

Some brain peptides regulating the secretion of digestive enzymes in the Indian meal moth, *Plodia interpunctella* (Lepidoptera: Pyralidae)

Seyede Mino Sajjadian*, Vahid Hosseininaveh, Khalil Talebi Jahromi

Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, P.O. Box 4111, Karaj, Iran

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Abstract: The Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) is a destructive polyphagous pest of many stored products. To interfere with the physiological processes, especially digestion, of the larval pest, more information on the regulatory mechanisms is needed. The brain extract from 1-day-old last instar larvae of *P. interpunctella* was examined. In the bioassays, the midguts were treated with the brain extract, and the carbohydrase and protease activities were measured. The brain extract showed increasing dose-dependent effects on α -amylase, α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, and trypsin secretion in the larval midgut. The extract was further characterised and partially purified using high performance liquid chromatography (HPLC). Several peptides were determined in the brain extract regulating hydrolase activities in the larval midgut of the pest.

Key words: digestive enzymes, HPLC, Lepidoptera, Pyralidae, regulating brain peptides

Introduction

The Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), is an important world-wide insect pest of various stored products (Mohandass *et al.* 2007). The pest causes significant quantitative and qualitative damage to stored products (Phillips *et al.* 2000). Chemical insecticides have been the primary means of controlling stored-product pests. However, because of many problems associated with the use of synthetic pesticides in integrated pest management approaches, use of chemicals to protect grains and grain products against stored-product pests is limited. More environmentally-benign alternatives are replacing chemical use (Hagstrum and Subramanyam 1996). One such approach is genetically modified plants or microorganisms bearing proteins or peptides targeting insect digestion.

In many insects, neuropeptides have been reported to exist in the brain and other nervous tissues (Verhaert and De Loof 1985). Immunological studies have shown the presence of peptides and hormones in the insect nervous system. Duve and Thorpe (1981) have reported gastrin/cholecystokinin (CCK)-like material in the brain of the blowfly, *Calliphora erythrocephala* (Diptera: Calliphoridae). They also did the mapping of gastrin/cholecystokinin-like peptides in the neuroendocrine system (subesophageal ganglion, thoracic ganglion, and corpus cardiacum) of the same insect species (Duve and Thorpe 1984). Immunocytochemical investigations by Verhaert and De Loof (1985) had proved the presence of P-like peptides in the central nervous system (brain, retrocerebral complex,

and subesophageal ganglia) of the American cockroach, *Periplaneta americana* (Blattodea: Blattellidae). They also reported that several substances, antigenically similar to vertebrate peptide hormones, are present in cerebral ganglia and contiguous neuronal structures of the same insect species (Verhaert *et al.* 1984; Verhaert and De Loof 1985, 1986). Insulin is a peptide hormone that has important roles in regulating the cellular metabolism and growth in vertebrates (Satake *et al.* 1997). Application of different methods show that insulin-like peptides also exist in invertebrates such as insects (Kramer 1985). The effects of an insect insulin-related peptide, bombyxin, on the metabolism of carbohydrates in the silkworm *Bombyx mori* (Lepidoptera: Bombycidae) were studied by Satake *et al.* (1997). More than 12 neuropeptides were isolated from neurosecretory system of the Madeira cockroach *Leucophaea maderae* (Dictyoptera: Blattellidae) and all of them, except one, apply a myotropic action (Holman *et al.* 1990). Leucokinins are a group of related neuropeptides that stimulate the gut motility and secretion of fluid by the Malpighian tube in insects (Harshini *et al.* 2002). This family of neuropeptides has been found largely among insects. A member of this family was isolated from adults of the fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae) (Terhzaz *et al.* 1999). In a study by Harshini *et al.* (2002), the effect of leucokinins, isolated from *L. maderae*, their related peptide analogues, and leucopyrokinin on release of amylase and protease from the midgut epithelial cells of the larvae of *Opisina arenosella* (Lepidoptera: Cryptophasidae) was determined. The presence of insu-

*Corresponding address:
minoo.sajjadian@ut.ac.ir

lin-, gastrin-, and pancreatic polypeptide-like immunoreactivity neurons in the brain of *B. mori* has been demonstrated by Yui *et al.* (1980). A brain peptide from the larvae of *Opisina arenosella* (Lepidoptera: Crytophasidae) was isolated by Harshini *et al.* (2003) They had partially purified this peptide and reported that it stimulates the release of amylase from the midgut tissue of the same insect species (Harshini *et al.* 2003).

Due to the importance of *P. interpunctella* as a serious pest insect, there has been considerable research on various aspects of the pest's developmental biology. The remarkable damage of the pest on the handling of numerous stored products makes the insect an important subject, particularly in terms of biochemical characterisation of digestive enzymes. The present study is an effort to survey the presence of brain peptides regulating the secretion of digestive enzymes such as α -amylase, α -glucosidase and β -glucosidase, α -galactosidase and β -galactosidase, and trypsin in the larva midgut of *P. interpunctella*.

