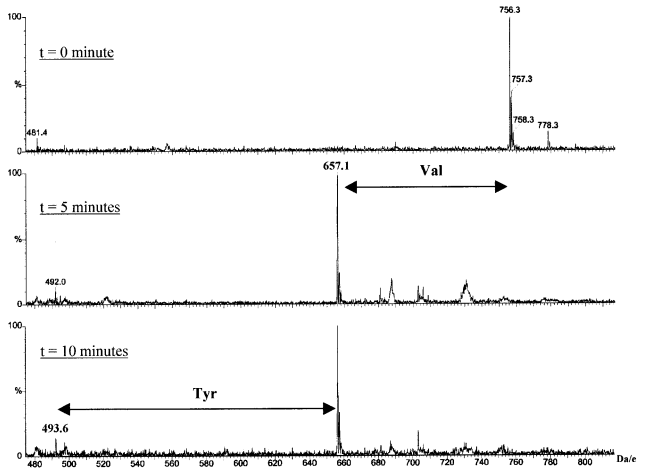

Fig. 3.: MALDI-TOF mass spectra after aminopeptidase digestion of the peptide at m/z 756.6. The mass differences allowed identification of the amino acid released by the hydrolysis of the peptide bond. The successive digestion times of 5 and 10 min demonstrated the following N-terminal sequence: H_2N-Val-Tyr-.

Comparison of the retention times of the native and synthetic peptide under the same chromatographic conditions confirmed the identity of the two peptides. The research performed in the data bases did not lead to any homologous amino acid sequence suggesting that VYSAPYG was a novel bioactive peptide as often observed in this class of mollusk.

3.3. Biological activities

Increasing concentrations of the synthetic peptide were successively applied various contractile muscles (Table 1). From 10^{-6} M, we observed a decrease of the amplitude of contractions of the esophagus (Fig. 5a). From 10^{-5} M, we observed a decrease of the tonus of funnel (Fig. 5b) and mantle muscular fibers (Fig 5c). We did not observe any effect on the other organs tested.

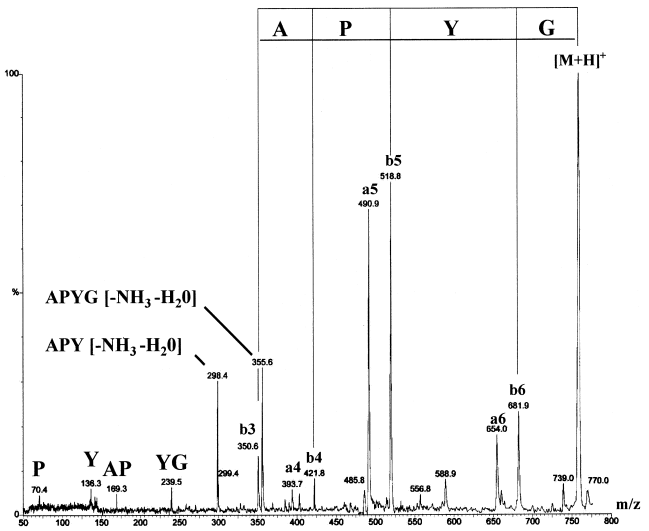

Fig. 4. MALDI-PSD TOF mass spectrum of the positive ion generated from the peptide at m/z 756.6. The C-terminal amino acid sequence determined from the mass difference between b ion series was Ala-Pro-Tyr-Gly. The m/z 298.4 and 355.6 confirmed the C-terminal sequence as well as ammonium ions of proline and tyrosine at m/z 70.4 and 136.3.

Table 1
Biological activities of synthetic VYSAPYG applied on various organs

Tested tissues	Activity	Concentration threshold
Mantle muscular fibers	Inh	10^{-5} M
Funnel muscular fibers	Inh	10^{-5} M
Arm muscular fibers	0	
Fin muscular fibers	0	
Esophagus	Inh	10^{-6} M
Vena cava	0	
Oviduct	0	

