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Identification of a tachykinin-related peptide with orexigenic properties in the German cockroach

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ABSTRACT

A number of evidences suggest that tachykinin-related peptides (TRPs) of insects can stimulate food consumption after being released from the midgut to the hemolymph. The idea of the present work has been to test this hypothesis in the anautogenous cockroach *Blattella germanica*. First, we have identified the peptide LemTRP-1 (APSGFLGVR-NH_2) from brain extracts, by means of an ELISA developed with a polyclonal antibody against this peptide. ELISA studies have also shown that, whereas brain LemTRP-1 levels were fairly constant, midgut levels increase to a maximum on day 3 after adult emergence, falling thereafter until the end of the gonadotrophic cycle. Interestingly, maximum values of food consumption are concomitant with the decrease of LemTRP-1 immunoreactivity in the midgut. Furthermore, starvation decreases LemTRP-1 immunoreactivity in midgut, whereas in the hemolymph it increases. Finally, injection of synthetic LemTRP-1 to adult females significantly stimulates food consumption. The whole observations suggest that LemTRP-1 is released from the midgut to the hemolymph when sustained food consumption is required to maintain vitellogenesis at the highest levels, and that LemTRP-1 in the hemolymph stimulates food consumption in these days.

1. Introduction

Tachykinin-related peptides (TRPs) constitute a family of invertebrate peptides with a characteristic carboxy-terminal sequence $FX_1GX_2R\text{-}NH_2$. The name comes from their relative sequence similarity with vertebrate tachykinins, which show a conserved C-terminal sequence FXGLM-NH_2. In addition, TRPs and vertebrate tachykinins share other characteristics, as their occurrence in both nervous system and gut tissues, and their stimulatory activity of gut musculature contractions [20].

The first peptides belonging to the TRP family (Lom-TK I and II) were purified from brain-corpora cardiaca-corpora allata-suboesophageal ganglion extracts of the locust, *Locusta migratoria*, by monitoring their myotropic activity on cockroach hindgut [21]. Since then, peptides belonging to TRP family have been identified in insects belonging to Orthoptera, Diptera, Dictyoptera and Hymenoptera orders [20,23]. Furthermore, cDNAs encoding TRP precursors have been cloned and sequenced in the fruit fly *Drosophila melanogaster* [22], the mosquito *Anopheles gambiae* [18], the honeybee *Apis mellifera* [23] and the cockroaches *Leucophaea maderae* and *Periplaneta americana* [17]. In all cases, the cDNA sequence confirmed the identity of the previously reported peptides.

Immunocytochemical studies in cockroaches have revealed the occurrence of TRP immunoreactivity in interneurons of the

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central nervous system, in the stomatogastric nervous system, in processes to the corpora cardiaca glandular lobe, in nerves innervating different gut areas and in midgut endocrine cells [10,13]. This wide distribution has been observed in all the species tested [14], and suggests that TRPs have multiple functions. Indeed, a remarkable variety of activities has been reported for these peptides in insects, including stimulation of visceral and skeletal muscle contractions, induction of adipokinetic hormone release by corpora cardiaca, neuronal depolarization, stimulation of urine production, and induction of pheromone biosynthesis [6,14,20].

Furthermore, it has been demonstrated that the midgut of *L. maderae* and *L. migratoria* incubated in vitro release TRPs in response to an increase of K^+ levels in the bathing solution [25]. In *L. migratoria*, starvation increases the concentration of TRP-immunoreactive material in the hemolymph, concomitantly with a decrease in the midgut, which suggested that TRPs are released as hormones from the midgut, and that this release could be linked to the nutritional status [25]. These results make TRPs good candidates for being tested as orexigenic factors, under the hypothesis that its release in starved specimens might stimulate food consumption.