Materials and Methods

Insects

To establish the colony, a collection was done of adults and larvae of *P. interpunctella* from infected pistachio. The insects were reared on an artificial diet according to Sait *et al.* (1997) under controlled conditions at $27\pm 1^\circ\text{C}$, $60\pm 5\%$ relative humidity (RH) and 12 : 12 h (light : dark) photoperiod. The diet consisted of 800 g of wheat bran, 160 g dried brewer's yeast, 200 ml honey, and 200 ml glycerol. The fourth larval instars or 1-day-old fifth larval instars were used in all experiments.

Preparation of the brain extract

The entire head capsule of each larva was isolated and transferred into the insect saline (10–200 head capsule/ml). The insect saline (pH 7.4) was composed of 130 mM NaCl, 5 mM KCl, and 1 mM CaCl_2 (Harshini *et al.* 2002). The head capsules within the insect saline were boiled for 1, 3, 5, and 10 min to denature the probable enzymes present. The boiled capsules were homogenised in a hand-held glass grinder and the homogenates were centrifuged at $10,000 \times g$ for 10 min. In the control, the head capsules were homogenised without boiling. The resulting supernatant was used as the incubation solution in the bioassays.

Enzyme sample preparation

The last larval instars were cold-immobilised, dissected under a stereoscopic microscope, and the midguts were removed and cleaned of unwanted adhering tissues. The midguts, including contents, were placed into 100 μl distilled water and homogenised using a hand-held glass grinder on ice. The homogenates were centrifuged at $16,000 \times g$ at 4°C for 15 min. The supernatants were passed through filter paper (Whatman®, No. 40), transferred to new tubes, and kept at -20°C for further use.

Hydrolase assay

α -Amylase activity was assayed according to Bernfeld (1955) using dinitrosalicylic acid (DNS) and 1% soluble starch as the substrate with slight modifications. Briefly, 10 μl enzyme extract was added to 85 μl 40 mM citrate-phosphate-borate buffer (pH 3 to 11) and 10 μl of soluble starch. The experiment was stopped with the addition of 50 μl DNS (44 mM) and by heating in boiling water for 10 min. The absorbance of the reaction mixture was read at 540 nm using a microplate reader (ELX 808). In the blanks, the enzyme was added to the reaction mixture after the addition of DNS.

The activity of α -glucosidase and β -glucosidase was detected according to Ramzi and Hosseinaveh (2010) and Tabatabaei *et al.* (2011) using *p*-nitrophenyl- α -D-glucopyranoside (*p*NaG, 5 mM), and *p*-nitrophenyl- β -D-glucopyranoside (*p*NbG, 5 mM) as the substrates, respectively. The assay was carried out by incubating 10 μl of the enzyme extract with 85 μl of 40 mM citrate-phosphate-borate buffer (pH 3 to 11), and 5 μl of the substrates for 30 min. The reaction was stopped by adding 50 μl of NaOH (1 M). The optical density was measured at 405 nm using a microplate reader (ELX 808). In the controls, the enzyme was added to the reaction mixture after the addition of NaOH. The same procedure was used for the measurement of α -galactosidase and β -galactosidase activity but with the substrates *p*-nitrophenyl- α -D-galactoside (*p*NP α Gal, 5 mM), and *p*-nitrophenyl- β -D-galactoside (*p*NP β Gal, 5 mM), respectively.

Trypsin activity was determined using BA p NA (N_α -benzoyl-L-arginine-*p*-nitroanilide, 20 mM) as the substrate. For determining trypsin enzyme activity, 5 μl of the substrate was added to 10 μl of the enzyme extract and 85 μl of the 40 mM citrate-phosphate-borate buffer at pH 6 to pH 11. The absorbance was measured continuously for 30 min with a time interval of 3 min at 405 nm.

Bioassay

To study the effects of the brain extract on the secretion of digestive enzymes, the whole alimentary canal of each larva was removed. The alimentary canal was transferred in a small volume of insect saline. Two ends of the midgut were tightly ligated with floss fibers, and the foregut and the hindgut were cut and discarded. One hundred ligated midguts were transferred to a petri dish (5 cm diameter) which held 5 ml of insect saline and 1 ml of the brain extract. During the incubation, a small stream of oxygen was bubbled into the solution. The bioassay apparatus was kept in a water bath at 37°C . In the controls, one milliliter of distilled water was used instead of the brain extract. The ligated midguts were homogenised and centrifuged. The resulted supernatants were used as the enzyme source in subsequent experiments. To prove the claim that the differences in the secretion of the enzymes come from the regulatory impact of the brain extract, the enzymatic activity of both boiled and non-boiled brain extract was also studied.

