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Locustatachykinin I and II, two novel insect neuropeptides with homology to peptides of the vertebrate tachykinin family

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Two myotropic peptides termed locustatachykinin I (Gly-Pro-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2) and locustatachykinin II (Ala-Pro-Leu-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2) were isolated from brain-corpora cardiaca-corpora allata-suboesophageal ganglion extracts of the locust, *Locusta migratoria*. Both peptides exhibit sequence homologies with the vertebrate tachykinins. Sequence homology is greater with the fish and amphibian tachykinins (up to 45%) than with the mammalian tachykinins. In addition, the intestinal myotropic activity of the locustatachykinins is analogous to that of vertebrate tachykinins. The peptides discovered in this study may just be the first in a whole series of substances from arthropod species to be identified as tachykinin family peptides. Moreover, both chemical and biological similarities of vertebrate and insect tachykinins substantiate the evidence for a long evolutionary history of the tachykinin peptide family.

Tachykinin; *Locusta migratoria*; Substance P; Insect neuropeptide; Myotropin; Muscle contraction; Homology; Locustatachykinin

1. INTRODUCTION

One of the most important aspects in insect endocrine research over the past few years is the realization that insects utilize vertebrate-type peptides for a variety of regulatory functions. In the nervous and intestinal system of insects, at least several dozen molecules are present which are recognized by antisera directed against peptides which have a hormonal function in vertebrates [1–4]. The difficulty of collecting sufficient amounts of tissue, the minute concentrations of the peptides present and the scarcity of adequate bioassays make the isolation of insect neuropeptides quite difficult. Only a few of such peptides which resemble to some extent vertebrate neuropeptides have recently been purified [4–11]. Tachykinins are a family of multifunctional peptides. The presence of vertebrate tachykinin-like immunoreactivity has been demonstrated in the neural [12–16] and intestinal [17] systems of insect species. But there is no biochemical or structural evidence for tachykinin-like peptides in insects. We report here for the first time the isolation, characterization and synthesis of two new neuropeptides, termed locustatachykinin I and locustatachykinin II or Lom-TK-I and -II, according to the new insect peptide nomenclature [18], from extracts of brain complexes of the migratory locust, *Locusta migratoria*. Both peptides exhibit sequence homologies with the vertebrate tachykinins, of which mammalian substance P is the best known member.

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2. MATERIALS AND METHODS

2.1. *Animals, tissue extraction and isolation procedures*

Locusta migratoria was raised under laboratory conditions [19]. About 9000 brain-corpora cardiaca-corpora allata-suboesophageal ganglion complexes were dissected manually one by one. The colonization of *Leucophaea maderae* and preparation of the *Leucophaea* hindgut bioassay have been described previously [20]. The preparation of the locust oviduct and foregut for bioassay is performed in a locust saline solution containing NaCl (9.82 g/l), KCl (0.48 g/l), $MgCl_2 \cdot 6H_2O$ (0.73 g/l), $CaCl_2 \cdot 2H_2O$ (0.47 g/l), NaH_2PO_4 (0.95 g/l), $NaHCO_3$ (0.18 g/l) and glucose (4 g/l).

The tissues were extracted in methanol/water/acetic acid (90:9:1) and subsequently prepurified on Sep-pak cartridges [21].

Operating conditions for columns (i–iv) on Waters ALC-100 HPLC system were as follows: *(i) Waters μ-Bondapak phenyl*, 4.6 mm × 30 cm (Waters Associates, Milford, MA). Solvent A, 0.1% trifluoroacetic acid (TFA) in water; Solvent B, 50% acetonitrile in 0.1% aqueous TFA. Conditions: 100% A for 8 min, then linear gradient to 100% B over 2 h; flow rate, 1.5 ml/min; detector, 2.0 absorption units full scale (AUFS) at 214 nm. *(ii) Rainin Microsorb C1*, 4.6 mm × 25 cm (Rainin Instrument Co., Woburn, MA). Solvent B, 25% acetonitrile in 0.1 aqueous TFA; Solvent A, 0.1% aqueous TFA. Conditions: 100% A over 8 min, then linear program to 100% B over 80 min. *(iii) Supelcosil-DB C8*, 4.6 mm × 15 cm (Supelco Inc., PA). Solvents A and B, same as μ-Bondapak phenyl. Conditions: 25% B for 8 min, then linear gradient to 100% B over 80 min; flow rate, 1.5 ml/min; detector, 1.0 AUFS at 214 nm. *(iv) Waters I-125 Protein-Pak*, 7.8 mm × 30 cm. Solvent A, 95% acetonitrile made to 0.01% TFA; Solvent B, 50% acetonitrile made to 0.01% TFA. Conditions: 100% A for 8 min then linear program to 100% B over 80 min; flow rate, 1.5 ml/min; detector, 0.2 AUFS at 214 nm.