A good model to test this hypothesis would be the German cockroach, *Blattella germanica*, given that it is anautogenous and show a well-defined feeding cycle paralleling that of vitellogenesis [16]. The feeding cycle suggests that food intake is finely regulated, and possible regulatory mechanisms have been already reported. Thus, the peptide perisulfakinin has been identified as a putative satiety factor [8], and a number of YXFGL-NH_2 allatostatins, W^2W^9-amide myoinhibitory peptides and leucomyosuppressin have been shown to inhibit food intake in this cockroach [1–3]. Nevertheless, no information about factors stimulating food intake has been reported.

The aim of the present work has been to identify TRPs in *B. germanica*, and to study whether they may play a stimulatory role in the regulation of food intake. The reference peptide to search native TRPs in *B. germanica* was LemTRP-1 (APSGFLGVR-NH_2), which had been already identified in the cockroaches *L. maderae* and *P. americana* [11,12,17].

2. Materials and methods

2.1. Insect rearing

Adult females of *B. germanica* (L.) were obtained from a colony reared on dog chow and water, at 30 ± 1 °C and 60–70% r.h. Freshly moulted adult virgin females were isolated and used at the appropriate physiological ages within the first gonadotrophic cycle. Physiological age was assessed by measuring the basal oocyte length [4]. For starvation experiments, animals were supplied only with water since the imaginal moult.

2.2. Synthesis of peptides and conjugates

Peptides LemTRP-1: APSGFLGVR-NH_2, LemTRP-2: APEESPK-RAPSGFLGVR-NH_2, LemTRP-4: APSGFMGMR-NH_2 and LemTRP-5: APAMGFQGVR-NH_2 [11] were synthesized using standard Fmoc solid phase methods [5]. The identity and purity (ca. 90%) of each peptide were assessed by amino acid analysis, matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra and HPLC. To raise antibodies against LemTRP-1, the peptide was synthesized with its N-terminus extended by two residues of 2-aminohexanoic acid and conjugated to keyhole-limpet-hemocyanin (KLH) (Sigma, St. Louis, MO, USA) according to [19]. For ELISA plate coating, LemTRP-1 was conjugated to bovine serum albumin (BSA) (Sigma), using glutaraldehyde [24]. The conjugates were dialyzed, lyophilized and stored at −20 °C.

2.3. Antibody production and titer test

Three male white New Zealand rabbits were used to raise antibodies using LemTRP-1-KLH as immunogen. Rabbits were injected subcutaneously with 100 μg of peptide, in the conjugated form, diluted in 500 μl of water emulsified with 500 μl of Freud's complete adjuvant (Sigma) on days 0 and 7, and with incomplete adjuvant on day 14. Blood samples were obtained on day 21. Rabbits were boosted again once a month using the same dose and incomplete adjuvant, and serum was obtained 1 week after each booster injection, during 6 months. Serum was added with 0.1% thimerosal (Serva, Heidelberg, Germany) and stored at −20 °C. The titer of serum from each rabbit was determined by measuring the binding of serial dilutions to microtiter plates coated with 1 μg/ml of LemTRP1-BSA. A two-dimensional titration protocol was used for the screening and determination of the optimum concentration of both coating antigens and antisera to be used later in the competitive experiments [7].

2.4. ELISA method

LemTRP-1-BSA at a concentration of 0.15 μg/ml in 0.1 M carbonate–bicarbonate buffer (pH 9.6) was used for coating polystyrene 96 wells microtiter plates (Nunc Maxisorp, Roskilde, Denmark), in a volume of 100 μl/well, and incubated overnight at 4 °C. The plates were washed five times with PBST buffer (0.2 M, phosphate-buffered saline solution containing 0.05% Tween 20, pH 7.4). The plates were blocked with 1% polyvinylpyrrolidone (Sigma) in PBST buffer. After 1 h, plates were washed again as described above. The immunological reaction was initiated by adding dilutions of the samples or standard peptide analyte in PBST buffer (from 10^{-6} M to 10^{-10} M) in volume of 50 μl/well followed by 50 μl/well of the antibody previously diluted 1/30,000 in PBST buffer (final dilution in the well: 1/60,000). After incubation at room temperature for 2 h, the plates were washed as described above, and 100 μl/well of a 1/6000 diluted goat antirabbit IgG peroxidase conjugated (Sigma) solution were added. After 1 h incubation and a washing step, 100 μl/well of substrate solution were added and incubated in the dark with gentle shaking. Substrate solution was prepared with 12.5 ml of citrate buffer (pH 5), 200 μl of 0.6% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide and 50 μl of 1% H_2O_2. Reaction was stopped by adding 50 μl/well of 2N H_2SO_4. Absorbance was read at 450 nm with a Titertek Multiscan Plus MKII spectrophotometer (Labsystems, Helsinki, Finland). The calibration curves were analyzed using a four parameter logistic equation.

