

Tachykinin stimulation effects on α -amylase, protease and lipase activities in midgut of American cockroach, *Periplaneta americana* (Blattodea: Blattidae)

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Abstract

The effect of a neuropeptide, the tachykinin on α -amylase, protease and lipase activities in the midgut of American cockroach, *Periplaneta americana* (Linnaeus) is evaluated. Immunohistochemical reactivities against tachykinin (tachykinin-ir) were detected in the midgut. Tachykinin-ir cells in three regions (anterior, median and posterior) of the midgut were counted in normally fed, starved for four weeks and re-fed cockroaches. The number of tachykinin-ir cells decreased during 4 weeks of starvation and increased 3 h after refeeding. The results of the competitive ELISA were consistent with the profile of immunohistochemical reactivity. Incubation of the dissected midgut with tachykinin led to an increase in α -amylase, protease and lipase activities while the buffer remained ineffective. Tachykinin injection into the hemocoel increased α -amylase, protease and lipase activities, but PBS injection showed no sign of effectiveness. The results suggest that nutrients upregulate midgut cells secreting tachykinin causing further activities of digestive enzymes.

Keywords: Tachykinin, Digestive enzymes, Midgut, *Periplaneta Americana*

چکیده

تاکیکینین: تحریک کننده فعالیت آنزیم‌های آلفا آمیلاز، پروتئاز و لیپاز در معده میانی سوسری آمریکایی (*Periplaneta americana*) (Blattodea: Blattidae)
اعظم میکانی

در این تحقیق به بررسی اثر تاکیکینین بر روی فعالیت آلفا آمیلاز، پروتئاز و لیپاز در معده میانی سوسری آمریکایی (*Periplaneta americana* (L.)) پرداخته شد. فعالیت ایمونوهیستوشیمیایی سلول‌های معده میانی (بخش‌های جلویی، میانی و عقبی) که تاکیکینین ترشح می‌کردند مورد تایید قرار گرفت. چهار هفته گرسنگی، تعداد سلول‌هایی را که عکس‌العمل ایمونوهیستوشیمیایی مقابل آنتی‌بادی تاکیکینین داشتند (Tachykinin-ir cells) را کاهش داد، در حالی که تغذیه دوباره پس از سه ساعت Tachykinin-ir cells را افزایش داد. آزمون الایزا نیز نتیجه فوق را تایید کرد. قرار دادن معده میانی در بافر حاوی تاکیکینین منجر به افزایش فعالیت آلفا آمیلاز، پروتئاز و لیپاز شد در حالی که بافر به تنهایی هیچ اثری بر روی فعالیت آنزیم‌های فوق نداشت. تزریق تاکیکینین به همولنف حشره منجر به افزایش فعالیت آنزیم‌های فوق در معده میانی شد، در حالی که تزریق بافر هیچ تاثیری بر روی فعالیت آنزیم‌ها نداشت. به طور کلی می‌توان نتیجه گرفت که تغذیه باعث ترشح تاکیکینین از سلول‌های معده میانی شده که آن نیز به نوبه خود فعالیت آنزیم‌های آلفا آمیلاز، پروتئاز و لیپاز را افزایش می‌دهد.

واژگان کلیدی: تاکیکینین، آنزیم‌های گوارشی، معده میانی، *Periplaneta americana*

Introduction

The American cockroach, *Periplaneta americana* (L.), is omnivorous meaning that its midgut has an ability to recognize and digest many kinds of foods due to presence of digestive enzymes such as α -amylase, protease, maltase, lactase and lipase (Matsui *et al.*, 2013).

