

Full Length Research Paper

Identification and sequence analysis of pyrokinin/PBAN peptide of psocids, *Liposcelis entomophila*

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Using an antiserum against *Helicoverpa zea* pheromone biosynthesis activating neuropeptide (PBAN), pyrokinin/PBAN-like immunoreactivity in the head and body of female of *Liposcelis entomophila* was detected by competitive ELISA. Pyrokinin/PBAN peptides were extracted and purified by reverse-phase HPLC, and the sequence were elucidated by a matrix-assisted laser desorption ionization time of flight mass spectrometer (MALDI-MS-TOF). The results showed that pyrokinin/PBAN neuropeptide was expressed in the heads of *L. entomophila* females. Meanwhile, it was not found in the abdomen of *L. entomophila* females and the head of *Liposcelis bostrychophila*. These results indicated that the peptide perhaps had the functions to regulate sex pheromone releasing. The sequence pyrokinin/PBAN family peptide in *L. entomophila* was SAGMFTPRL-NH₂. The result of BLASTp in NCBI web showed that its sequence had largely homologous sequences with other PBAN/pyrokinin peptides sequence in other insects that were published and share a highly conserved sequence FXPRLamide in the C terminal. This result indicated that the identified peptide in *L. entomophila* belong to the pyrokinin/PBAN family. The purification and identification of this peptide have an important instructional meaning for cloning the gene of pyrokinin/PBAN and elucidating the release mechanism of the sex pheromone of *L. entomophila*.

Key words: *Liposcelis entomophila*, pyrokinin/PBAN, peptide.

INTRODUCTION

Sex pheromone release in most of the moth species is regulated by pheromone biosynthesis activating neuropeptide (PBAN). The PBAN is a member of the pyrokinin/PBAN family of neuropeptides, have 8 - 4 amino acids in length with a common C-terminal motif Phe-X-Pro-Arg-Leu-NH₂ (X = Gly, Ser, Thr or Val) (FXPRLamide) and has diverse functions in several species of insects (Gade, 1997; Rafaeli, 2002; Rafaeli

and Jurenka, 2003) as well as a crustacean (Torfs et al., 2001). The FXPRLamide common sequence forms a type I β -turn, required for receptor recognition, it is the critical portion that determines bioactivity as was shown in all previous physiological assays (Nachman et al., 1991; 1993, 2009; Teter et al., 2002). The mode of action of PBAN through neurohormonal activity (Rafaeli and Jurenka, 2003) and the genes of PBAN or receptor were well researched (Wei et al., 2008; Bober et al., 2010) in Lepidopteran moths. Functions of the pyrokinin/PBAN family include stimulation of sex pheromone biosynthesis (Choi and Jurenka, 2004; Rafaeli and Jurenka, 2003; Wei et al., 2004), mediation of key functions associated with feeding (gut muscle contractions) (Predel and Nachman, 2001), development of embryonic diapause, pupal diapause (Zhang et al., 2009), pupariation (Zdarek et al., 1998) and defense (melanin biosynthesis) in a variety of insects (moths, cockroaches, locusts and flies) (Suwan,

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Abbreviations: MALDI-MS-TOF, Matrix-assisted laser desorption ionization time of flight mass spectrometer; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; PBAN, pheromone biosynthesis activating neuropeptide; LC, liquid chromatography.

1994; Raina et al., 2003). Interesting phenomena is that the pyrokinin/PBAN family members showed cross reactivity among different members (Choi et al., 2009).

Beyond lepidopteran moths, only in few insects so far has the presence of PBAN family peptides been researched and documented. Based on the genome wide research, the PBAN/pyrokinin peptides were found in the red flour beetle, *Tribolium castaneum* (Li et al., 2008) and honeybee (*Apis Mellifera*) (Hummon et al., 2006). However, the physiological function of the PBAN/pyrokinin peptides in these insects was not clearly established. Choi et al. (2009) demonstrated the presence of pyrokinin/PBAN-like peptides in the central nervous system of the fire ant, *Solenopsis invicta*. These investigations indicated that the functions of pyrokinin/PBAN-like peptides were widely distributed among insecta. Up till now, there have been no reports on pyrokinin/PBAN peptides in Psocoptera.

