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Identification of leucomyosuppressin in the German cockroach, *Blattella germanica*, as an inhibitor of food intake

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Abstract

The feeding pattern of the adult female of *Blattella germanica* peaks in the middle of the vitellogenic cycle. Following the hypothesis that a factor inhibiting gut peristalsis also inhibits food intake and is involved in the regulation of feeding, we searched for the most powerful myoinhibitory peptide in brain extracts from *B. germanica* females collected after the peak within the feeding cycle. Through HPLC purification and sequence analysis, we obtained the peptide leucomyosuppressin (LMS): pQDVDHVFLRFamide. LMS elicited a powerful myoinhibitory effect on *B. germanica* foregut and hindgut, with ED_{50} values around 10^{-10} M. In addition, it inhibited food intake in vivo in a dose-dependent manner at doses between 5 and 50 μg. The study of the distribution of ingested food in the foregut, midgut and hindgut of *B. germanica* females treated with LMS showed that food accumulates in the foregut, which may be due to the myoinhibitory effects of the peptide. We propose that this accumulation inhibits food intake because of the persistence of the signals from gut stretch receptors.
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Keywords: Blattella germanica; German cockroach; Leucomyosuppressin; Feeding regulation; Antimyotropic action

1. Introduction

Antagonistic muscular sets in the insect gut are arranged as bands of longitudinal, circular and oblique muscles. They create peristaltic movements in the gut that mix food with enzymes and regulate the passage of food bolus through the digestive tract and the time spent by it in each gut region. Volumetric feedback provided by stretch receptors located in different areas of the gut wall has been described among the mechanisms contributing to regulation of food intake [1]. Therefore, a reduction in gut movements may produce an accumulation of food which, in turn, may inhibit food intake because of the persistence of the signals from gut stretch receptors. Accordingly, a strong inhibitor of gut contractions would hinder food intake.

A great number of insect neuropeptides have been purified through their stimulatory or inhibitory activity upon the contraction of gut musculature, although most have been attributed other activities besides myomodulation. Among the myostimulatory peptide families of insects we can find proctolin, sulfakinins, kinins, pyrokinins, tachykinin-related peptides, periviscerokinins and corazonin. Peptide families with myoinhibitory members include allatostatins, myoinhibitory peptides and myosuppressins [2–5].

Blattella germanica can be considered as an anautogenous species, given that the female starts vitellogenesis only after feeding. In addition, the adult female shows a feeding cycle almost parallel to the vitellogenic cycle. In our laboratory conditions, food consumption in the adult female starts on day 1, peaks on day 4 and steadily decreases thereafter until day 7, when oviposition occurs [6]. This pattern suggests that food intake is finely regulated and in a recent first approach to study this regulation in *B. germanica*, the peptide perisulfakinin has been identified as a putative satiety factor in this species [7].

The study of perisulfakinin has also revealed that brain extracts from 5- to 7-day-old *B. germanica* females (thus, when food consumption decreases) show the highest inhibitory activity in food intake assays [7]. Therefore, according to the hypothesis that a factor inhibiting gut peristalsis contributes to regulating feeding inhibition, we searched

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for the most powerful myoinhibitory peptide in brain extracts from 5- to 7-day-old females which may be involved in the decrease of food intake detected between these days.

2. Materials and methods

2.1. Insect rearing and tissue collection

Adult females of *B. germanica* were obtained from a colony fed on dog chow and water, and reared in the dark at 30 ± 1 °C and 60–70% r.h. Brains from 5- to 7-day-old adult virgin females were freed of optic lobes and adhering fat body, and homogenized in methanol/water/acetic acid (87:8:5, v/v/v), using a mechanical homogenizer Eurostar digital (Ika labortechnik, Spain), designed for 1.5 ml tubes. After centrifugation (8000 × *g* for 10 min at 4 °C), the supernatant was collected and stored at − 20 °C until use.