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2.2. *Amino acid analysis and sequencing*

Amino acid composition of the peptide was determined by Waters Pico Tag amino acid analysis. Samples were hydrolyzed in vacuo with gaseous HCl at 150°C for 1 h, neutralized with 20 μl of a methanol/water/triethanolamine (2:1:1) solution, vacuum lyophilized and converted to the phenylisothiocyanate (PITC) derivatives with 20 μl of an ethanol/triethanolamine/water/PITC (7:1:1:1) solution, vacuum lyophilized and analyzed according to the instructions supplied by the manufacturer [21].

Sequence analysis was performed through Edman chemistry with the Applied Biosystems Gas/Liquid Phase Sequencer (470a). Phenylthiohydantoin (PTH)-derivatives were transported to the on-line PTH-analyzer (120a) as described [22].

2.3. *Peptide synthesis*

Both Lom-TKs were synthesized by solid phase peptide synthesis on a Biosearch Model 9500 automated peptide synthesizer using the standard tBoc-chemistry as developed by the manufacturer for this instrument. The 4-methylbenzhydrylamine (MBHA) resin was used for the synthesis of the Lom-TKs to yield the C-terminal amides of the presumed sequences. The following amino acid derivatives were used when side chain protection was mandated: Boc-*O*-benzyl-L-serine, Boc-*O*-(2-bromo)benzyl-oxycarbonyl-L-tyrosine, and Boc-N^9-tosyl-L-arginine. At the conclusion of the synthesis on the instrument, each peptide resin was dried thoroughly in a vacuum desiccator over P_2O_5 prior to cleavage and deprotection. Conditions for the cleavage were under anhydrous HF (9 ml/g of resin) in the presence of excess anisole (1.5 ml/g resin) and ethanedithiol (0.75 ml/g resin) at −15°C for 2 h followed by 30 min at 0°C. Following removal of HF under vacuum, the resin was washed thoroughly with diethyl ether to remove excess scavenger and residual HF. Peptides were extracted with water then 10% acetic acid and lyophilized.

3. RESULTS

The isolation of the new neuropeptides from *Locusta migratoria* was based upon a simple single response bioassay [20], the most critical step in any isolation procedure. After each purification step, biological activity was detected by observing the myotropic effect of fractions, that is changes in frequency or amplitude of spontaneous contractions of the cockroach (*Leucophaea maderae*) proctodeum (hindgut). The present study proves that this heterologus bioassay is an excellent detection method not only for the isolation of cockroach peptides, but also for the isolation of peptides from heterologous insect species. We isolated the Lom-TKs from methanol/water/acetic acid (90:9:1) extracts of 9000 brain-corpora cardiaca-corpora allata-suboesophageal ganglion complexes by sequential elution through a series of 4 columns having different separation characteristics (μ-Bondapak phenyl, Rainin Microsorb C1, Supelcosil-DB C8 and Waters I-125 Protein-Pak columns). Two biologically active fractions eluting at 52–54 min and at 62–64 min, respectively, from the μ-Bondapak phenyl column, were further purified.