2.5. HPLC procedures

A total of 1440 brains from 5- to 7-day-old females were dissected out under saline solution (NaCl 9 g/l; KCl 0.2 g/l; $NaHCO_3$ 0.2 g/l; $CaCl_2$ 0.2 g/l), and homogenized in methanol/water/acetic acid (87/8/5, v/v/v), using a mechanical homogenizer Eurostar digital (Ika labortechnik, Staufen, Germany) 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. Brain extract was processed in five consecutive HPLC steps. LemTRP-1-immunoreactive fractions were detected using the above described ELISA and used for further purification. Steps 1, 2, and 3 were carried out with a Merck–Hitachi (Darmstad, Germany) low-pressure system, L-6200A pump with a L-4200 UV–vis detector. Steps 4 and 5 were carried out with a Waters (Milford, MA, USA) low-pressure system, 626 pump with a 600S controller and 996 PDA detector.

Step 1: Waters DeltaPak semi-preparative C_{18} column (300 mm × 7.8 mm, 15 μm, 300 Å). Linear gradient of CH_3CN/0.1% TFA. The gradient change was 1.67%/min and the flow rate 1.5 ml/min. HPLC fractions were analyzed with the ELISA described above, which revealed immunoreactive material in fractions eluting between 20.0% and 23.3% CH_3CN.

Step 2: Column and solvents as in step 1. The gradient change was 0.5%/min and the flow rate 1.5 ml/min. Immunoreactive material was detected in fractions eluting between 21.0% and 22.5% CH_3CN.

Step 3: Merck LiChroCART C_{18} column (125 mm × 4 mm, 5 μm, 100 Å). Linear gradient of CH_3CN/0.1% TFA. The gradient change was 0.25%/min and the flow rate 1.5 ml/min. Immunoreactive material was detected in fractions eluting between 18.5% and 19.2% CH_3CN.

Step 4: Waters DeltaPak C_{18} column (150 mm × 2 mm, 5 μm, 300 Å). Linear gradient of CH_3CN/0.05% TFA. The gradient change was 0.25%/min and the flow rate 0.2 ml/min. Immunoreactive material was detected in fractions eluting between 16.4% and 17.0% CH_3CN.

Step 5: Column and solvents as in step 4. The gradient change was 0.16%/min and the flow rate 0.2 ml/min. A single peak of immunoreactive material eluted at 21.1% CH_3CN.

For the chromatographic separation of hemolymph from 3-day-old fed and starved females, the HPLC system and column described for steps 1 and 2 of the brain extract separation were used. Samples of 155 μl and 229 μl of hemolymph from fed and starved females, respectively, were used in the HPLC separation. A linear gradient of CH_3CN/0.1% TFA with a linear change of 1%/min and a flow rate of 1.5 ml/min was carried out, and HPLC fractions were analyzed using the ELISA described above.

2.6. MS and sequencing

An aliquot of the purified LemTRP-1-immunoreactive peak was analyzed using an Applied Biosystems (Foster City, CA, USA) Voyager DE-RP MALDI-TOF mass spectrometer. The amino acid sequence of the purified factor was determined by Edman degradation using an Applied Biosystems Procise instrument.

2.7. Myotropic assay

LemTRP-1 was tested on foregut and hindgut of *B. germanica* females prepared in a standard organ bath as previously described [8]. 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. Myotropic 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.