2.2. HPLC purification of the active factor

A total of 5300 brains from 5- to 7-day-old females were extracted and purified in five consecutive HPLC steps, as summarized in Fig. 1. Steps 1, 2 and 3 were performed with a Merck-Hitachi (Darmstadt, Germany) low-pressure system, L-6200A pump + L-4200 UV–VIS detector. Steps 4 and 5 were carried out with a Waters (Milford, MA, USA) low-pressure system, 626 pump + 600S controller + 996 PDA detector. Details of gradients and columns used in each separation are summarized in Fig. 1.

2.3. Mass spectrometry and sequencing

An aliquot of the fraction responsible for the biological activity was analyzed using an Applied Biosystems Voyager DE-RP matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer (Foster City, CA, USA). This ensured the purity of the selected fraction. Thereafter, peptide sequence was obtained using two approaches. First, the peptide was sequenced on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, Finnigan MAT, San Jose, CA, USA) equipped with a nanospray source (Protana, Odense, Denmark). The spray voltage applied was 0.85 kV and the capillary temperature was 110 °C. For MS/MS experiments, the isolation window was 3 mass units wide and the relative collision energy was in the range 20–45% depending on the charge of the precursor ion. After the removal of the pyroglutamyl residue, the amino acid sequence was determined by Edman degradation using a Beckman LF-3000 sequencer (Palo Alto, CA, USA).

2.4. Removal of the pyroglutamic acid residue

An aliquot (approximately 500 ng) containing the purified peptide was dried out and then dissolved in 21 μl of buffer reaction containing 50 mM Na_2HPO_4, 10 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), and 200 μU pyroglutamyl aminopeptidase (Calbiochem, La Jolla, CA, USA), pH 7.0. After 3.5 h incubation, the sample was fractionated using the same chromatographic system as described for steps 4 and 5 in the following conditions: column, Waters DeltaPak C_{18}, 150 × 2 mm, 5 μm, 300 Å; solvents, H_2O 0.05% TFA and 80% CH_3CN 0.05% TFA; flow rate, 0.2 ml/min and gradient, 0.25% CH_3CN/min.

2.5. Myotropic bioassay

Peptides were tested on the foregut and hindgut of *B. germanica* females in a standard organ bath as described elsewhere [7]. The composition of the bath was: 154 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 22 mM glucose and 5 mM HEPES, pH 6.8. An FSG-01 transducer (Experimetria, Budapest, Hungary) was used for isometric recording. The activity was calculated as the difference of the mean of the force produced by the tissue 1 min after and 1 min before the treatment.

2.6. Feeding bioassay

The feeding bioassay was performed as previously reported [7]. Accordingly, freshly ecdysed adult females were starved for 48 h, injected with saline or with the testing peptide and provided carrot ad libitum. They were then fed for 5 h (which ensures that carotenoids would not yet appear in the faeces), after which the whole gut was dissected out and extracted with methanol. Carotenoid concentration in the methanolic extracts was estimated by spectrophotometric measurement of the absorbance at 450 nm. The total weight of carrot ingested was estimated by interpolation on a standard curve constructed using methanolic extracts of increasing amounts of lyophilized carrot. For the experiments of peptide effects on food distribution along the gut, the three gut regions were separated by sectioning just before the gastric caeca and at the point of evagination of the Malpighian tubules.

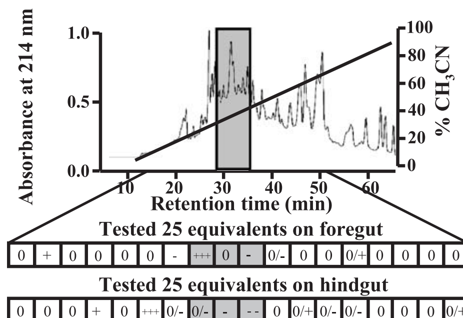
2.7. Synthetic peptides

The myosuppressins, schistoFLRFamide (PDVDHVFLR Famide) [8], neomyosuppressin (NMS) (TDVDHVFLRFamide) [9] and manducaFLRFamide (pQDVVHSFLRFamide) [10], and the allatostatin BLAST-2 (DRLYSFG