This study describes a new member of pyrokinin/PBAN peptide in *Liposcelis entomophila* (Psocoptera: Liposcelididae), an important and emerging stored product pest. The *L. entomophila* is a small (<2 mm in length) cosmopolitan insect species, originally identified on trees in tropical Africa (Badonnel, 1931) and is found in various processed and unprocessed dry foods in households, granaries, and warehouses (Tang et al., 2008; Nayak and Daglish, 2007). The insect had developed strong resistance to phosphine and almost for all currently registered grain protectants (Nayak and Daglish, 2007). Psocid management is reported to be difficult through aeration regulation, biological control or other fumigated methods that were researched (Wang et al., 2001; Opit et al., 2008a, b). Mass attraction through pheromonal substances could be a good alternative approach for psocids management. Identification and understanding of the mechanism involved in pheromone release and response is a pre-requisite for such approaches and the present study is therefore aimed at describing the PBAN peptides present with *L. entomophila*.

MATERIALS AND METHODS

Insects

The colony of *L. entomophila* was established from nymphs collected at a wheat warehouse in Zhongxiang, HuBei Province, China in 2005. Under laboratory conditions, the colonies were reared in glass bottles (250 ml) using an artificial diet consisting of whole wheat flour, skim milk and yeast powder (10:1:1) at 27 ± 2°C temperature, 75 - 85% relative humidity and a scotoperiod of 24 h. The sexes of the psocids were identified at nymphal stage using color and size (Bai et al., 2008) and were kept in isolation.

Extraction and purification of the peptide

The heads and abdomens of about 2000 *L. entomophila* females were dissected aseptically and stored in microfuge tubes at -70°C.

The dissected tissues of heads and abdomens were sonicated in 200 ml of methanol/water/trifluoroacetic acid (90:9:1, v/v/v) solution at room temperature. After centrifugation at 16000 rpm for 20 min, the pellets were subjected to extraction twice as above. The collected supernatants were pooled and evaporated to dryness.

Peptide purification was performed on a HPLC-system consisting of two Model 302 piston pumps, a manometric modul 802, a model 811 mixing chamber (Waters Corporation, USA) and a LKB 2151 variable wavelength detector set at 214 nm. An Apple II computer was used to build up the desired gradient using the Gilson Gradient software. All separations were carried out on RP-C18 columns (250 mm × 4.6 mm, 100 Å, 5 mm), the fractions were collected manually and dried by vacuum centrifugation. The manually fractions were purified as the method described in Predel et al 2001.

Preparation of Hez-PBAN antibody

In order to identify the presence of the FXPRamide family neuropeptides in the purified fractions of HPLC, the fragments of Hez-PBAN (24 - 33) (SRTKYFSPRL-NH₂) (Raina et al., 1989) (U08109) were synthesized (Shanghai Sangong Company, Shanghai, P.R.China). The synthesized PBAN was coupled to bovine serum albumin (BSA), (Sigma, USA) by glutaraldehyde, and the PBAN-BSA conjugate was further emulsified with Freund's complete adjuvant before injecting into rabbits at multiple sites. Two booster injections were administered at 4 weeks intervals, starting first dose. The anti-Hez-PBAN serum used in the present study was obtained 2 weeks after the final booster dose as described in Wei et al. (2004).

Competitive ELISA

Competitive ELISA was performed as in Marco et al (1996). Briefly, microtiter plates were coated with BSA using dimethylpimelidate dihydrochloride (DMP) (0.3 µg/mL in coating buffer, 50 µL/well) overnight at 4°C. Then, the plates were washed with PBST, blocked with 3% nonfat milk for 1 h at room temperature and washed again. The samples and standards (eight different concentrations from 10,000 to 3.2 µM and 0), which had been preincubated with the antibody (1 /25,000 diluted in PBST) overnight at 4°C, were added to the wells (50 µL/well). After incubation for 1 h more at room temperature, horseradish peroxidase-conjugated anti-rabbit Hez-PBAN (1/6,000 in PBST, 50 µL/well) was added to the wells and incubated for 1 h at room temperature. The color was developed with tetramethylbenzidine (0.01%) H₂O₂ 0.004% in citrate buffer, 50 µL/well). The titer of antibody Hez-PBAN was evaluated by ratio of absorption of antibody and peptides and blank at 490 nm. The immunoreactivity of different peptide extractions and purification peaks were expressed by absorbance (490 nm) of their response complex with antibody of Hez-PBAN. The data were statistically analyzed using the Duncan multiple comparison in SAS software.