2.8. Sampling for physiological observations

Brains and midguts were dissected under saline solution and homogenized in PBS. Then, samples were boiled for 5 min and centrifuged at 16,000 × *g* for 10 min. Supernatants were collected and the pellets were re-extracted, centrifuged again and the supernatants were pooled together with the previous ones, lyophilized and stored at −20 °C until use. Hemolymph samples to determine peptide concentration by HPLC separation and ELISA were obtained by cutting off one leg of the animal and applying gentle pressure to the abdomen. A pool from 110 and 150 fed and starved females, respectively was used for the determination. The hemolymph was collected and diluted in methanol/water/trifluoroacetic acid (90/10/0.1, v/v/v). After evaporation of organic solvent and lyophilization, samples were stored at −20 °C until use.

2.9. Measurement of food consumption

Food consumption was measured as reported previously [16], with some modifications. Briefly, individual specimens were provided with a weighed food (dog chow) portion, and 24 h later the remaining food was dried out in an oven and weighed again. The water lost by evaporation of food placed in a control box, containing only the water vial, was used as a correction factor. In this case, instead of using the oviposition time for realigning the results to a 8 days scale as in Ref. [16], food consumption was quantified in chronological periods of 24 h throughout the gonadotrophic cycle.

2.10. Feeding bioassay

The feeding bioassay was carried out as previously reported [8], although with some modifications. Freshly ecdyzed adult females were fed with carrot for 24 h, after which they were injected with saline or with the testing peptide and provided again carrot ad libitum. After 5 h, the gut was dissected out and the foregut, midgut and hindgut regions were separated by sectioning just before the gastric caeca and at the level of malpighian tubules evagination. Each gut region together with its content was extracted with methanol, and carotenoid concentration was estimated by spectrophotometric measurement of the absorbance at 450 nm. Total weight of carrot ingested was quantified by interpolation on a standard curve constructed using methanolic extracts of increasing amounts of lyophilized carrot.

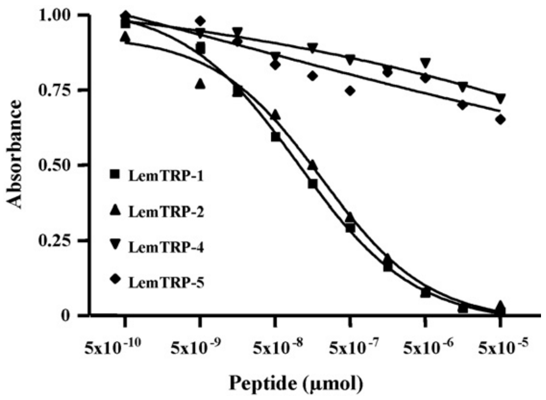

Fig. 1 – Test of the cross-reactivity of LemTRP-1 antiserum to synthetic peptides: LemTRP-1, 2, 4 and 5. Each measurement was in duplicate.

3. Results

3.1. ELISA characterization

Fig. 1 shows the sensitivity curve obtained with different concentrations of LemTRP-1, and the antiserum used at a dilution of 1:60,000. The ED_{50} (concentration required for 50% displacement of binding to conjugate) was 1.15×10^{-7} µmol.

In order to study the selectivity of the antibody, cross-reactivity assays using anti-LemTRP-1 serum with synthetic LemTRP-2, -4 and -5 were carried out (sequences indicated in Section 2.2). Results indicate that antiserum shows high cross-reactivity with LemTRP-2, comparable to that with LemTRP-1. Conversely, cross-reaction with LemTRP-4 and -5 is very low (Fig. 1). The limit of detection of standard LemTRP-1 was around 5×10^{-9} µmol.

3.2. Purification and identification of LemTRP-1

A crude extract of 1440 brains from adult females of *B. germanica* was processed through five consecutive HPLC steps, using LemTRP-1 immunoreactivity for monitoring the fractions of interest. In the last step, an immunoreactive homogeneous peak resulted in the nonapeptide APSGFLGVR-NH_2, identical to LemTRP-1 previously identified from the cockroaches *L. maderae* and *P. americana*. The final yield was approximately 110 pmol. We presume that the C-terminus was amidated on the basis of the MS analysis, which showed a molecular mass of 902.52 (MH^+). The identification was further confirmed by the coelution in HPLC of the native peptide with synthetic APSGFLGVR-NH_2.