Insect neuropeptides are involved in most physiological and developmental processes, including molting, growth, metabolism (Wielendaele *et al.*, 2013) and feeding (Maestro & Bellés, 2006). Crustacean cardioactive peptide (CCAP) up-regulates digestive enzyme activities in response to nutrient ingestion in *P. americana* (Sakai *et al.*, 2006). In contrast, short neuropeptide F (sNPF) injection into the hemocoel led to a decrease in α -amylase, protease and lipase activities, whereas PBS injection had no effects (Mikani *et al.*, 2012). Tachykinin-like peptides

constitute a large and diverse family, found in both vertebrates and invertebrates. Substance P, the major mammalian tachykinin, was discovered in 1931 as a factor causing stimulation of intestinal muscle contractions (Von Euler & Gaddum, 1931). Since then, many other tachykinins have been characterized from a large variety of vertebrate and invertebrate species. A number of tachykinins have been isolated from locust, cockroach, blowfly and mosquito (Nassel, 1999). As we mentioned before, the first bioactivity demonstrated for the insect tachykinins was stimulation of contractions in locust oviduct and cockroach hindgut (Sliwowska *et al.*, 2000). Later, many other functions were shown for tachykinin family including neurostimulatory action on identified neurons in locust thoracic ganglia (Lundquist & Nassel, 1997), stimulatory effect on release of adipokinetic hormone in locust corpora cardiaca (Nassel *et al.*, 1995) and

myostimulatory effect on Malpighian tubules (Coast., 1998). Later numerous tachykinin immunoreactivity (tachykinin-ir) cells were found in the midgut of the cockroach, *Leucophaea maderae* (Winther & Nassel, 2001). Tachykinins have been isolated from many insects including locusts, cockroaches, mosquitos and blow flies. Based on immunocytochemical data, insect tachykinins are predominantly located in interneurons within the central nervous system and in endocrine cells of the intestine (Kwok *et al.*, 2005), suggesting a role in neuromodulation and in gut function. The first bioactivity demonstrated for the insect tachykinin was stimulation of contractions in locust oviduct (Schoofs *et al.*, 1993). Later it has been shown that cockroach hindgut contractions are also stimulated by tachykinin (Sliwowska *et al.*, 2001).

The insect midgut endocrine cells contain a multitude of peptides which modulate digestive processes and gut motility (Pabla & Lange, 1999). Tachykinins are reported as midgut neuropeptides that regulate midgut contractions (Lange & Orchard, 1998). Here, for the first time, we investigated the influence of tachykinin on digestive enzyme (α -amylase, protease and lipase) activities in the American cockroach, *Periplaneta americana*. We performed *in vivo* and *in vitro* tachykinin treatments and then measured the levels of digestive enzyme activities to show the effect of tachykinin on digestive enzyme activities.

Materials and Methods

Insects

Laboratory cultures of *P. americana* were maintained at 25°C under a light/dark period of 12:12 h, fed with an artificial diet (MF; Oriental Yeast, Tokyo, Japan) and water and libitum. Adult males 3-5 days after emergence were starved and kept individually for 4 weeks in clear plastic cups (10.0 cm diameter, 4.5 cm in height) with water. After 4 weeks of starvation, they were re-fed for 3 h. Cockroaches were anesthetized by cooling on ice before dissection. The midgut from each cockroach was dissected for subsequent experiments.

Digestive enzyme assay: α -amylase, protease and lipase

α -Amylase assay

α -Amylase activity of the midgut was measured as described previously (Mikani *et al.*, 2012) using an

α -amylase measuring kit (Kikkoman Corp. Chiba, Japan). Briefly, after dissection of the cockroach midgut in 50 mM Tris-HCl (pH 7.4) and removing of lumen content, it was incubated in 50 mM Tris-HCl (pH 7.4) at room temperature for 30 min in the presence or absence of different concentrations of tachykinin in order to release α -amylase activity into the medium. Samples (50 ml) were incubated at 37°C for 10 min, with 250 ml substrate buffer which contains 2-chloro-4-nitrophenyl 65-azido-beta maltopentaoside (N3-G5-CNP) and 250 ml co-working enzyme solution which contains glucoamylase and beta-glucosidase. Then, the reaction was stopped by adding 2.0 mL of stop solution containing sodium carbonate. In all assays, α -amylase activity was calibrated proportionally to protein concentration. One unit (U) of α -amylase activity was defined as the amount of enzyme that produces 1 μ mol 2-chloro-4-nitrophenol (CNP) from N3-G5-CNP for 1 min at room temperature. The absorbance for CNP was measured at 400 nm.