HPLC fractions were centrifuged at 12,000 rpm for 5 min, and the supernatant was dried down and stored at -80°C until the ELISA analysis was complete.

MALDI-TOF mass spectrometry

The sequence of peptides was analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) on a Voyager system 4095 mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser emitting at 337 nm. Samples were analyzed in reflectron mode using a delayed extraction time of 175 ns and an accelerating voltage of 20 kV. The procedures of MALDI-TOF mass spectrometry were described in Nachman et al. (2002).

Table 1. The evaluation of titer of rabbit serum antibody detected by synthesized Hez-PBAN antigen (concentration of coated antigen was 10 μ g/m).

Number of rabbit	1:100	1:1000	1:10000	1:100000
1	10.21 \pm 1.23 a	5.48 \pm 1.62 b	1.62 \pm 0.78 c	1.04 \pm 0.36 c
2	7.98 \pm 2.25 a	2.32 \pm 0.25 b	1.13 \pm 0.84 c	1.75 \pm 0.61 b

Data in the table represents the mean \pm SD of three repeats. Within each column, data followed by different letters are significantly different ($P < 0.05$) (Duncan multiple comparison, SAS).

Table 2. The comparison of absorbance in OD₄₉₀ of different peptides extraction as antigen in comparative ELISA with antibody Hez-PBAN (concentration of coated antigen was 10 μ g/mL).

Number of Rabbit	Different peptides extraction	Serum dilution factors		Blank (CK)
		1:100	1:1000	
No.1	Head of female in <i>L. entomophila</i>	0.265 \pm 0.062 a	0.142 \pm 0.026 a	0.071 \pm 0.006 a
	Abdomen of female in <i>L. entomophila</i>	0.148 \pm 0.013b	0.106 \pm 0.015 b	0.044 \pm 0.002 a
	Head of <i>L. bostrychophia</i>	0.126 \pm 0.009 b	0.102 \pm 0.062b	0.053 \pm 0.002 a
No.2	Head of female in <i>L. entomophila</i>	0.220 \pm 0.019 a	0.109 \pm 0.005 a	0.052 \pm 0.001 a
	Abdomen of female in <i>L. entomophila</i>	0.154 \pm 0.011 b	0.081 \pm 0.001 b	0.047 \pm 0.003 a
	Head of <i>L. bostrychophia</i>	0.130 \pm 0.006 b	0.096 \pm 0.003 b	0.066 \pm 0.002 a

Data in the table represents the mean \pm SD of three repeats. Within each column, data followed by different letters are significantly different ($P < 0.05$) (Duncan multiple comparison, SAS).

Table 3. The comparison of absorbance in OD₄₉₀ of manual collecting fractions as antigen in comparative ELISA with antibody Hez-PBAN (concentration of coated antigen was 10 μ g/ml).

Number of rabbit	Different manual collecting fraction	Serum dilution factors		Blank (CK)
		1:100	1:1000	
No.1	Manual collecting fraction of peak1	0.286 \pm 0.079 a	0.162 \pm 0.027 a	0.076 \pm 0.001a
	Manual collecting fraction of peak2	0.108 \pm 0.035 b	0.085 \pm 0.012 b	0.049 \pm 0.02 a
No.2	Manual collecting fraction of peak1	0.248 \pm 0.10 a	0.149 \pm 0.043 a	0.064 \pm 0.04 a
	Manual collecting fraction of peak2	0.104 \pm 0.008 b	0.081 \pm 0.005 b	0.057 \pm 0.005 a

Data in the table represents the mean \pm SD of three repeats. Within each column, data followed by different letters are significantly different ($P < 0.05$) (Duncan multiple comparison, SAS).