3.3. Myostimulatory activities on gut tissues

Given that tachykinins and TRPs have been described as myotropic peptides in both vertebrates and insects, we first assessed this biological effect in our insect model. Thus, we studied the effects of LemTRP-1 on foregut and hindgut motility in *B. germanica*, using a standard organ bath system. Results (Fig. 2) showed that LemTRP-1 elicits myostimulatory activity on both gut regions, with an ED_{50} for the force increase within the nanomolar range.

3.4. LemTRP-1 levels and feeding rhythm

LemTRP-1 levels were quantified by ELISA in extracts of individual brains and midguts of virgin females during the first gonadotrophic cycle. The profile of LemTRP-1 immunoreactivity in the brain (Fig. 3) shows fairly constant levels (between 40 pg/brain equivalent and 60 pg/brain equivalent), throughout the whole period. In contrast, LemTRP-1 levels in the midgut starts to rise from day 1, reaches the maximal value on day 3 (ca. 70 pg/midgut equivalent), and decreases thereafter, showing relative low levels until day 7, when chorionation and oviposition occurs (Fig. 3).

Quantification of food ingestion during the first gonadotrophic cycle indicates that *B. germanica* females undergo a food consumption cycle, with maximum values (ca. 10 mg/ 24 h) observed between days 3 and 6, decreasing thereafter until the time of oviposition and ootheca formation (Fig. 3).

3.5. Effect of starvation and feeding on LemTRP-1 levels

To study the effect of starvation, brain, midgut and hemolymph from fed and starved 3-day-old *B. germanica* females were extracted, and LemTRP-1 immunoreactivity was measured by ELISA. Results show no differences in LemTRP-1 immunoreactivity between brains from fed and starved females (Fig. 4A). Conversely, 57% significant reduction in LemTRP-1 levels was observed in midgut from starved females when compared to the midgut of fed controls (Fig. 4A).

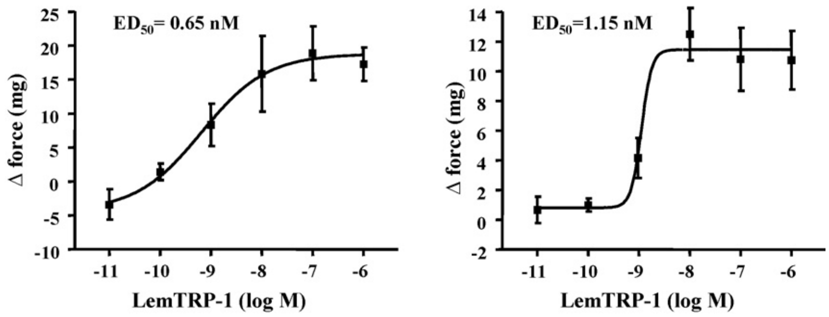

Fig. 2 – Stimulatory effect of LemTRP-1 on foregut (left) and hindgut (right) motility in *B. germanica* females. Results (mean ± S.E.; *n* = 4–7) are expressed as the difference of the mean of the force produced by the tissue during 1 min after and before the treatment. The ID_{50} for each tissue is indicated.