Protease assay

Protease activity of the midgut was measured by digestion of azocasein according to the method of Elpidina *et al.* (2001) with some modifications. Each cockroach midgut was dissected in 50 mM Tris-HCl (pH 7.4). After removal of luminal contents, the midgut was incubated in 50 mM Tris-HCl (pH 7.4) at room temperature for 20 min in presence or absence of different concentrations of tachykinin. Sample (300 μ l) was incubated with 300 μ L of 0.5% (w/v) azocasein solution in Tris-HCl (pH 7.4) at 37°C for 30 min. The reaction was stopped by adding 800 μ L of 20% trichloroacetic acid (TCA) and cooled on ice for 10 min. Then, the mixture was centrifuged (4000 \times g at 4°C, 15 min) to remove the precipitated azocasein. The absorbance of the supernatant was measured at 335 nm. One unit (U) of hydrolytic activity of the protease was defined as the amount of enzyme required to cause an increase of 0.01A₃₃₅ units per min in 1ml of reaction mixture. BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA) with bovine serum albumin F-V (BSA, Nacalai Tesque Inc., Kyoto, Japan) as a standard was used for determination of protein for each sample.

Lipase assay

Lipase activity of the cockroach midgut was measured using a lipase measuring kit (Quantichrom™ Lipase Assay Kit, BioAssay System, USA) as described previously (Mikani *et al.*, 2012). Each cockroach midgut was dissected in phosphate-buffered saline (PBS; 145 mM NaCl, 1.45 mM NaH_2PO_4 , 8.55 mM Na_2HPO_4 , pH 7.5). After removing luminal contents, the midgut was incubated in cold PBS for 30 min in the presence or absence of tachykinin. Lipase activity released into the medium was quantified. In order to preparing working reagent, 5 mg of color reagent was mixed in 140 μL of assay buffer and 8 μL of dimercaptopropanol tributryrate (BALB) reagent. 10 μL of each sample was mixed well with 140 μL of working reagent. The assay was based on an improved BALB method, in which SH groups formed from lipase cleavage of BALB react with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The absorbance was measured at 412 nm using a microplate reader (SH-9000, Corona Electric, Ibaraki, Japan). One unit of enzyme catalyzed the cleavage of 1 μmol of substrate per minute under the assay conditions.

Tachykinin injection

Different concentrations of tachykinin which was purchased from Chinapeptides Co., Ltd. in 4 μL PBS was injected into the hemocoel of adult cockroaches using a Hamilton syringe (Hamilton, NV, U.S.A). Control insects were injected with 4 μL PBS. The puncture made by the injection was sealed with the instant adhesive, Aron Alpha (Toagosei, Tokyo, Japan). The injection was applied for assessing the effect of tachykinin on digestive enzyme activities.

Competitive Elisa

Competitive ELISA was performed as described previously (Mikani *et al.*, 2012). Briefly, cockroaches were anesthetized by cooling on ice before dissection. The midgut was isolated and food particles were removed. Then the isolated midguts were homogenized individually in Tris buffered saline (TBS; 135 mM NaCl, 2.6 mM KCl, 25 mM Tris-HCl, pH 7.6). After centrifugation ($4000 \times g$, 4 °C, 15 min), the supernatant was used for competitive ELISA. A tachykinin-BSA conjugate was prepared by coupling tachykinin to BSA with dimethyl suberimidate (Aldrich, Switzerland). The Polystyrene microtiter