RESULTS

Pyrokinin/PBAN family neuropeptide exists in the head of female *L. entomophila*

The results of the competitive ELISA reaction involving extractions of head and abdomen of female *L. entomophila* and antibody of Hez-PBAN showed the positive reaction (Tables 1 and 2). The ratio of absorption of antibody and blank at 490 nm were more than 2.5 when the serum dilution factors were 1:1000 and 1:100 (Table 1). This result indicated that antibody Hez-PBAN had significantly conjugated with the synthesized Hez-PBAN antigen. The extract of heads of *L. entomophila* female showed the most significant effective conjugation with the antibody of Hez-PBAN peptide (Table 2). The extract of abdomen of *L. entomophila* female and the heads of *L. bostrychophia* female showed the least binding affinity to the Hez-PBAN peptide. These results

indicated that the FXPRLamide family of neuropeptides was prevalent only in heads of *L. entomophila* female.

Isolated and purified peptide from extracts of head of female *L. entomophila* had the specific reactivity with Hez-PBAN

The remaining time for fraction of standard Hez-PBAN in the HPLC was 38.89 min (Figure 2A). There were two similar peaks (36.25 and 40.85 min) (Figure 2B) in fraction of the extract from heads of *L. entomophila* females. The purified fractions of those extracts from female heads also produced similar signal peak (Figure 2C). The fractions of the two peaks collected manually tested for their reactivity in ELISA also showed conjugative reactions with anti-Hez-PBAN serum (Table 3). The fraction of peak1 had the highest and peak 2 had

little immunoreactivity the anti-Hez-PBAN serum. These results indicated that fraction from the peak1 perhaps was the pyrokinin/PBAN peptides and its sequence was subjected for analysis by MALDI-TOF-MS.

Sequence of pyrokinin/PBAN peptides identified by MALDI-TOF mass spectrometry and analysis through BLAST in NCBI

The manually collected peak1 fraction of female heads of *L. entomophila* from HPLC was subjected to several step purification processes (Predel and Nachman 2001) to achieve the purified (over 95%) peptides (Figure 1C1). The purified peptide was freeze-dried at -40°C. The sequence of the LC fraction of heads (brain) of *L. entomophila* females was identified as SAGMFTPRL-NH₂ (Figure 2). When compared with the sequence of synthesized antibody SRTKYFSPRL-NH₂, the LC fraction peak1 had only four same amino acid conjunct sites (FXPRL). The sequence in the C-terminal had the FTPRLamides; meanwhile, the sequence of Hez-PBAN C-terminal had the FSPRLamides (Figure 3). Comparative homology analysis of amino acid sequences of the peptide with other Pyrokinin/PBAN neuropeptides showed that the peptide had the C-terminal sequence FXPRLamide (Figure 3). The results confirmed that the peptide isolated from the head (brain) of *L. entomophila* females had the same C-terminal sequence as that of Hez-PBAN peptide and was a pyrokinin/PBAN neuropeptide.

DISCUSSION

We have successfully purified one peptide that contained the C-terminal sequence of FXPRLamide. This neuropeptide is the first of the members of the pyrokinin-family isolated from the psocid, *L. entomophila*. In contrast to previous purifications of myotropic peptides from other insect species, the novel peptides discussed here were directly isolated from the head that included the suboesophageal ganglion (SG) complex, which is the major storage and release centre of neuro-secretions produced. The occurrence of the pyrokinins in these neurohemal organs is suggestive of their role as a hormone. The individuals of *L. entomophila* were so small that the retrocerebral complex could not be isolated and hence our present approach of using the head of females as unit to extract the peptide worked significantly to obtain the extraction successfully. The members of the FXPRLamide family have diverse biological functions such as regulation of pheromone and pigment synthesis, muscle activity and induction of diapause (Grade et al., 1996; Schoofs, et al., 1996; Choi et al., 2009; Herbert et al., 2009) in different insects. A pyrokinin (leucopyrokinin) from cockroach *Leucophaea maderae* was first identified

(Holman et al., 1986) as a myotropin, with subsequent myotropic peptides being identified from various insect orders (Nachman et al., 1986). The individuals of *L. entomophila* were so small that we could not inject the synthesised peptide into their body and hence the biological functions were not researched this time.