Fig. 1. Flow diagram summarizing the conditions for HPLC purification of the myoinhibitory factor from *B. germanica* brains. Fractions used in successive purification steps are highlighted. Activity of each fraction is expressed as the increase (+) or decrease (−) of foregut and hindgut force in myotropic assay. Foregut: 0 (0 mg); 0/ − or 0/+ (3–8 mg); − or + (8–15 mg); − (15–25 mg); − − − or +++ (25–30 mg); − − − − (>30 mg). Hindgut: 0 (0 mg); 0/ − or 0/ + (2–4 mg); − or + (4–6 mg); − − (6–8 mg); +++ (15–25 mg). TFA, trifluoroacetic acid; NH_4Oac, ammonium acetate.

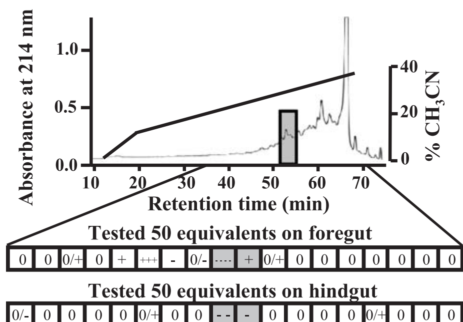
STEP 1

Waters DeltaPak C_{18}
300 x 7.8 mm, 300 Å, 15 µm
CH_3CN / 0.1% TFA
1.67 %/min, 1,5 ml/min



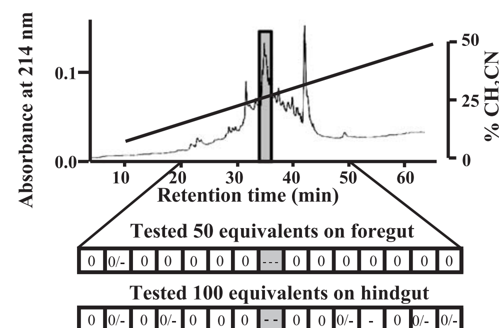
STEP 2

Waters DeltaPak C_{18}
300 x 7.8 mm, 300 Å, 15 µm
CH_3CN / 0.1% TFA
0.5 %/min, 1,5 ml/min



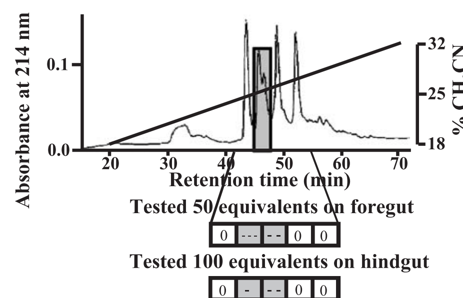
STEP 3

Merck LiChroCART C_{18}
125 x 4 mm, 100 Å, 5 µm
CH_3CN / 10 mM NH_4OAc
0.75 %/min, 1 ml/min



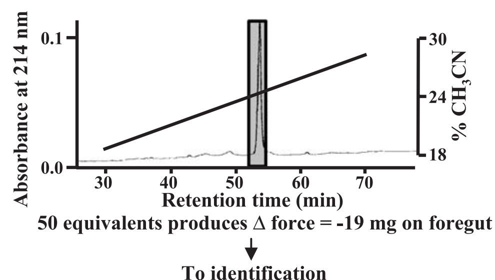
STEP 4

Waters DeltaPak C_{18}
150 x 2 mm, 300 Å, 5 µm
CH_3CN / 0.05% TFA
0.25 %/min, 0,2 ml/min



STEP 5

Waters DeltaPak HPI C4
150 x 2 mm, 300 Å, 5 µm
CH_3CN / 0.05% TFA
0.25 %/min, 0,2 ml/min



50 equivalents produces Δ force = -19 mg on foregut

To identification

Fig. 1.