plates (96 wells) (Corning Incorporated, USA) were prepared with tachykinin-BSA (0.6 $\mu\text{g}/\text{ml}$ per well) in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.0) for 3 h and then unwanted adsorption was blocked with 250 μL of 2% skimmed milk to each well followed incubation at room temperature for 1 h. Standard peptides (0.01–100 nmol/well) or midgut supernatant samples were added in a volume of 50 $\mu\text{L}/\text{well}$. Subsequently, 50 μL of the diluted antiserum against tachykinin (1:9,500 final concentrations in TBS with 2% skimmed milk) were added to the wells. The plate was incubated overnight at 4 °C with gentle shaking. After incubation, the plate was washed three times with TBS containing 0.5% Tween-20 (TBS-Tw) and then incubated with 100 μL of secondary antibody solution containing goat immunoglobulin anti-rabbit IgG labeled with alkaline phosphatase at 1:1000 in TBS at room temperature for 1 h. After three times washes, 100 μL of substrate solution (1 mg/ml p -nitrophenylphosphate disodium salt hexahydrate in 10mM diethanolamine buffer, pH 9.5) was added to each well and incubated for 1 h. The color reaction was stopped by adding 50 μL of 4M NaOH. Finally, the absorbance was read at 405 nm (SH-9000, Corona Electric, Ibaraki, Japan).

Immunohistochemistry

Immunohistochemistry was performed as described previously (Sehadova *et al.*, 2007). The midgut of young adult males were dissected in PBS buffer and was fixed at 4 °C overnight in Bouin solution (15 vol. picric acid, 5 vol. formalin, 1 vol. acetic acid). Standard histochemical techniques were used for tissue dehydration, embedding in paraffin, sectioning to 7 μm , deparaffinization and rehydration as previously described (Sehadova *et al.*, 2007). The sections were blocked with 1.5% normal goat serum diluted in Tris-buffered saline (TBS) for 30 min at room temperature (RT). Primary antibody used was rabbit anti-tachykinin (used at 1:2000 dilution; Genemed Synthesis, Inc., CA, USA). The sections were incubated with primary antibody diluted with blocking serum (1:2000) in a humidified chamber overnight at 4 °C. After three times rinses with TBS (10 min each time), the sections were incubated with biotinylated anti-rabbit IgG that was diluted with blocking serum (1:200) for 1.5 h at room temperature. After three times rinses with TBS (10 min \times 3) the sections were incubated for 30 min with

VECTASTAIN ABC reagent (Vectastain ABC KIT PK-6101). After the slides were rinsed three times with TBS (each time, 10 min) and once with 0.1 M Tris-HCl, pH 7.5, for 10 min, immunoreactivity was visualized by rinsing the sections in 144 mL of diaminobenzidine tetrahydrochloride (DAB) solution (DAB; 0.25 mM in 0.1 M Tris-HCl, pH 7.5, 144 mL, 30% H_2O_2 , 30 μL) for 8 min. The sections were dehydrated through an ethanol-xylene series and then mounted in Bioleit medium (Kouken Rika, Osaka, Japan). Finally the slides were observed using a BX50F4 microscope (Olympus, Tokyo, Japan).

Morphometric analysis

The immunoreactive cells were assessed using the point counting method (Sakai *et al.*, 2006). Ten sections of the midgut from each cockroach were randomly selected from 50 sections. A grid lattice was put on the image of immunostained midgut sections and the number of points covering immunoreactive cells counted. Data was reported as the number of tachykinin-endocrine cells per 100 points of its falling on the midgut epithelium.

2.7. Statistical analysis

The results are expressed as mean \pm SEM. $p < 0.05$ was considered the level of significant difference between means by one-way ANOVA (Fishers, LSD).

Results

Tachykinin in the midgut

Fig. 1A shows tachykinin-ir cells in the epithelium of normally fed cockroach. The number of the cells decreased after 4 weeks of starvation (Fig. 1B).

The number of the tachykinin-ir cells in three regions of the midgut were counted in normally fed, starved for four weeks and refeed cockroaches. The result showed that the number of tachykinin-ir cells decreased during 4 weeks of starvation and increased 3 h after refeeding (Fig 2).

The results of the competitive ELISA, to evaluate tachykinin titer in the midgut homogenates of individual normally fed, starved for four weeks and refeed cockroaches were consistent with the profile of immunohistochemical reactivity. Tachykinin titer in the midgut extracts were sharply dropped after four weeks of starvation. The titer increased 3 h after refeeding (Fig. 3).