The structure and pheromone activity of PBAN of *Helicoverpa zea* was identified by Rain et al. (1989) and subsequently the PBAN sequence of *H. zea* (34 amino acids), particularly the terminal sequence (24 - 33) was synthesized to identify the pyrokinin/PBAN bioactivity of other insects. The neuropeptide activity was studied by injecting the synthetic peptide into the brain of insects or used as an antibody in ELISA (Jurenka et al., 1995; Altstein et al., 1999; Rain et al., 2003, Hariton et al., 2009). In the present study, we could not find the immunoreactivity of PBAN-like peptide in the head of another psocid *L. bostrychophila* female. *L. bostrychophila* is a parthenogenetic insect species and there are only female adults present and did not release sex pheromones (Mikac and Clarke, 2006). Hence, the present finding that the presence of PBAN-like peptide activity in the female *L. entomophila* adults indicated that the sex pheromone released by females of *L. entomophila* attract the male to mate with it (Shao et al., 2006). Perhaps the pyrokinin/PBAN peptide had the functions to regulate the release of sex pheromone of *L. entomophila*. Matrix-assisted laser desorption/ionization times-of-flight mass spectrometry (MALDI-TOF MS) has proven to be very useful for the detection of endogenous peptide patterns, at unmatched sensitivity levels, which is at the level of single organs, cells and even cell organelles (Clynen et al., 2003). A MALDI-TOF mass spectrum of the head extracts of female adults showed almost only masses corresponding to FXPRLamides (Clynen et al., 2003; Predel et al., 2006; Herbert et al., 2009). Based on the sequence information of this peptide, the gene of pyrokinin/PBAN peptide or the receptor of this peptide would now be studied in detail with *L. entomophila*.

In fact, direct use of neuropeptides for insect control is impractical as the insect cuticle contains polar lipid matrix that inhibits penetration of polar compounds like peptides, and the insect gut, hemolymph and membranes of a number of tissues contain peptidases that rapidly degrade the peptides (Nachman et al., 1995; 1999). The development of agonists and antagonists with enhanced peptidase-resistance and cuticle/gut penetrability can overcome these limitations and represents a key step in the development of control techniques employing analogs of insect neuropeptides capable of disrupting critical life processes. The analogs of neuropeptides that penetrate the insect cuticle using the pyrokinin/PBAN class of insect neuropeptides as models are emerging (Nachman et al., 1998, 2003, 2009; Teal et al., 2002). Such analogs may provide leads in the development of novel insect-specific and environmentally favorable pest management agents capable of disrupting PK/PBAN-regulated physiological systems.