On a Microsorb C1 column, the 52–54 min fraction eluted at 44–48 min and this fraction was further purified on a Supelcosil-DB C8 column. A single active peak at 25 min was isolated. Final purification was effected on a Waters I-125 Protein-Pak and a pure peptide, designated as locustatachykinin I or Lom-TK-I, eluted at 48 min (fig.1). Amino acid analysis revealed the molar ratio composition as Ser (1), Gly (3), Arg (1), Pro (1), Tyr (1), Val (1), Phe (1). Gas phase sequence analysis yielded the amino acid sequence Gly-Pro-Ser-Gly-Phe-Tyr-Gly-Val-Arg, which accounted for all the

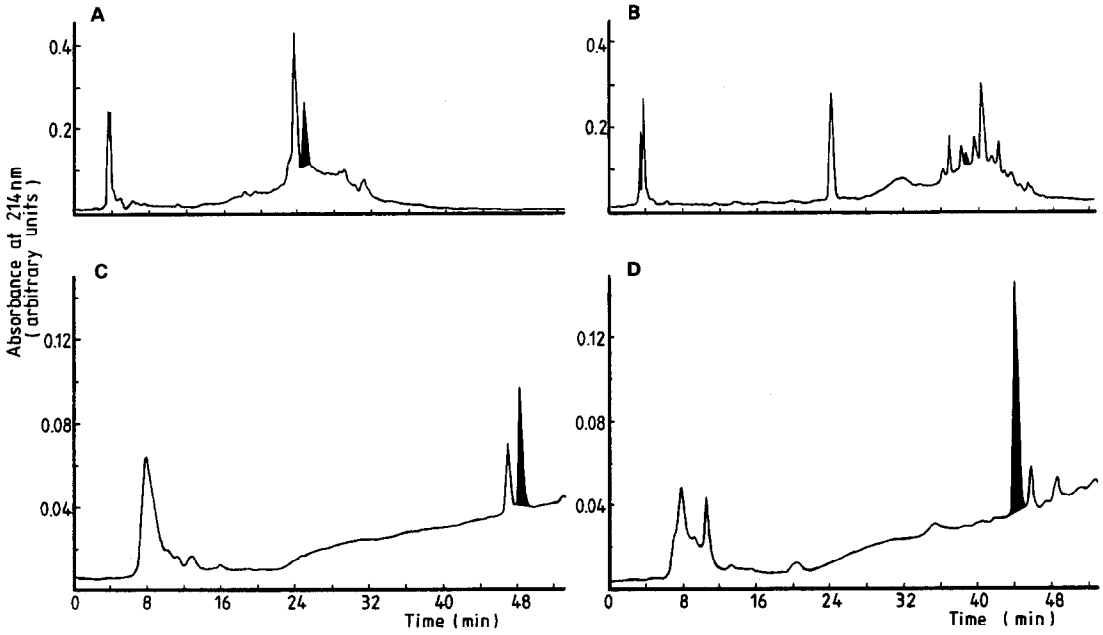

Fig.1. Separation of myotropic activity by HPLC. (A,B) Fractionation on Supelcosil-DB C8 of area eluting at 44–48 min (A) and of area eluting at 48–52 min (B) from Microsorb C1. Peaks eluting at 25 min (A) and between 38 and 39 min (B) contained myotropic activity. (C,D) Fractionation on Waters-I-125 Protein column of active peaks collected in (A,B). Biological activity eluted in a peak at 48 min (C) and in a peak eluting at 44 min (D).

amino acids (table 1). The second fraction (62–64 min) eluted at 48–52 min on the Microsorb C1 column. This fraction yielded an active peak between 38 and 39 min on the Supelcosil-DB C8 column. Final purification on the Waters Protein-Pak column revealed a peak at 44 min. The molar ratio composition of this second peptide (Lom-TK-II) was Ser (1), Gly (2), Arg (1), Ala (1), Pro (1), Tyr (1), Val (1), Leu (1), Phe (1). Gas phase sequence analysis yielded the primary structure Ala-Pro-Leu-Ser-Gly-Phe-Tyr-Gly-Val-Arg, which accounted for all the amino acids (table 2).

A total of 1.9 μg of Lom-TK-I and 5.3 μg of Lom-TK-II was isolated from 9000 locust brain complexes. This was sufficient material to obtain exopeptidase digestion data, amino acid analyses, threshold activity concentrations and sequence analyses data, but insufficient to obtain dose-response curves. The contents of Lom-TK-I and Lom-TK-II were approximately 0.22 ng and 0.6 ng, respectively, per brain-CC-CA-SOG complex (0.00023 nmol and 0.00055 nmol, respectively, per complex) in the insect.