Table 1
Sequences of myosuppressin-type peptides characterized in insects

Sequence	Common name	Species	Order
pQ**DV**D**H**V**FLRFa**	leucomyosuppressin	*Leucophaea maderae*	Dictyoptera
		Diploptera punctata	
		Periplaneta americana	
P**DV**D**H**V**FLRFa**	schistoFLRFamide	*Schistocerca gregaria*	Orthoptera
		Locusta migratoria	
A**DV**G**H**V**FLRFa**	–	*Locusta migratoria*	Orthoptera
pQ**DV**V**H**S**FLRFa**	manducaFLRFamide	*Manduca sexta*	Lepidoptera
	pseudaletiaFLRFamide	*Pseudaletia unipuncta*	
T**DV**D**H**V**FLRFa**	neomyosuppressin	*Neobellieria bullata*	Diptera
	dromyosuppressin	*Drosophila melanogaster*	

The conserved residues in all species have been highlighted in bold. pQ indicates pyroglutamic acid. See references in the Discussion.

Lamide) [11] were synthesized using standard Fmoc chemistry. Leucomyosuppressin (LMS) (pQDVDHVFLRFamide) was from Bachem (UK) (St. Helens Merseyside, England), and perisulfakinin (Pea-SK) (EQFDDY(SO_3H)GHMRFamide) was from Bachem (Bubendorf, Switzerland).

3. Results

3.1. Isolation of a myoinhibitory peptide from brain extracts

Starting from an extract of 5300 brains from 5- to 7-day-old adult females of *B. germanica*, five consecutive HPLC separation steps were required to obtain a homogeneous peak consisting of the searched peptide with high myoinhibitory activity in gut tissues (Fig. 1). In each purification step, fractions showing the strongest myoinhibitory effect on foregut and hindgut preparations were selected. In the first three steps, biological activity in the myotropic assay was tested in each 2-min fraction, and the most active inhibitory fractions were collected and used for the next chromatographic step. In the fourth and fifth steps, myotropic assays were carried out on isolated peaks. The fifth purification step resulted in the isolation of a homogeneous active peak eluting at 23.9% of CH_3CN. The number of brain equivalents used in each step, their results and the percentage of CH_3CN corresponding to the active fractions are summarized in Fig. 1.

3.2. Identification of the peptide

The analysis with tandem MS/MS of the homogenous peak from the fifth purification step gave the sequence pQDVDHVFL/IRFamide. Because of the uncertainty of the mass spectrometry result at the eighth position (Leu or Ile), we carried out an additional Edman degradation analysis, after removing the pyroglutamic acid (pQ) residue of the N-terminus through enzymatic cleavage with pyroglutamyl aminopeptidase. The resulting products were separated by HPLC and the digested peptides were collected and used for Edman degradation analysis, which identified Leu as the residue at the eighth position and confirmed the other residues identified by MS/MS. Therefore, the complete sequence of the myoinhibitory peptide isolated from brain extracts was identified as pQDVDHVFLRFamide.

This peptide belongs to the FMRFamide-related peptide family and is identical to leucomyosuppressin (LMS), which has been described in the cockroach *Leucophaea maderae* [12] as a myoinhibitory factor. Similar decapeptides have been obtained in other insects (Table 1), all of them possessing myotropic properties. In addition to the decapeptides depicted in Table 1, two longer peptides with 24 (named