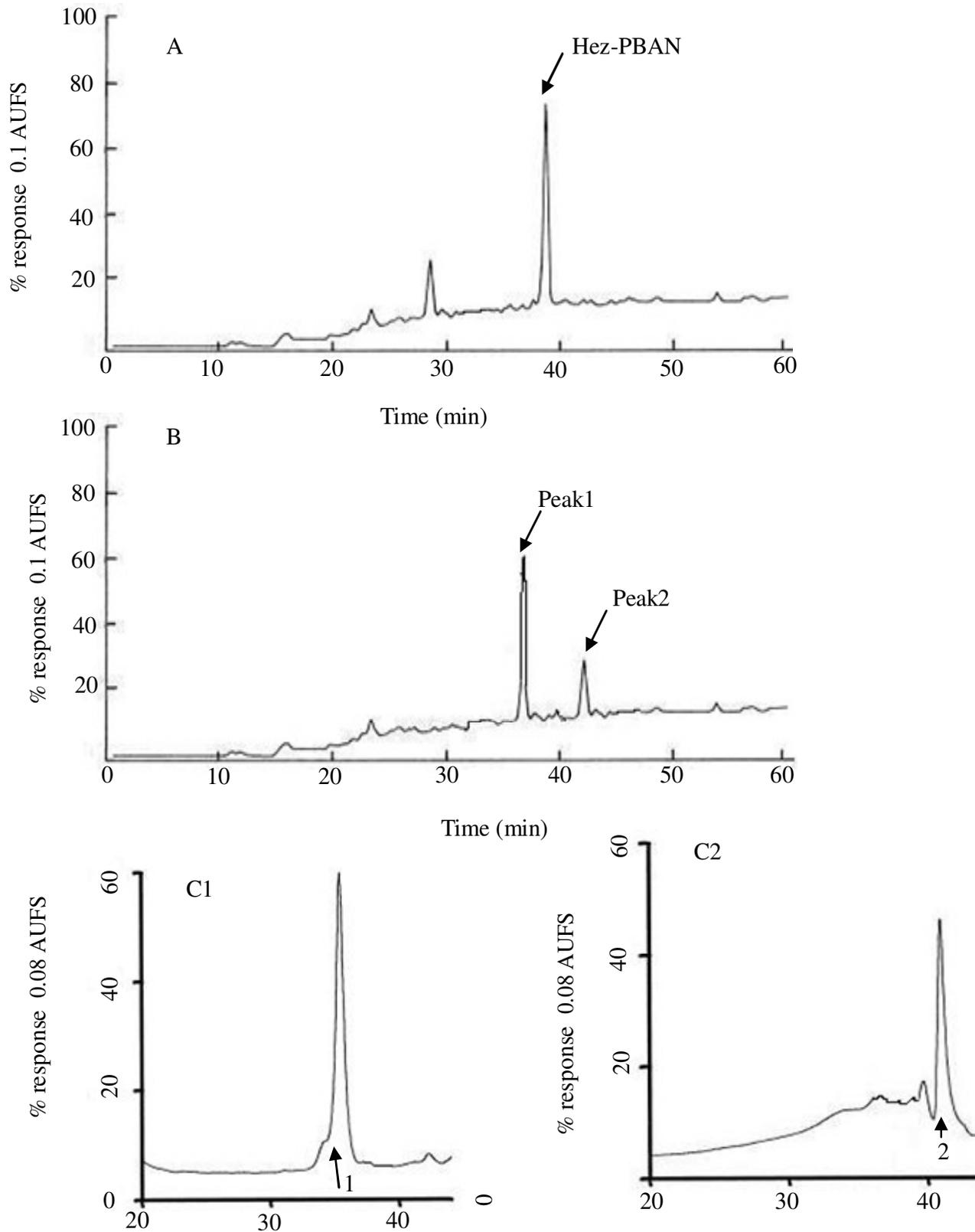


Figure 1. Purification of pyrokinin from brain extraction of female in *L. entomophila*. Peaks were collected manually and aliquots were tested in the ELISA bioassay. (A) Chromatogram of the standard peptide of Hez-PBAN. (B) Chromatogram of the third purification step of brain extraction of female in *L. entomophila*. (C1) The chromatogram of the purification of manual collecting fraction in 36.25 min. (C2) Chromatogram of the purification of manual collecting fraction in 40.85 min.