Table 1

Amino acid yields obtained at each step of the sequence determination of Lom-TK-I

Residue	Assignment	Amino acids present	Amount (pmol)
1	Gly	–	31
2	Pro	–	31
3	Ser		9
		Ser′	19
4	Gly	–	20
5	Phe	–	20
6	Tyr	–	21
7	Gly	–	15
8	Val	–	11
9	Arg	–	15

An aliquot (200 pmol) of the pure sample was subjected to amino acid sequence analysis

Table 2

Amino acid yields obtained at each step of the sequence determination of Lom-TK-II

Residue	Assignment	Amino acids present	Amount (pmol)
1	Ala	–	68
2	Pro	–	53
3	Leu	–	50
4	Ser		15
		Ser′	19
5	Gly	–	31
6	Phe	–	29
7	Tyr	–	36
8	Gly	–	23
9	Val	–	19
10	Arg	–	25

An aliquot (400 pmol) of the purified peptide was sufficient material for the sequence analysis

HPLC comparison of synthetic peptides in the amide and acid forms revealed that both peptides were C-terminal amides. Amino acid analyses of both synthetics confirmed that the amino acid residues and molar ratios were identical to those of the natural locustatachykinins. Both synthetic peptides co-chromatographed with their corresponding natural products.

The synthetic replica of Lom-TK-I caused an increase in the spontaneous contraction of the cockroach hindgut at a threshold concentration of $2.7 \pm 0.48 \times 10^{-10}$ M (SD, $n = 5$), a value approximately identical to that recorded for the natural product, $2.8 \pm 0.44 \times 10^{-10}$ M (SD, $n = 5$). The synthetic analog of Lom-TK-II caused an increase in spontaneous contractions of the cockroach hindgut at a threshold concentration of $1.5 \pm 0.18 \times 10^{-10}$ M, again nearly identical to that recorded for the natural product ($1.4 \pm 0.18 \times 10^{-10}$ M). The synthetic Lom-TKs stimulate the contractions of the foregut and the oviduct of *Locusta*. Lom-TK-I and Lom-TK-II caused an increase in spontaneous contractions of the locust foregut at threshold concentrations of $5.5 \pm 0.6 \times 10^{-9}$ M and $3.2 \pm 0.3 \times 10^{-10}$ M, respectively. Lom-TK-I and Lom-TK-II stimulate the locust oviduct contractions at concentrations higher than $3.7 \pm 0.5 \times 10^{-8}$ M and $2.4 \pm 0.3 \times 10^{-9}$ M, respectively.

Under the same conditions (same saline solution), the Lom-TKs had no effect on the contraction pattern of the hindgut of *Locusta*.

4. DISCUSSION

The newly discovered nona- and decapeptides are structurally very similar; they have a Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2 carboxy-terminal in common. In addition, the novel locust peptides exhibit sequence identity to the vertebrate tachykinins. The amino acid sequences of various peptides belonging to the tachykinin family are shown in fig.2. As it is typical for the peptides of the tachykinin family, Lom-TK-I and -II are peptide amides with a Phe-residue at position 5 and a Gly-residue at position 3 from the C-terminus.

All currently identified tachykinins influence the motility of various smooth muscles of vertebrates [23]. The intestinal myotropic activity of the newly identified peptides is analogous to that of the vertebrate tachykinins. Lom-TK-I shows a greater resemblance to the mammalian tachykinins than does Lom-TK-II. Residues Pro^2, Phe^6, Gly^8 of the Lom-TK-I sequence match positions 4, 7 and 9 of substance P. Thus 3 of the 9 amino acids (33%) are identical with those of substance P.