In the dose-response assay, the brain extract at concentrations of 10, 50, 100, and 200 head capsules in 1 ml insect saline was prepared. The effects of the different

concentrations of the brain extract on the secretion of digestive enzymes were bioassayed and were compared with the control.

High-performance liquid chromatography (HPLC) fractionation

The heads of 1,000 larvae were homogenised in 5 ml of cold NaCl (2%, w/v). The homogenates were centrifuged at 16,000 \times g for 15 min. Ice-cold acetone was then added to the resulted supernatants at a final saturation level of 70% aqueous acetone and maintained at 4°C for 90 min. The solution was centrifuged at 10,000 \times g for 20 min at 4°C, and ice-cold acetone was added to the supernatant at a final saturation level of 80% aqueous acetone. After a few hours at 4°C, the microtubes were centrifuged at 18,000 \times g for 20 min at 4°C. The precipitates were collected in the distilled water (HPLC grade) and were injected into the HPLC according to Harshini *et al.* (2003). In the case of the control, the entire process of preparing the brain peptides was performed on distilled water. The conditions for HPLC operating was configured as following:

- Stationary phase: C18 column, 4.6 mm \times 25 cm;
- Mobile phase: Solvent A – 0.01 M ammonium acetate buffer (pH 6.1); Solvent B – 25% acetonitrile in water, according to the following time table:

Time [min]	Solvent A [ml]	Solvent B [ml]
0	100	0
8	100	0
38	75	25
60	25	75
65	100	0

- Flow rate: 1.25 ml/min;
- Operating temperature: 40°C;
- Detector range: 0.00299 AU at 214 nm.

After the injection of the brain extract to the HPLC, the output liquid from the device was collected in the 1.5 ml microtubes every minute. According to the chromatograph, putative fractions bearing peptides were chosen (fractions number 9, 13, 16, 20, and 24). The putative fractions were diluted to 10% in the insect saline. The effect of the fractions was studied on the secretion of digestive trypsin and β -glucosidase as described before. A sample containing the insect saline was used as the control. A sample containing 50% acetonitrile was also used in the bioassays.

Statistical analysis

Data were analysed using STATGRAPHICS computer software, Version 5.1. Independent *t*-tests were used to compare the average of the treatments and controls. The software SigmaPlot® Version 10.0 was used to plot the graphs.

Results

Hydrolase activity

The greatest α -amylase (Fig. 1a) and trypsin (Fig. 1d) activity of the larval midgut of *P. interpunctella* was obtained at a pH of 9.0. However, α -glucosidase and β -glucosidase activity was optimum at a pH of 6.0 (Fig. 1b). Optimal α -galactosidase and β -galactosidase activity occurred at a pH of 5.0 (Fig. 1c).