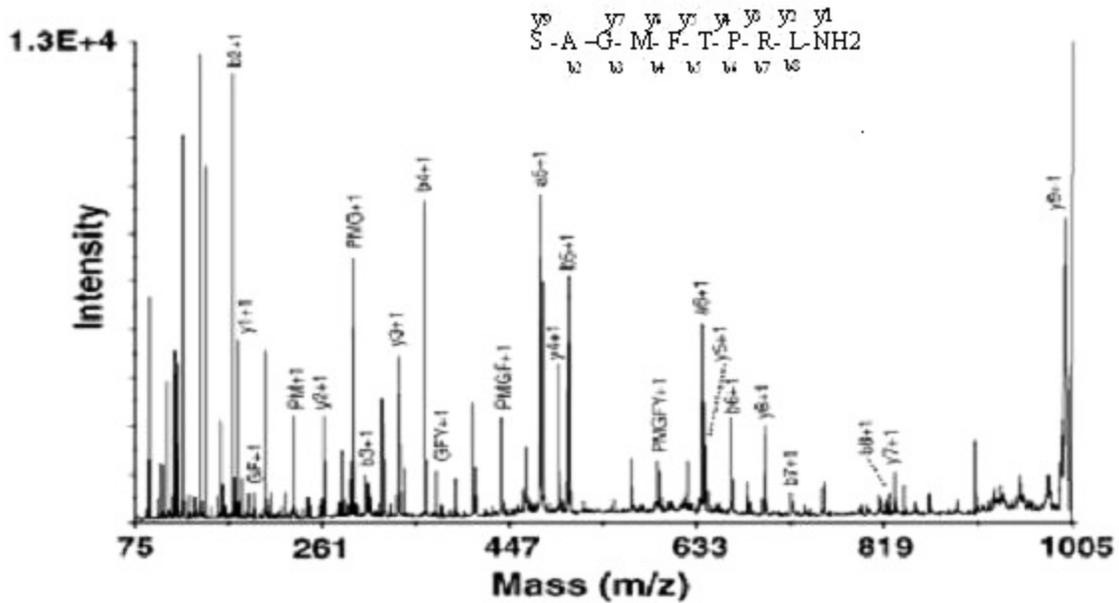


Figure 2. Tandem mass spectrometry (MS/MS) fragmentation spectra of analysis of the pyrokinin of *L. entomophila* brain HPLC fraction.

Lie-PK	SAGM ^F TPRI ^F
Lom-PK-II	OSVPT ^F TPRI ^F
Lom-PK-1	QDSGDGWPOQP ^F VPRL ^F
Lem-PK	QTS ^F TPRI ^F
Pea-PK-1	HTAG ^F IPRI ^F
Drm-PK-2	SVP ^F KPRI ^F
Sac-PBAN	LTEDMPATPTDQE--MFDODPEQIDTR-TRY ^F SPRI ^F
Mas-PBAN	ISEDMPATPSDQ ^F EYPMYHPDPEQIDTR-TRY ^F SPRI ^F
Anp-PBAN	LSDDMPATPKDQ ^F E--MYHQDPEQVDTR-TRY ^F SPRI ^F
Bom-PBAN	LSEDMPATPADQ ^F E--MYQPDPEEMESR-TRY ^F SPRI ^F
Har-PBAN	LSDDMPATPADQ ^F E--MYRQDPEQIDSR-TKY ^F SPRI ^F
Hez-PBAN	LSDDMPATPADQ ^F E--MYRQDPEQIDSR-TKY ^F SPRI ^F
Hea-PBAN	LSDDMPATPADQ ^F E--MYRQDPEQIDSR-TKY ^F SPRI ^F
Hev-PBAN	LADDMPATPADQ ^F E--MYRQDPEQIDSRRTKY ^F SPRI ^F
Mab-PBAN	LADDMPATPADQ ^F E--MYRPDPEQIDSR-TKY ^F SPRI ^F
Agi-PBAN	LADDTPATPADQ ^F E--MYRPDPEQIDSR-TKY ^F SPRI ^F
Spl-PBAN	LADDMPATPADQ ^F E--LYRPDPDQIDSR-TKY ^F SPRI ^F
Lyd-PBAN	LADDMPATMADQ ^F E--VYRPEPEQIDSR-NKY ^F SPRL ^F

Figure 3. Homology comparison to other FXPRL peptides *Lie*-PK manes the results of this research Lom, *Locusta migratoria* PKII (AAB29407.1),PK-1(P20404)Lem, *Leucophaea . maderae* (P13049) *Pea* , *Periplaneta . americana* (P82691); *Drm* *Drosophila melanogaster* (41547) *Sac*, *Samia cynthia ricini* (AY286544); *Mas*, *Manduca sexta* (AY172672); *Anp*, *Antheraea pernyi* (AY445658); *Bom*, *Bombyx mori* (D28764); *Har*, *Helicoverpa armigera* (AY280543); *Hez*, *Helicoverpa zea* (U08109); *Hea*, *Helicoverpa assulta* (U96761); *Hev*, *Heliothis virescens* (AY173075); *Mab*, *Mamestra brassicae* (AF044079); *Agi*, *Agrotis ipsilon* (AJ009674); *Spl*, *Spodoptera littoralis* (AF401480); *Lyd*, *Lymantria dispar* (P43511).

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