Both Lom-TKs are more related to the amphibian and fish tachykinins than to mammalian substance P. Residues Phe^5, Tyr^6, Gly^7 of Lom-TK-I and Phe^6, Tyr^7, Gly^8 of Lom-TK-II match positions 7, 8 and 9 of

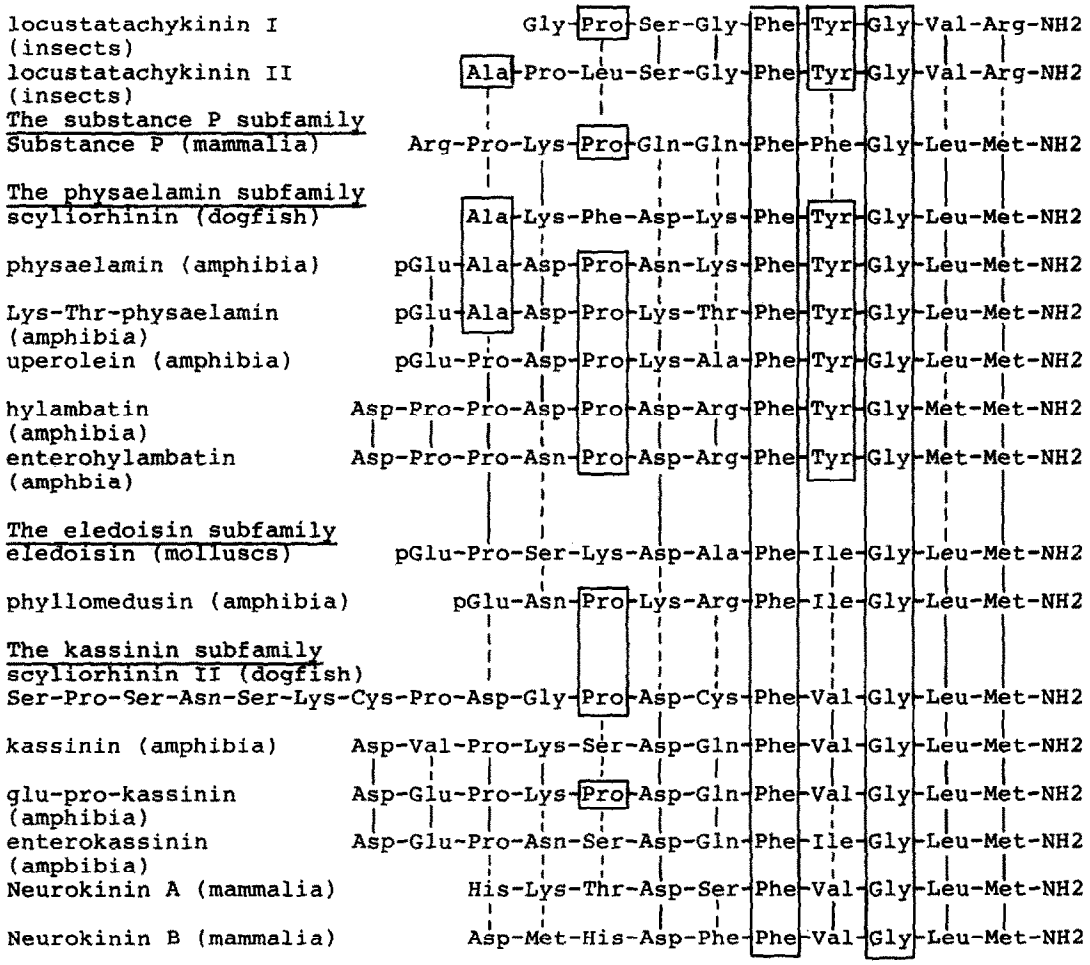

Fig.2. Sequence homologies between Lom-TK-I, Lom-TK-II and other vertebrate and invertebrate tachykinin peptides belonging to 4 distinct subfamilies. The boxed sections indicate identical amino acids to the locustatachykinins. The sequence similarity of Lom-TK-I and -II with vertebrate counterparts is 20–45%, whereas that among vertebrate tachykinins is 40–90%. Solid lines indicate direct homology and the dashed lines correspond to residues of which the nucleotide codons could differ by a single nucleotide.