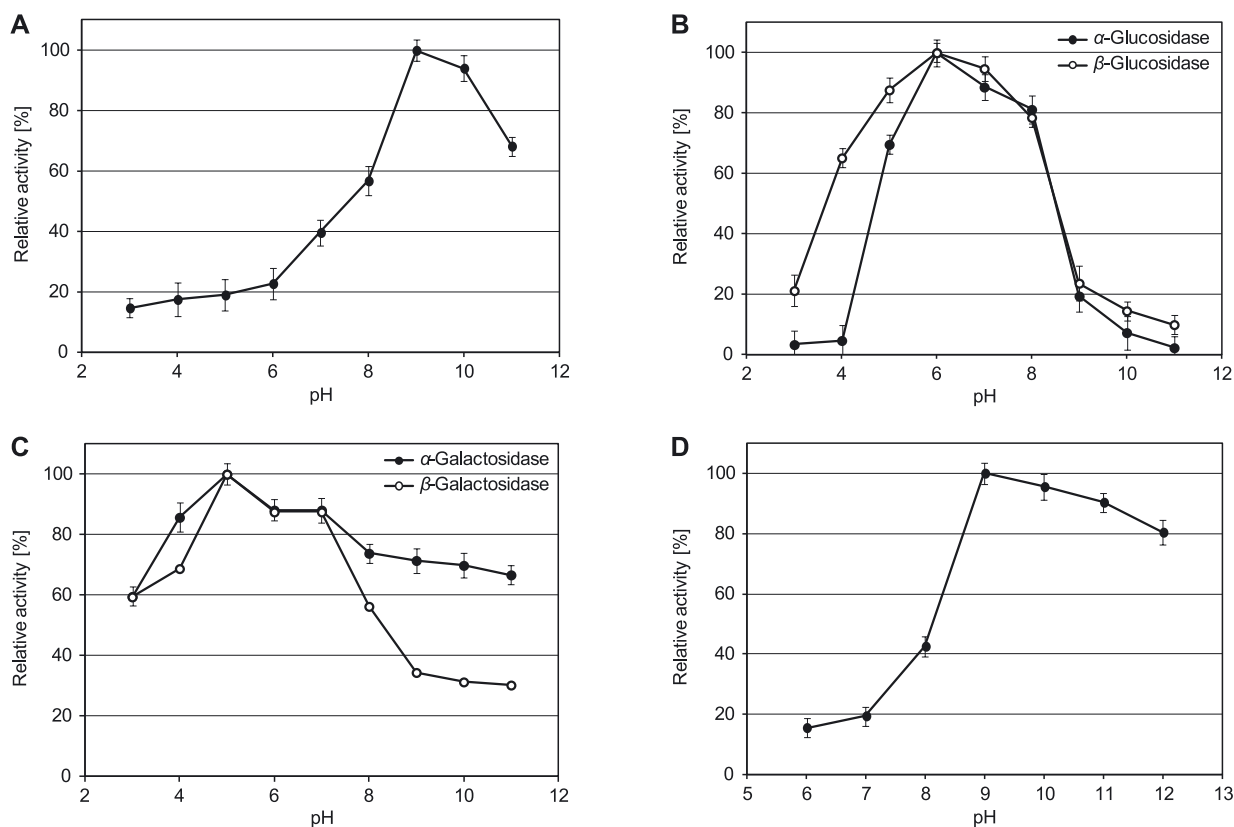


Fig. 1. The effect of pH on the activity of α -amylase (A), α -glucosidase and β -glucosidase (B), α -galactosidase and β -galactosidase (C), and trypsin (D) from the midgut of larval *P. interpunctella*. The vertical bars are standard errors of the means of three replicates

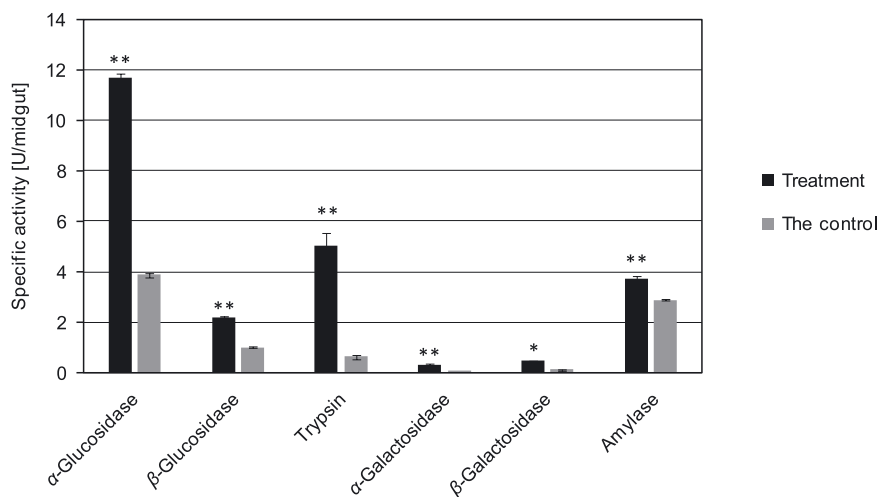


Fig. 2. Effect of the brain extract on the secretion of digestive enzymes in larval *P. interpunctella* (** $p < 0.01$; * $p < 0.05$). The vertical bars represent standard errors of the means of three replicates