Stimulatory effect of tachykinin on digestive enzyme activity in the midgut

Incubation of the dissected midgut with tachykinin at concentrations equal or greater than 10^{-7} M induced significant increase in α -amylase activity (Fig. 4 A). The effect of incubation of the midgut with tachykinin on protease and lipase activities were also evaluated. Tachykinin also increased protease and lipase activities at concentration equal or greater than 10^{-8} M, whereas the buffer alone had no effect on these digestive enzyme activities (Fig 4B, C).

Effect of tachykinin injection into the hemocoel on digestive enzyme activity in the midgut

Tachykinin injection equal to or greater than 10^{-12} moles increased α -amylase activity more than twice (Fig. 5 A). The injection of 10^{-11} moles tachykinin also led to an increase in protease and lipase activities whereas PBS injection had no effect on enzyme activities (Fig. 5 B, C).

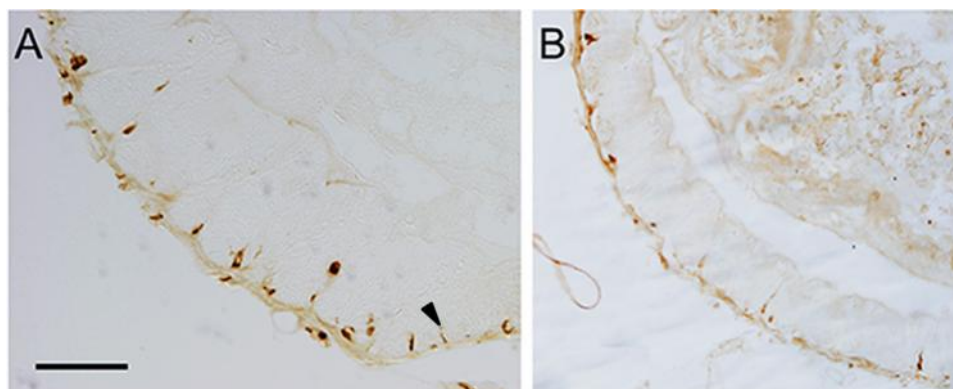


Fig. 1. Tachykinin-ir cells in the midgut epithelium. A, tachykinin-ir in normally fed adult male cockroach. B, tachykinin-ir cells in the midgut epithelium of starved for 4 weeks adult male cockroach. Scale bars, 48.0 μm .