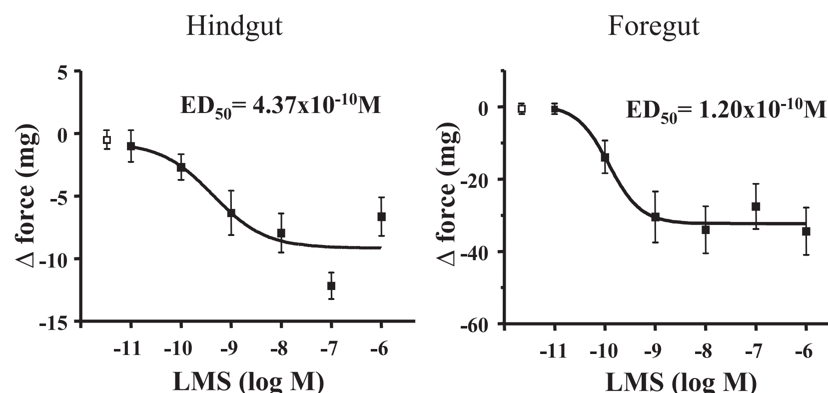


Fig. 2. Inhibitory effect of synthetic leucomyosuppressin (LMS) on hindgut and foregut motility in *B. germanica* females. Results (mean ± S.E.M.; $n = 5-7$) are expressed as the difference of the mean of the force produced by the tissue during one min after and before the treatment. ED_{50} for each tissue is also shown. Empty squares indicate values for water control experiments.

F24) and 39 (F39) residues have been purified from midgut extracts of the Lepidopteran *Manduca sexta* parasitized by the Hymenopteran *Cotesia congregata* [13]. The C-terminal region of F39 includes the sequence of F24, and both bear the typical sequence QDVVHSFLRFamide at the C-terminus, which suggests that the two longer forms are precursors of the bioactive decapeptide.

Given the occurrence of myosuppressin peptides in gut tissues, our LMS was expected to be found in gut extracts of *B. germanica*. However, using an extract of 1250 midguts from 4- to 6-day-old females, which is the age at which crude midgut extracts gave the highest antialimentary activity, we failed to detect LMS using the myotropic assay to monitor the active fractions, and the retention time of synthetic LMS as a reference.

3.3. Myoinhibitory activities on gut tissues

To confirm the antimyotropic properties of LMS in *B. germanica*, we studied the dose–response effect of a synthetic version of the peptide on the foregut and hindgut myotropic assay. Synthetic LMS showed strong myoinhibitory activity in terms of frequency (not shown) and amplitude, with an ED_{50} at the nanomolar range (Fig. 2).

The foregut myotropic bioassay of *B. germanica* was then used to study the activity of myosuppressin-type peptides from insects representing other orders, like schistoFLRFamide (PDVDHVFLRFa) from Orthopterans, manducaFLRFamide (pQDVVHSFLRFa), from Lepidopterans, and neomyosuppressin (NMS) (TDVDHVFLRFa) from Dipterans (Table 1). The ED_{50} value for LMS was in the range of 10^{-10} M, whereas those corresponding to the other peptides were around one order of magnitude higher, pointing to high species-specificity (Fig. 3).

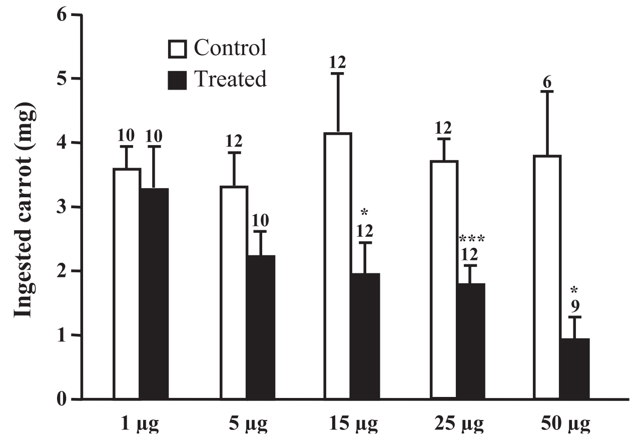


Fig. 4. Inhibitory effect of leucomyosuppressin (LMS) on food intake in *B. germanica*. Results are expressed as mean ± S.E.M. The number of replicates is indicated at the top of each bar. Asterisks indicate significant differences (Student's *t*-test) with respect to control (*$P<0.05$; ***$P<0.001$).