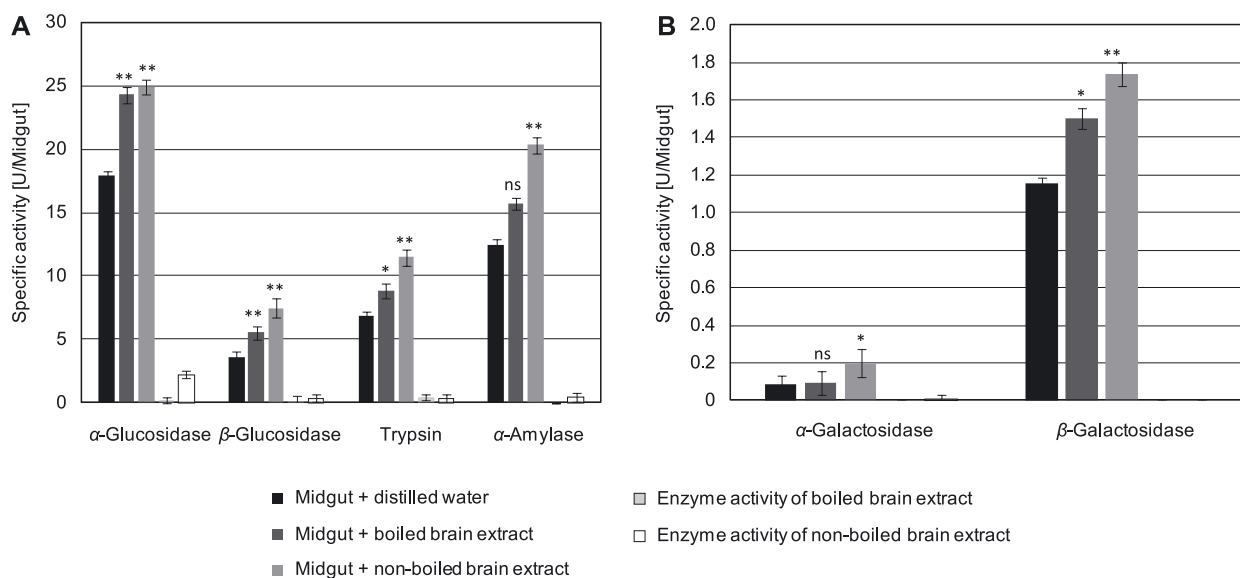


Fig. 3. Effect of high temperature (boiling water) on the brain extract effects on the secretion of digestive enzymes [** $p < 0.01$; * $p < 0.05$; ns (not significant): $p > 0.05$]. The vertical bars are standard errors of the means of three replicates

Effect of the brain extract on hydrolase secretions

The brain extract influenced the secretion of the hydrolases of the larval midgut of *P. interpunctella* (Fig. 2). The extract had a significant increasing impact on the secretion of α -galactosidase (~15.6-fold), trypsin (~7.9-fold), β -galactosidase (~3.7-fold), α -glucosidase (~3-fold), β -glucosidase (~2.2-fold), and α -amylase (~1.3-fold).

Temperature stability of the brain extract

The enzyme activities were enhanced when the midgut was treated with the boiled and non-boiled brain extract, compared to the control (Fig. 3a, b). However, slightly higher enzyme activities were determined in the treatment of the midgut with the non-boiled brain extract than that of the boiled brain extract.

The brain extract boiled in a water bath for 1, 3, 5, and 10 min showed a smaller enhancing impact on the secretion of α -amylase, β -glucosidase, and trypsin than that of the non-boiled brain extract (Fig. 4a, b, c). In the treatment of the midgut with the brain extract boiled for 5 min (3 min in the case of β -glucosidase), the enzyme activities increased. When the brain extract was boiled more than 5 min (3 min in the case of β -glucosidase), it was less effective at enhancing the secretion of the enzymes.

Dose-effect of the brain extract

Incubation of the midgut preparations with different concentrations of the brain extract linearly enhanced the activity levels of α -amylase ($R^2 = 0.94$), β -glucosidase ($R^2 = 0.90$) and trypsin ($R^2 = 0.97$) levels in the lumen contents compared with the control (Fig. 5a, b, c).