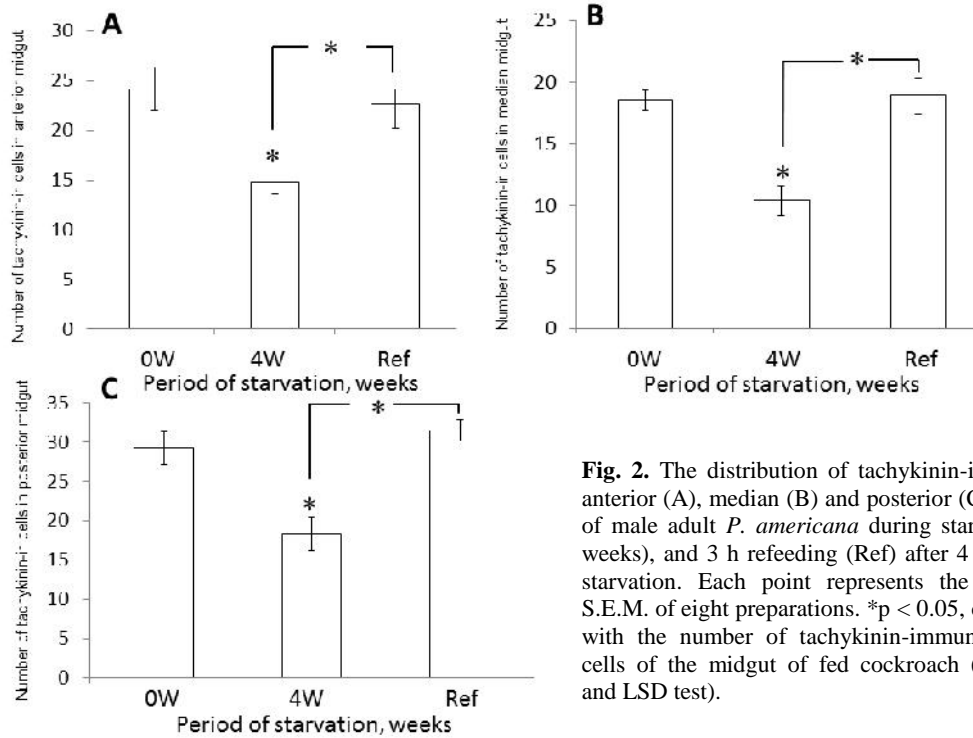


Fig. 2. The distribution of tachykinin-ir cells in anterior (A), median (B) and posterior (C) midgut of male adult *P. americana* during starvation (4 weeks), and 3 h refeeding (Ref) after 4 weeks of starvation. Each point represents the mean \pm S.E.M. of eight preparations. *p < 0.05, compared with the number of tachykinin-immunoreactive cells of the midgut of fed cockroach (ANOVA and LSD test).

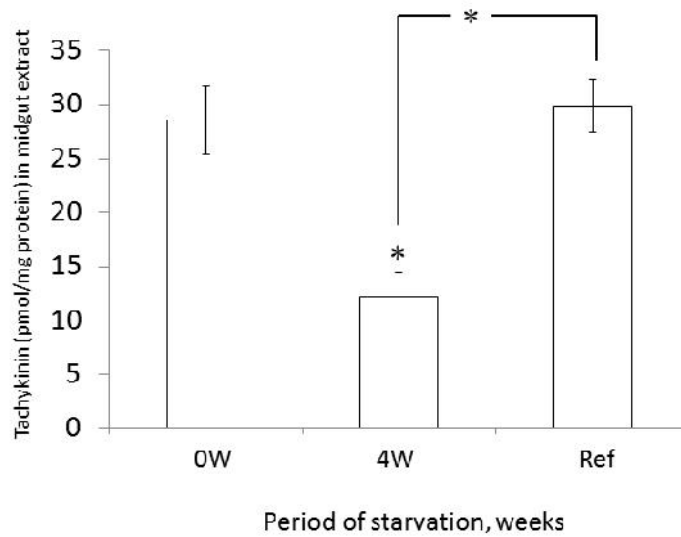


Fig. 3. A competitive ELISA detected tachykinin titer in the supernatant of midgut of male *P. americana* starved for 4 weeks and refeed for 3 h. Values are expressed as average tachykinin of 10 replicates. Each point represents the mean \pm S.E.M. *p < 0.05, compared with the value of the fed cockroach (LSD test).