3.4. Effects of LMS on food intake

Quantification of food intake in treated and control females of *B. germanica* (Fig. 4) showed a dose-dependent

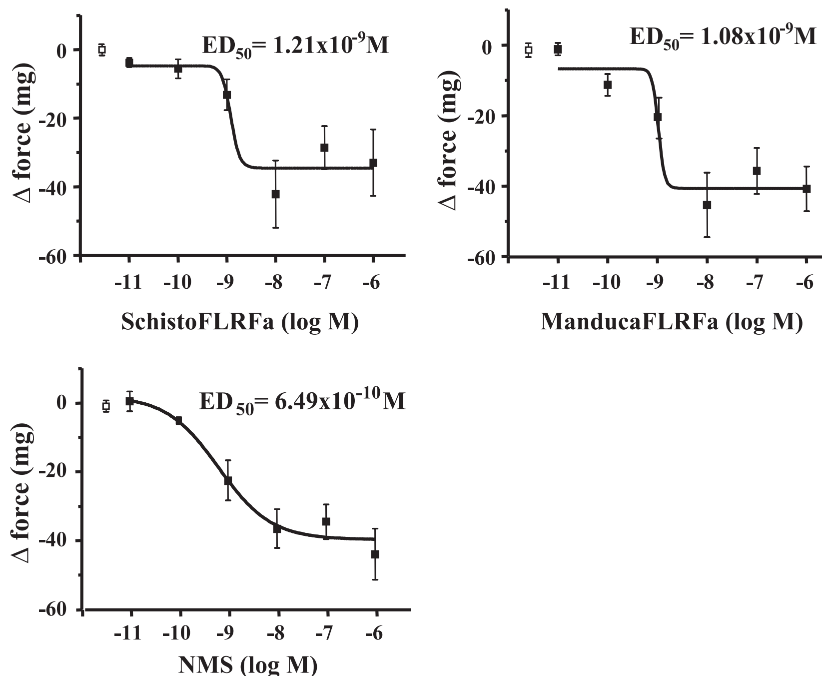


Fig. 3. Inhibitory effect of myosuppressin homologues of *Schistocerca gregaria* (schistoFLRFa), *Neobellieria bullata* (neomyosuppressin, NMS) and *M. sexta* (manducaFLRFa) on foregut motility in female *B. germanica*. Results (mean ± S.E.M.; $n=5-7$) are expressed as the difference of the mean of the force produced by the tissue during one min after and before the treatment. ED_{50} for each myosuppressin is also shown. Empty squares indicate values for water control experiments.

inhibitory effect of LMS, resulting in ca. 50% significant inhibition at doses of 15 and 25 μg and ca. 75% significant inhibition at 50 μg. The 1 μg dose was inactive and that of 5 μg indicated a tendency to inhibit food intake, but the difference between treated and control was not significant.

3.5. Pattern of food distribution within the digestive tract

The myoinhibitory activity of LMS in the foregut and hindgut, and its inhibitory effect on food consumption led us to predict that consumed food accumulates in the anterior regions of the digestive tract. To test this hypothesis, we studied the effects of LMS on the pattern of food distribution among the three gut regions, foregut, midgut and hindgut. The amount of food in the three gut compartments of control animals showed a gradient: foregut > midgut > hindgut, which corresponds to the volume gradient of these regions. In animals treated with effective doses of LMS, however, the percentage of food in the hindgut was severely reduced, and food accumulated in the gut anterior regions (Fig. 5), in agreement with the above hypothesis.