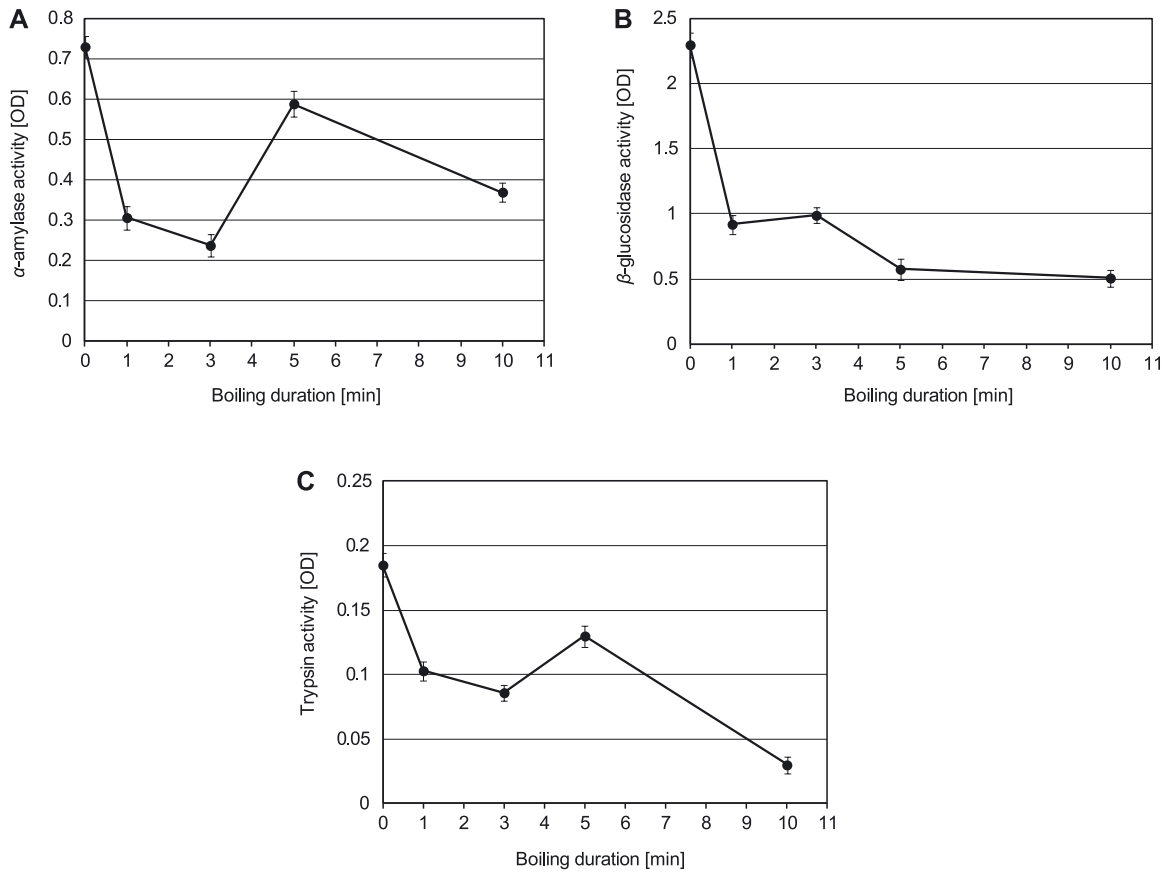


Fig. 4. Temperature stability of the brain extract and its effect on the secretion of α -amylase (A), β -glucosidase (B), and trypsin (C) from the midgut larval *P. interpunctella*. The vertical bars are standard errors of the means of three replicates

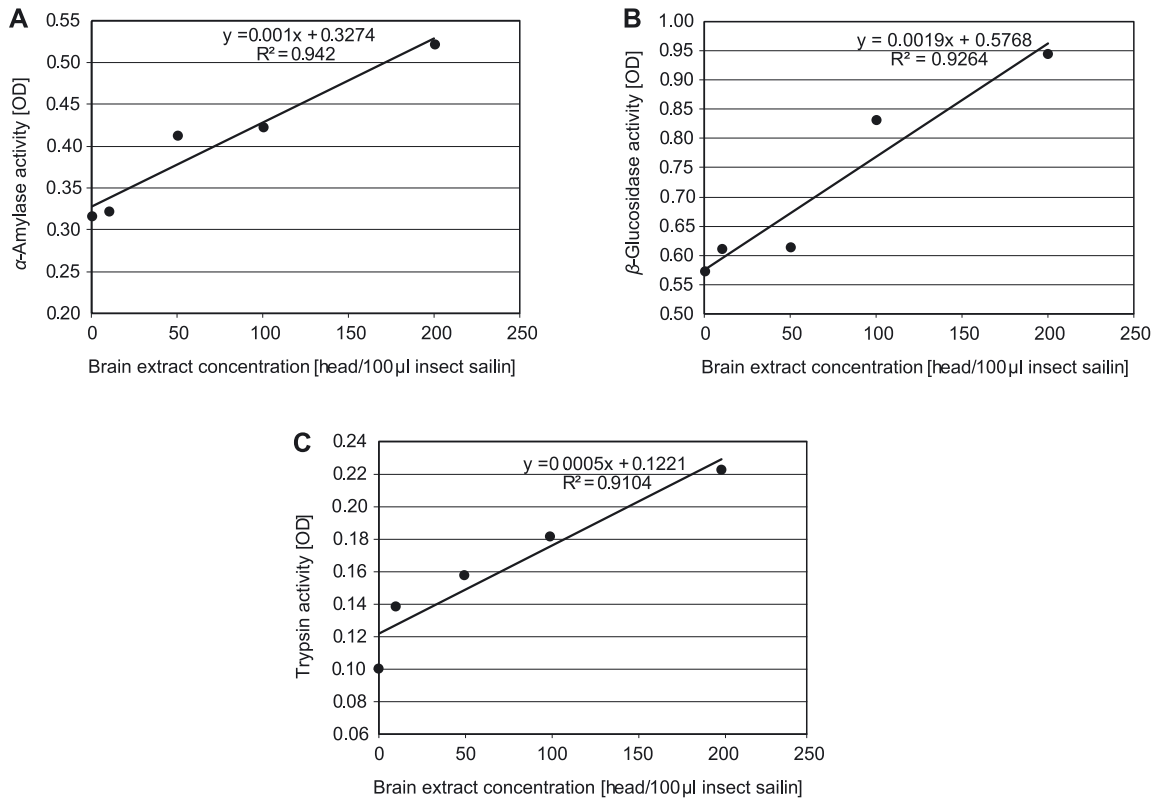


Fig. 5. Dose-response manner on the effect of different concentrations of the brain extract on the activity of α -amylase (A), β -glucosidase (B), and trypsin (C) in midgut preparations of larval *P. interpunctella*