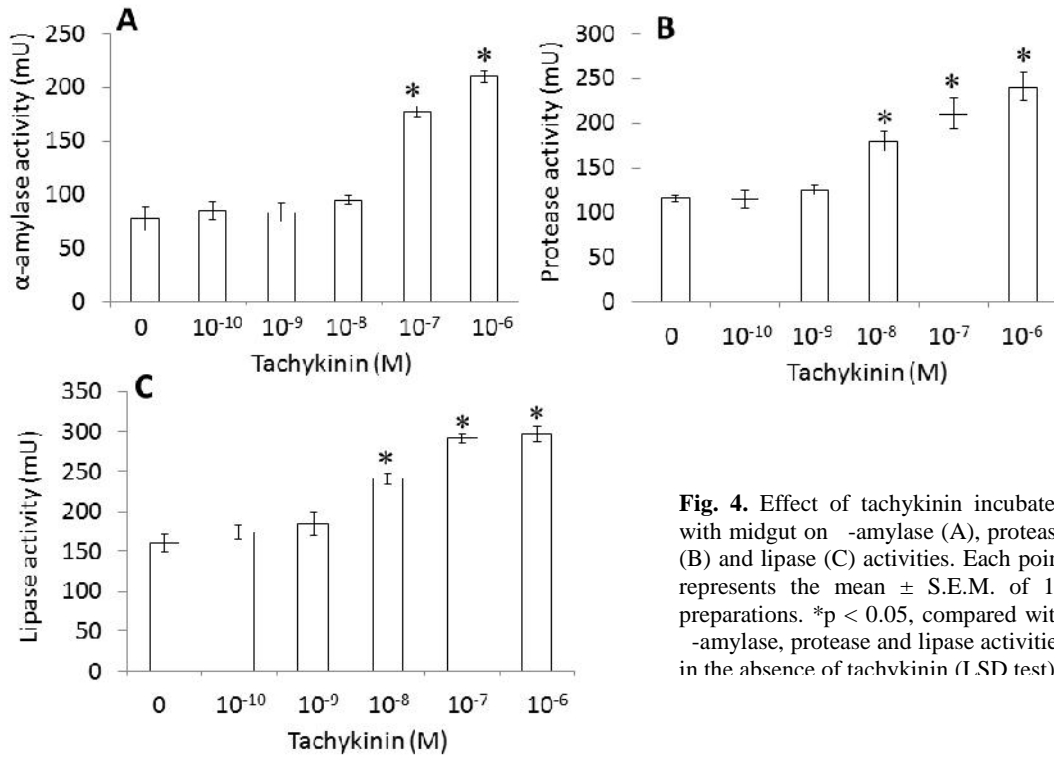


Fig. 4. Effect of tachykinin incubated with midgut on α -amylase (A), protease (B) and lipase (C) activities. Each point represents the mean \pm S.E.M. of 10 preparations. * $p < 0.05$, compared with α -amylase, protease and lipase activities in the absence of tachykinin (LSD test).