physaelamin (Amphibia) [24]. These residues are also present in the dogfish scyliorhinin I [25] and in the amphibian tachykinins, Lys-Thr-physaelamin, uperolein, hylambatin and enterohylambatin which belong to the same tachykinin subfamily [23]. Furthermore, the proline residue in position 8 from the C-terminal of Lom-TK-I is identical to the residue in the same position of the amphibian tachykinins of the physaelamin subfamily. In addition, the Ala^1 of Lom-TK-II matches the residue in the same position of scyliorhinin I (dogfish) and the residue in positions 2 of physaelamin and Lys-Thr-physaelamin (amphibians). Hence, both Lom-TK-I and -II share 4 of 9, respectively 10 residues (45% and 40%, respectively) with the dogfish and amphibian tachykinins of the physaelamin subfamily, which is considerably high since between some vertebrate tachykinin sequences (i.e. between amphibian uperolein and dogfish scyliorhinin), homologies of only 50% occur. Moreover, the 30–45% sequence homology between the Lom-TKs and the vertebrate tachykinins could possibly be greater, since some differences in amino acid residues could arise from single base substitution in the nucleotide codons (fig.2).

The Lom-TKs show also limited homology (20–30%) to neurokinins A and B (mammals) [26], phylomedusin and kassinins (Amphibia), and to scyliorhinin II (dogfish), which belong to different tachykinin subfamilies [23,24]. Lom-TK-I shows more resemblance to eledoisin, a tachykinin isolated from octopus salivary glands [27], than does Lom-TK-II. Residues Phe^5 and Gly^7 are identical to those in analogous positions 7 and 9 of eledoisin. Lateral homologies exist between Pro^2-Ser^3 and the corresponding amino acid pair in position 2 and 3 of eledoisin.

The sequence homology and analogous biological activity exhibited by the Lom-TKs, eledoisin and the vertebrate tachykinins suggests that their carboxyterminal regions are conserved because they include the minimum fragment needed for biological activity. The discovery of tachykinin-related peptides in insects substantiates the evidence for a widespread and early evolutionary origin of the tachykinin superfamily. If

the vertebrate tachykinins and the insect tachykinins are derived from the same ancestral tachykinin molecule, this study has demonstrated that evolutionary pressure has operated only to conserve the C-terminal regions, which are believed to interact with the tachykinin receptors. Based on relative potency of effect on a number of biological activities, Erspamer [23] has succeeded in classifying the vertebrate tachykinins into 4 subfamilies. Although the locustatachykinins have important characteristics in common with the various tachykinins of mammals, amphibians, fish and molluscs, they possess a Val-Arg C-terminal, a new feature not found in any of these peptides. This indicates that the locustatachykinins are another branch of a relatively ancient superfamily of tachykinin-like hormones. Tachykinins may be even much older than insects, since substance P-like material has been detected in the nerve cells of a coelenterate [28], which have the simplest nervous system of the animal kingdom. The presence of multiple tachykinins in invertebrate as well as in vertebrate species suggests that these peptides play a most important, well-conserved physiological role.

All of the tachykinins identified by now stimulate various smooth muscles of vertebrates and also display other biological actions [23], such as regulation of blood pressure, modulation of actions of other hormones in the gut or in the nervous system. Contraction of insect visceral muscle promoted by the Lom-TKs is analogous to the tachykinin-induced motility in mammalian smooth muscle. The investigated insect visceral muscles (*Leucophaea* hindgut and locust foregut and oviduct) are more sensitive to Lom-TK-II than to Lom-TK-I. The hindgut of *Leucophaea* and the foregut of *Locusta* are more sensitive to the Lom-TKs than the locust oviduct. No other activities for the Lom-TKs have as yet been described. However, the analogous vertebrate neuropeptides are multifunctional, having many biological activities. The commercial availability of synthetic locustatachykinins should accelerate the discovery of additional activities. There is already evidence that substance P is a neurotransmitter in the vertebrate brain [29]. The demonstration of tachykinins in the insect brain and their established structures now makes it possible to study the peptides' role at the cellular level.

Recent data have shown that mammalian tachykinins are derived from large precursors, encoded by two similar genes [30]. Further recombinant DNA technological and immunohistochemical work will determine whether the insect tachykinins are derived from the same biosynthetic precursor.

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