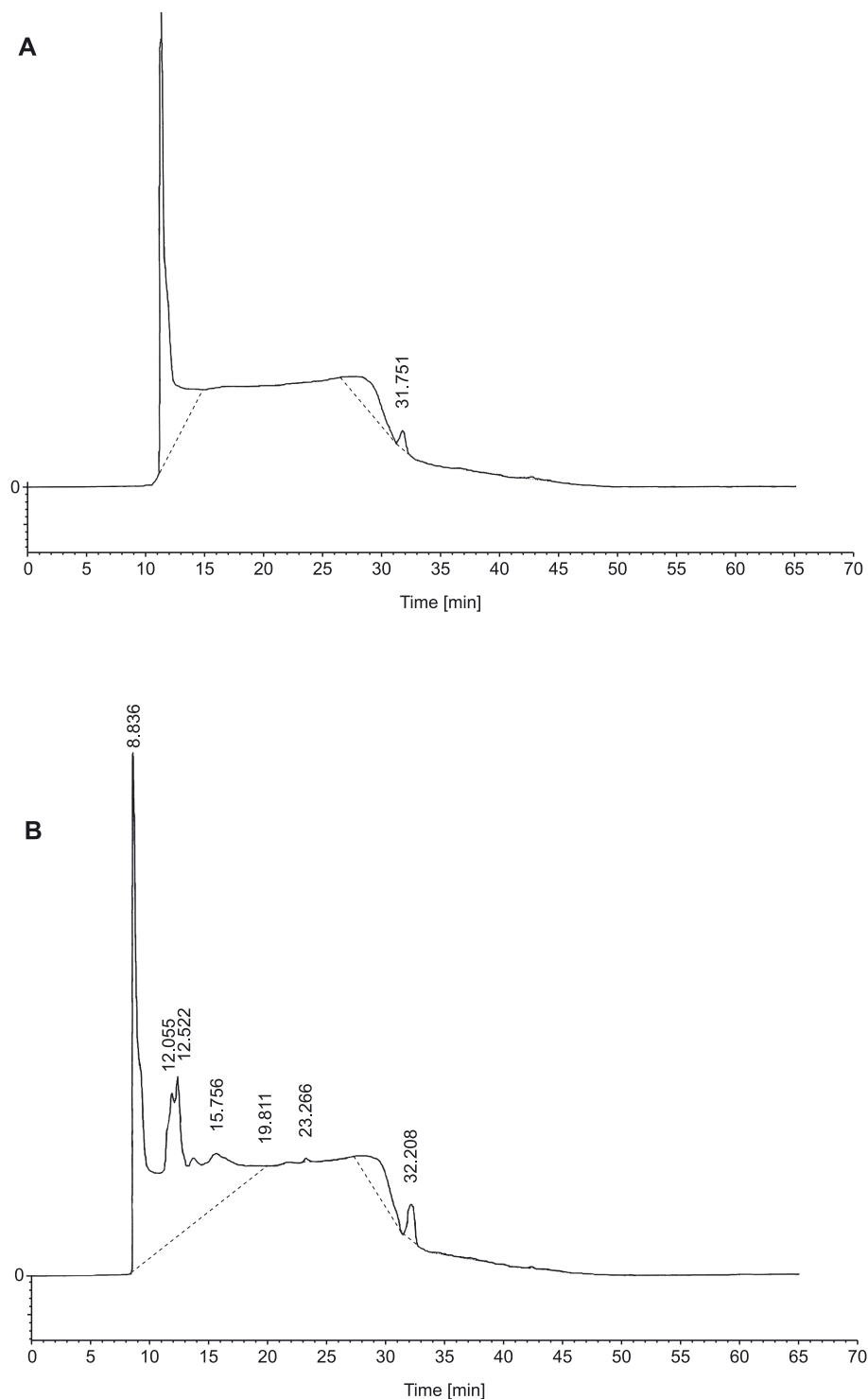


Fig. 6. HPLC chromatographs of the brain peptide treatment (A) and of the control (B)

HPLC

There were some differences in some points of the HPLC chromatographs of the brain extract (Fig. 6b) and the control (Fig. 6a). The different peaks were probably due to the putative brain peptides. The fractions corresponding to the peaks of interest were collected and used as treatment of the midgut. The peptide present in fraction No. 9 had a significant enhancing and declining effect on the

activity levels of β -glucosidase and trypsin, respectively. The peptide present in fraction No. 13 showed a significant decreasing effect on trypsin activity in the midgut. Fraction No. 16 did not have any significant effect on the enzyme activity levels. Fraction No. 20 significantly decreased the activity of β -glucosidase (Fig. 7a). Fraction No. 24 significantly increased trypsin activity (Fig. 7b).