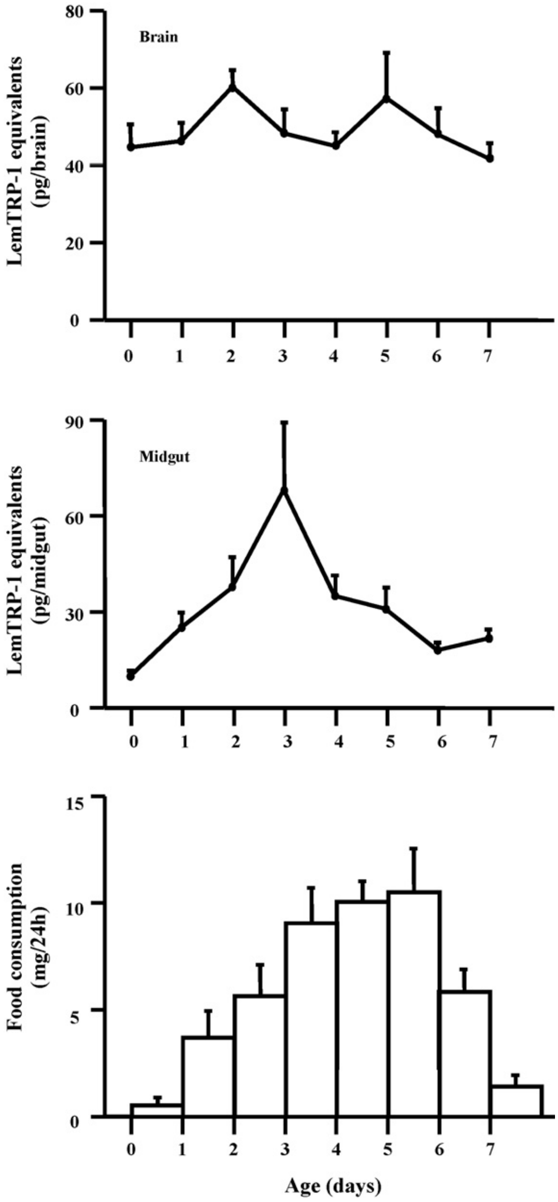

Fig. 3 – **LemTRP-1 equivalents in brain (n = 7–14) and midgut (n = 8) from *B. germanica* females throughout the first gonadotrophic cycle. Food consumption during the same period (n = 11). Results are expressed as the mean ± S.E.**

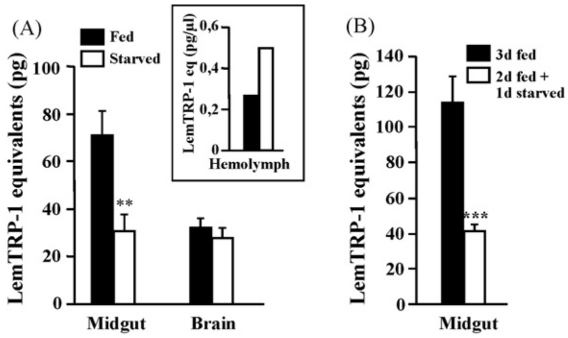

Fig. 4 – (A) LemTRP-1 equivalents in brain and midgut from 3-day-old fed and starved *B. germanica* females (n = 15–17). Inset: LemTRP-1 equivalents/µl of hemolymph, in immunoreactive fractions from HPLC separations of 155 µl of hemolymph from 3-day-old fed, and 229 µl from 3-day-old starved *B. germanica* females. The retention time of the immunoreactive fractions coincided with that of synthetic LemTRP-1. (B) LemTRP-1 equivalents in midgut from 3 days fed and 2 days fed + 1 day starved *B. germanica* females (n = 8). For A and B results are expressed as the mean ± S.E. Asterisks indicate significant differences (Student's t-test) ($P < 0.005$; ***$P < 0.0005$).**

Hemolymph samples were separated by HPLC before being submitted to ELISA quantification. A single peak of LemTRP-1 immunoreactive material, coeluting with the synthetic LemTRP-1, was detected in the HPLC separation of hemolymph from both fed and starved 3-day-old females. The amounts of LemTRP-1 equivalents per µl of hemolymph were 0.266 pg and 0.496 pg for fed and starved females, respectively (Fig. 4, inset).

In further experiments, we compared LemTRP-1 immunoreactivity in midguts from 3-day-old fed control females with midguts from 3-day-old females which have fed for the first 2 days, and starved during the third day. Results showed 64% reduction on LemTRP-1 levels in midguts from the group that had been starved the third day (Fig. 4B).