We then analyzed the effects on food distribution of other myoactive peptides inhibiting food intake in *B. germanica*, namely perisulfakinin Pea-SK [EQFDDY(SO_3H)GHMRFamide] and the allatostatin BLAST-2 (DRLYSFGLamide). Perisulfakinin is a myostimulatory peptide of gut tissues that inhibits food intake in *B. germanica* [7]. It produced the expected global food intake inhibition (Fig. 5), but the pattern of distribution of ingested food among the three compartments of the digestive tract was similar to that of control. The allatostatin BLAST-2, not only inhibits juvenile hormone production [11], but also shows myoinhibitory activity in the hindgut and inhibits food intake in *B. germanica* [14]. BLAST-2 elicited the expected fasting effects, but the pattern of food distribution among the three compartments of the digestive tract suggested differential accumulation in the midgut (Fig. 5).

4. Discussion

The search for strong gut myoinhibitory peptides in brain extracts of the adult female of *B. germanica* led to the identification of the peptide pQDVDHVFLRFamide, which was unambiguously determined by MS/MS sequencing and pyroglutamyl aminopeptidase digestion followed by Edman degradation analysis. This peptide belongs to the FMRFamide-related peptide family, in particular to the subfamily of myosuppressins, showing a characteristic DVX_1HX_2 FLRFamide (X_1 = D, G or V; X_2 = V or S) C-terminus [15]. It was first isolated from nervous tissues of the cockroach *L. maderae*, by monitoring its ability to inhibit spontaneous hindgut contractions, and was named leucomyosuppressin (LMS) [12]. Later, LMS was isolated from an extract of corpora cardiaca and corpora allata of the cockroach *Periplaneta americana* [5] and a cDNA coding for this peptide was reported in the cockroach *Diploptera punctata* [16]. Peptides belonging to the myosuppressin family have been identified in locusts [8,17,18], flies [9,19] and moths [10,13,20]. All myosuppressins show a

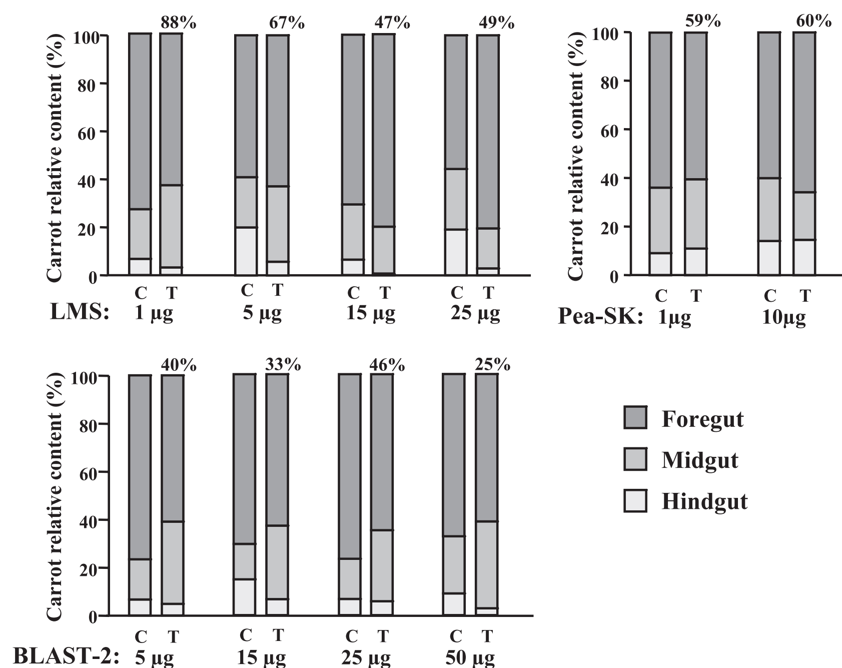


Fig. 5. Distribution of food (carrot) within the foregut, midgut or hindgut in *B. germanica* adult females treated with myoactive peptides: leucomyosuppressin (LMS), perisulfakinin Pea-SK, and the allatostatin BLAST-2. C: control specimens; T: treated specimens. Values at the top of "T" columns indicate the percentage of total carrot ingested by treated with respect to control animals (n = 10–12).

powerful inhibitory activity in visceral muscle contractions, e.g. in several gut regions of cockroaches [5,21], and in the oviduct of the migratory locust [17,22].