O: no activity
(Inh): inhibition

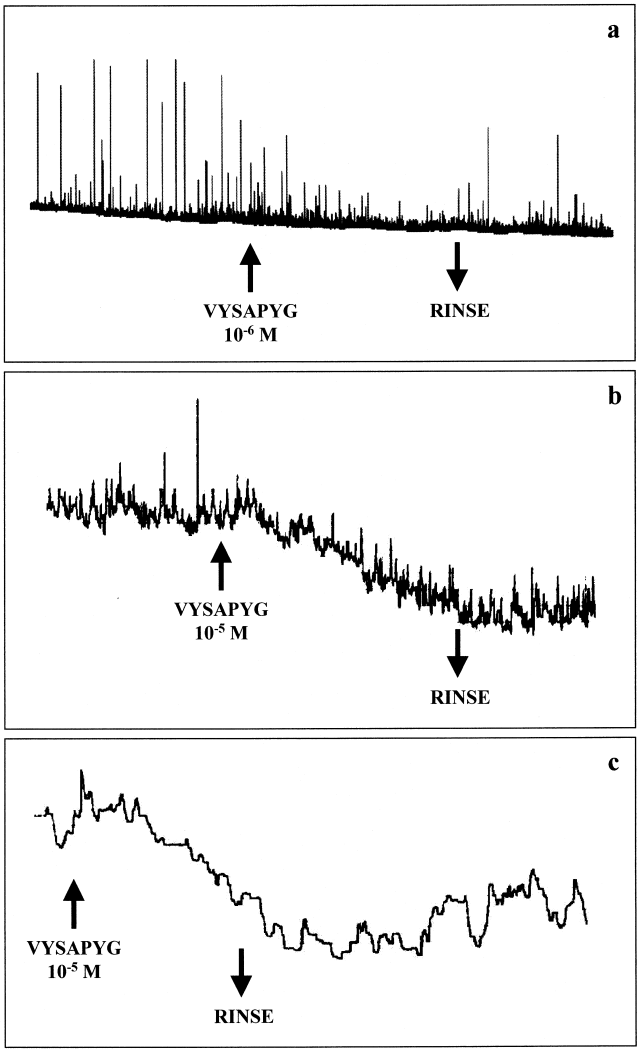

Fig. 5. Recording of biological activities with the myotropic bioassay. Decrease of the amplitude of the esophagus contractions (a). Decrease of the tonus of the funnel muscle fibers (b) and the mantle muscle fibers (c).

Table 2
Tissue mapping of VYSAPYG performed by ESI-MS

SEM	SBEM	STG	GFII	GFIII	STN	PN	FN	ABG	MBG	PBG
nd*	nd	d*	d	d	nd	d	d	nd	nd	nd

* nd: not detected
* d: detected
SEM, Supra esophageal mass; SBEM, Sub-esophageal mass; STG, Stellar ganglia; GFII, Giant fibers II; GFIII, Giant fibers III; STN, Stellar nerves; PN, Palleal nerve; FN, Fin nerve; ABG, Anterior buccal ganglia; MBG, Medium buccal ganglia; PBG, Posterior buccal ganglia.

3.4. Tissue mapping of VYSAPYG using ESI-MS

The distribution of the VYSAPYG is summarized in Table 2. The peptide was found in the stellar ganglia, in the afferent nervous bundle containing the giant fibers II and the palleal nerve, in the fin nerve and in the efferent giant fibers III. VYSAPYG was not detected in the buccal ganglia, the supra-esophageal mass, the sub-esophageal mass or the hemolymph.

4. Discussion

The present study shows that combination of exopeptidase digestion and mass spectrometry is a powerful strategy to elucidate the amino acid sequence of unknown newly isolated peptides. The level of purity is less crucial for mass spectrometry, particularly for MALDI-TOF, than for Edman N-terminal degradation, in order to obtain sequence information. Obviously, the number of amino acids identified will be directly related to the initial amount of pure peptide. For short chain regulatory peptides, as are often seen in invertebrate models, a very low amount of peptide could be sufficient to elucidate the whole amino acid sequence. In the case of regulatory peptides having a C-terminal amide or/and an N-terminal pyroglutamate, it will be possible to perform a fragmentation by MALDI-PSD TOF to elucidate the amino acid sequence.

Thus, the fragmentation of a small peptide (<20 aa) alone or associated with peptidase digestions, can provide partial or complete determination of the amino acid sequence even if the analyte is extracted from a very complex mixture. In the case of VYSAPYG, the two strategies, exopeptidase digestion followed by MS analysis and PSD analysis, were necessary to elucidate the complete amino acid sequence of the purified peptide. Owing to the small size of the peptide and to the absence of C- and N-terminal blockage, the amino acid sequence could be fully elucidated from a very low amount of material.

The tissue mapping of VYSAPYG revealed that its distribution is restricted to discrete areas of the nervous system suggesting a possible involvement in the regulation of specific physiological mechanisms. VYSAPYG was found in the stellar ganglia, in the giant fibers III, and in the nervous bundle containing the giant fibers II and the palleal nerve (Table 2), but was not detected in the hemolymph, suggesting a release by the nerve endings next to the target, as already described for the FaRPs (FMRFa-related peptides) at the level of the accessory sex gland in the mature females of *Sepia officinalis* [3]. Nevertheless, the biological activities recorded from the esophagus and the muscle fibers suggest that these organs are not the main targets of this neuropeptide. Indeed, the threshold of activity was very high i.e.: 10^{-5} M for funnel and mantle muscular fibers and 10^{-6} M for the esophagus. Mollusk neuropeptides such as APGWa-RPs, are capable of inducing the contraction of the pedal retractor muscle of *Mytilus edulis* at concentrations as low as 10^{-9} M [4] and 10^{-8} M for the mature oviduct of *Sepia officinalis* [2]. In the giant snail *Achatina fulica*, APGWa acts as an inhibitory neurotransmitter on *Mytilus* ABRM from 10^{-7} M [8]. FMRFa, a neuropeptide widely distributed in the different classes of mollusk, provokes contractions of the mature oviduct of *Sepia officinalis* at concentration of 5×10^{-6} M compared to 10^{-8} M for the mature nidamental glands [3]. Thus, we cannot exclude the hypothesis of the expression of a complex cocktail of VYSAPYG-related peptides in which the VYSAPYG would be one of the less active, as observed for the FaRPs in *Lymnaea stagnalis* [11]. The release of different combinations of VYSAPYG-RPs could allow a slight modulation of the target activities. It has been demonstrated for neuropeptides as APGWa-RPs and FaRPs that elongation of the N-terminal amino acid sequence can alter the biological properties or even completely modify the initial activity [9,6]. For example in *Locusta migratoria*, the N-terminally elongated FaRPs inhibits the contractions of the midgut [5] whereas other FaRPs have no activity [6]. Thus, the possible expression of shorter VYSAPYG-RPs which could exert activities at lower thresholds will have to be checked by means of mass spectrometry or cDNA screening.

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