3.6. *Effects of LemTRP-1 on food intake*

The effects of synthetic LemTRP-1 were tested on adult females at doses of 25 µg and 50 µg per specimen on food intake in *B. germanica*, using the carrot feeding bioassay. Both doses resulted in ca. 120% significant increase of food content in the foregut. This asymptotic level of response suggests that the increase observed is the maximum that can be reached in our experimental conditions. Conversely, no significant differences were observed either in the midgut or in the hindgut (Fig. 5), as expected, given the short duration (5 h) of the assay.

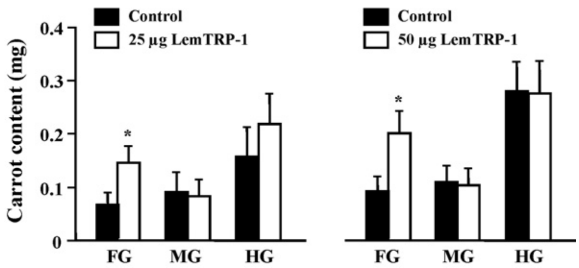

Fig. 5 – Food (carrot) content within the foregut (FG), midgut (MD) and hindgut (HD) in control and LemTRP-1-treated *B. germanica* adult females. Results are expressed as the mean ± S.E. (n = 24). The asterisk indicates significant differences (Student's t-test) ($P < 0.05$).

4. Discussion

In order to identify TRPs in the cockroach *B. germanica*, we developed an ELISA using a polyclonal antibody raised against the peptide LemTRP-1 (APSGFLGVR-NH_2). The limit of detection of LemTRP-1 was around 5×10^{-9} μmol (Fig. 1), which allowed the quantification of LemTRP-1 immunoreactivity in crude extracts of individual brains and midguts. Cross-reactivity tests show that the LemTRP-1 antiserum is specific to the C-terminal tetrapeptide -LGVR-NH_2, given that it cross-reacts with LemTRP-2, which is a C-terminal extended version of LemTRP-1, whereas cross-reaction with LemTRP-4 or -5, which have a different N-terminal sequence (sequences indicated in Section 2.2), is very low (Fig. 1). The ELISA helped to isolate the peptide LemTRP-1 from brain extracts of the cockroach *B. germanica*. The peptide LemTRP-1 had previously been identified in midgut and brain of the cockroaches *L. maderae* and *P. americana* [11,12,17].

In the adult female of *B. germanica*, LemTRP-1 stimulates foregut and hindgut contractions, with ED_{50} values within the nanomolar range for both gut regions (Fig. 2). This is not surprising given that TRPs were originally detected in the locust *L. migratoria* using *L. maderae* hindgut myotropic assay [21]. Nevertheless, although ED_{50}s for LemTRP-1 were similar to the values found for proctolin and sulfakinins tested in *B. germanica* tissues [8], the absolute values of force increase are lower for LemTRP-1 than for those peptides [8]. The stimulatory activity of LemTRPs on *L. maderae* hindgut contractions gave an ED_{50} around 10 nM [27]. In the cockroach *P. americana*, TRPs stimulate foregut muscle contractions, with an ED_{50} around 5 nM, but TRPs failed to activate foregut contractions in the cockroaches *L. maderae* and *Nauphoeta cinerea* [15]. These results point to foregut sensitivity differences between the more primitive cockroach families Blattidae (*P. americana*) and Blattellidae (*B. germanica*) and the more modified Blaberidae (*L. maderae* and *N. cinerea*).

ELISA studies showed that LemTRP-1 levels in the brain of *B. germanica* females are quite constant throughout the first gonadotrophic cycle (Fig. 3). In *L. maderae*, a large number of TRP-immunoreactive interneurons in the proto-, deuto- and tritocerebrum, supplying processes to most part of the brain, in addition to a few number of protocerebral neurons which send immunoreactive processes to the glandular lobe of the corpora cardiaca, have been reported [13]. The distribution of TRP immunoreactivity in the cockroach brain suggests that these peptides may play neuromodulatory roles [13], which seems compatible with the fairly constant expression observed in *B. germanica* brain.