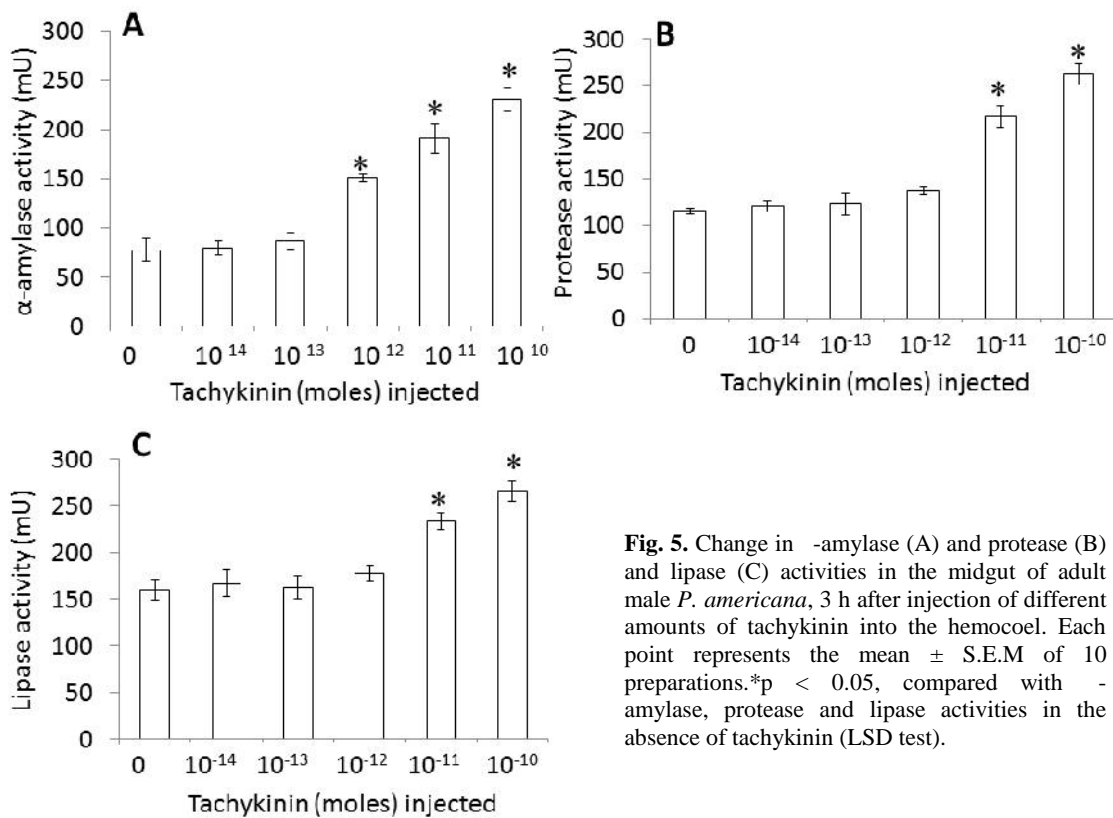


Fig. 5. Change in α -amylase (A) and protease (B) and lipase (C) activities in the midgut of adult male *P. americana*, 3 h after injection of different amounts of tachykinin into the hemocoel. Each point represents the mean \pm S.E.M. of 10 preparations. * $p < 0.05$, compared with α -amylase, protease and lipase activities in the absence of tachykinin (LSD test).

Discussion

Here for the first time, we showed tachykinin-ir cells in the midgut of the cockroach *P. americana* (Fig. 1). Previously some other neuropeptides like CCAP-ir, sNPF-ir and allatostatin-ir cells were observed in the midgut of the cockroaches (Sakai *et al.*, 2006., Matsui *et al.*, 2013, Mikani *et al.*, 2012).

The regulation of feeding in insects is very complex, and involves interactions between number of mechanisms, one of which is the release of neuropeptides (Mikani *et al.*, 2015). There are many neuropeptides that show myoactivity on gut, because stretching the foregut followed by contraction of the gut muscles are very important in food intake, that the control of gut motility is involved in the regulation of feeding (Wei *et al.*, 2000). These neuropeptides include FMRFamide-related peptides, the allatoregulatory peptides and tachykinins (Audsley & Weaver., 2009).

The midgut contains paraneuronal cells, some of which may monitor the nutrient content and stretching of gut to trigger the release of digestive enzymes (Fuse *et al.*, 1999). We showed before that incubation of dissected midgut with short neuropeptide F (sNPF) inhibited α -amylase, protease and lipase activities in *P. americana* (Mikani *et al.*, 2012). In contrast, incubation of the dissected midgut with CCAP stimulated α -amylase and protease activities (Sakai *et al.* 2006). Interestingly co-administration of CCAP and Allatostatin (AST) to the midgut caused increases of 1.5-fold and 1.4-fold for α -amylase and protease activities, respectively, compared with application of either peptide (Matsui *et al.*, 2013). Here we showed that incubation of the dissected midgut with tachykinin

also induced significant increase in α -amylase, protease and lipase activities (Fig. 4).

We previously showed that sNPF injection into the hemocoel led to a decrease in α -amylase, protease and lipase activities (Mikani *et al.*, 2012). We first here introduced tachykinin as a neuropeptide with stimulatory effect on these enzyme activities in *P. americana* (Fig. 5).

The number of tachykinin-ir cells decreased in the midgut of roaches being starved for 48 h. However it increased after 3 h of refeeding (Fig 2). The competitive ELISA also confirmed the present results (Fig. 3). It seems starvation/refeeding not only changed the digestive enzymes activities in the midgut (Mikani *et al.*, 2012) but also changed the tachykinin-ir cells population in the midgut epithelium (Figs. 2, 3) which showed stimulatory effect on digestive enzyme activities (Figs 4, 5). The nutritional input up-regulates epithelial paraneurons secreting tachykinin. Starvation mobilizes the reverse course of tachykinin regulation to reduce digestive activities of the midgut. It is most likely that the tachykinin produced in the midgut (Fig 1) is released to neighboring cells as a paracrine factor to stimulate the release or synthesis of digestive enzymes from columnar cells.

It is concluded that tachykinin stimulates α -amylase, protease and lipase activities in the midgut of *P. americana*.

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