Immunocytochemical and in situ hybridization studies have revealed the occurrence of myosuppressins in interneurons and neurosecretory cells of the nervous system, neurohemal organs, like the corpora cardiaca and midgut endocrine cells [3,15,16,23]. The occurrence of myosuppressins in neurons innervating gut musculature is consistent with the myomodulatory role of these peptides. Moreover, other myosuppressin activities have been described, including the inhibition of adipokinetic hormone release in *Locusta migratoria* [24] and the stimulation of α-amylase enzymatic activity in the midgut lumen of the beetle *Rynchophorus ferrugineus* [25] and α-amylase and invertase enzymatic activities in the midgut lumen of the cockroach *D. punctata* [26]. LMS did not elicit any effect on α-amylase activity in the midgut lumen of *B. germanica* when tested at concentrations from 10^{-5} to 10^{-11} M, following the methodology described elsewhere [14] (results not shown).

In the adult female of *B. germanica*, LMS inhibited both the frequency (not shown) and amplitude of foregut and hindgut contractions. Both tissues were very sensitive to LMS action, showing ED_{50} in the 10^{-10} M range and complete inhibition of tissue contractions at doses higher than 10^{-9} M. Using the *B. germanica* foregut assay, we compared LMS with myosuppressins of other species, namely schistoFLRFamide [8] from Orthopterans, manducaFLRFamide [10] from Lepidopterans and neomyosuppressin (NMS) [9] from Dipterans. Although the sequences of these peptides and that of LMS differ by between 1 and 3 amino acids out of 10, the ED_{50} of LMS was 5- to 10-fold lower than for the peptides from other insect orders, which points to the relatively high specificity of the LMS receptor.

To test the hypothesis that a strong gut myoinhibitory factor produces the accumulation of food in the gut, which, in turn, inhibits food intake because of the persistence of the signals from gut stretch receptors, we studied the effect of LMS on *B. germanica* food intake. Results showed a dose-dependent activity with significant and considerable (50%) inhibition of food intake at a dose of 15 μg. The antialimentary activity of LMS in *B. germanica* was similar to that of perisulfakinin [7] and BLAST-2 allatostatin [14].

We then aimed to determine whether LMS-treated insects accumulate food in some compartment of the digestive tract as a consequence of the myoinhibitory action of the peptide. To this end, we estimated the pattern of food distribution among the foregut, midgut and hindgut of *B. germanica* adult females treated with effective doses of LMS. Food accumulated in the foregut and decreased in the hindgut in a dose-dependent manner, which agrees with the above hypothesis. In contrast, other myoactive peptides, like perisulfakinin and BLAST-2 allatostatin, which also show antialimentary activities [7,14], did not cause significant accumulation of food in the foregut, suggesting that they inhibit food intake through a mechanism different from that of LMS. Indeed, animals treated with BLAST-2 rather accumulated the ingested food in the midgut, which is consistent with the myoinhibitory action of these peptides in the hindgut [14]. In perisulfakinin-treated animals, the pattern of food distribution among the three compartments of the digestive tract is similar to that of controls, which suggests that this peptide behaves as a satiety factor through the central nervous system [7].

In *B. germanica*, LMS was obtained from brain extracts, which suggests that it is released into the hemolymph through the corpora cardiaca, and acts either humorally upon the digestive tract, or directly through the neurons that innervate gut musculature, or both. Although we failed to detect LMS in gut extracts, we cannot rule out its occurrence in gut endocrine cells, which would allow the direct paracrine action of the peptide in gut tissues. Myosuppressins have generally been detected in the digestive tract by immunocytochemistry and in situ hybridization [3,15], although the myosuppressin precursors F24 and F39 have been obtained by purification from gut extracts in *M. sexta* [13]. These data suggest that myosuppressins in gut tissues are scarce, subjected to fast metabolic turnover or both, which may explain our failure to detect them in gut extract analyses.

Acknowledgements

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