In contrast, LemTRP-1 immunoreactivity in the midgut shows marked changes during the gonadotrophic cycle. In the midgut, LemTRP-1 levels increase from the day of emergence to a maximum on day 3, and then, they steadily decrease until day 6 (Fig. 3). In *L. maderae*, TRP-immunoreactivity has been detected in a fairly dense supply of varicose fibres in the wall of the midgut, which originated in the stomatogastric nervous system, and in numerous midgut endocrine cells [13].

B. germanica females show a cyclic pattern of feeding throughout the gonadotrophic cycle (Fig. 3). Maximum values are observed between days 3 and 6 (with food consumption values of ca. 10 mg/day, Fig. 3), a period coincident with the maximum of vitellogenin production [9], which is one of the most energy-consuming process in the adult female. Interestingly, maximum values of food consumption are concomitant with TRP-like immunoreactivity decrease in the midgut. A dual hypothesis arises from these observations: (1) TRPs are released from the midgut to the hemolymph when sustained food consumption is required to maintain vitellogenesis at the highest levels (between days 3 and 6 of the gonadotrophic cycle), and (2) TRPs in the hemolymph stimulate food consumption in these days.

In support of that hypothesis we found that while LemTRP-1 immunoreactive brain levels do not change in starved animals (Fig. 4A), starvation decreases LemTRP-1 immunoreactivity in midgut, whereas increases it in the hemolymph (Fig. 4A and inset), and that 24 h of starvation are enough to produce that effect (Fig. 4B). In *L. maderae*, TRPs have not been detected in typical neurohemal organs, and, therefore, the source of TRPs detected in the hemolymph seems to be the midgut endocrine cells [13,25]. Congruently with that, it has been demonstrated that increasing levels of K^+ in the bathing saline, elicited the release of TRPs from isolated midguts of *L. migratoria* and *L. maderae* [25]. In *L. migratoria* starvation also resulted in a decrease of TRPs in the midgut and an increase in the hemolymph [25]. In the locust, although the concentration increase in the hemolymph might be a consequence from the reduced hemolymph volume in starved specimens, it was concluded that TRPs were released from midgut in relation with nutritional stress [25]. In the present work, starved *B. germanica* females were provided with water ad libitum, and the hemolymph volumes obtained when sampling fed and starved animals were similar, which indicates that hemolymph volumes were comparable in fed and starved specimens.

Also in support of the above dual hypothesis are the stimulatory effects of LemTRP-1 on food consumption observed in our quantitative carrot feeding assays. Food content in the foregut was significantly higher in LemTRP-1-treated animals, which indicates a stimulation of food intake during the 5-h treatment period (Fig. 5). The similar results between treated and controls observed in midgut and hindgut contents (Fig. 5) are explained by the short duration of the assay. Carrot contents in these compartments correspond to food ingested before the treatment. The fact that LemTRP-1 immunoreactivity in the hemolymph of *B. germanica* females corresponds to the peptide LemTRP-1 is suggested by the occurrence of a single immunoreactive peak in the hemolymph HPLC separations, and the coincidence of the retention time of this peak with the retention time of synthetic LemTRP-1.

Thus, our observations in *B. germanica* strongly suggest that TRPs are released from midgut to the hemolymph, and that circulating TRPs contribute to maintain high levels of food consumption, especially in periods of high energetic demand, like during full vitellogenesis. This does not rule out, of course, the possibility that TRPs are involved in regulating other processes. For example, in *D. melanogaster*, the use of a RNAi construct to silence TRP gene expression specifically in the nervous system has demonstrated that these peptides modulate odor perception and locomotory activity [26]. Although odor perception may be closely related to food intake, knocking-down of TRPs gene expression on midgut would provide a more specific approach to demonstrate orexigenic roles for TRPs in insects.

Finally, the stimulation of food consumption induced by LemTRP-1 in *B. germanica* is mirrored by the inhibitory effects induced by other peptides, like leucomyosuppressin [1] and perisulkakinin [8] in the same cockroach. This suggests that food consumption in *B. germanica* is finely tuned by the concerted regulatory actions of stimulatory and inhibitory factors.

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