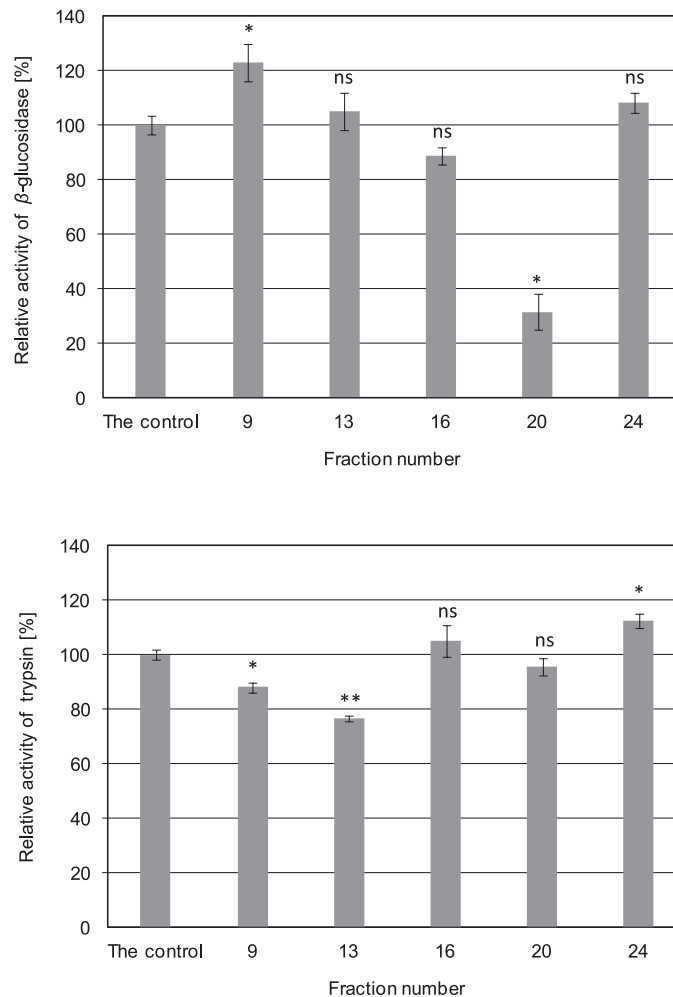


Fig. 7. Bioassay with the peptides obtained from HPLC fractionation. Effects of different fractions on the secretion of β -glucosidase (A), and trypsin (B) from midgut tissue of larval *P. interpunctella* [** $p < 0.01$; * $p < 0.05$; ns (not significant): $p > 0.05$]. The vertical bars are standard errors of the means of three replicates

Discussion

Neurosecretions of the insect brain are involved in the regulation of digestive enzyme secretion (Prabhu and Sreekumar 1994). The present *in vitro* study has shown that incubation of the midgut of larval *P. interpunctella* with the larval brain extract causes an increase in activity of hydrolases in the midgut lumen. Muraleedharan and Prabhu (1979) have shown that extirpation of neurosecretory cells in *Dysdercus cingulatus* causes a decrease in the secretion of the protease and amylase enzymes. Harshini *et al.* (2003) have also observed that the brain extract obtained from the larvae of *O. arenosella*, stimulates release of α -amylase from the midgut tissue of larvae, which is consistent with the findings of the present study. The increasing effect of the brain extract on the secretion of hydrolases from midgut cells is dose dependent. These results are consistent with an earlier study that showed that an increase in brain extract concentration caused an increase in the release of α -amylase from the midgut tissue of larvae of *O. arenosella* (Harshini *et al.* 2003). Another study showed that a midgut peptide or probably hormone has a similar action in the larvae of *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) (Sreekumar and Prabhu 1988).

In the present study, the stimulating factor of the secretion of the hydrolases from the *P. interpunctella* larva head was partially purified with 80% aqueous acetone. It was shown through HPLC analyses that there were several peaks in the peptide sample. In some related studies, some vertebrate-like gut peptides were separated and purified from the nervous system and tissues, such as the insulin-like material in *Calliphora vomitoria* (Diptera: Calliphoridae) (Duve *et al.* 1979), and the gastrin-like peptide in *Manduca sexta* (Lepidoptera: Sphingidae) (Kramer *et al.* 1977), and *C. vomitoria* (Dockray *et al.* 1981). In one study on *O. arenosella*, an elution profile of OCF2:3 showed PK No. 3 and PK No. 4 separated by a time interval of 0.107 min (Harshini *et al.* 2003). In another study, it was observed that HPLC analyses of two midgut epithelial fractions called OMF1:1 and OMF3:1 stimulated enzyme release in the same insect species (Harshini and Sreekumar 2001). However, regarding the important functional role of these peptides, any conclusion without elucidation of all their various activities in different physiological and biological systems is impossible (Holman *et al.* 1987).

In this research, some peptides from the brain of larval *P. interpunctella* were extracted. The larval midgut in the presence of the extracted peptides was stimulated to produce more hydrolases including trypsin, galactosi-

dases, glucosidases, and amylase. The stimulating factors ranged from 16 to 20 kDa. Identification of regulatory peptides can help us in designing new strategies for controlling stored-product insect pests.

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