Pergamon

Insect Biochemistry and Molecular Biology 30 (2000) 489–498

Insect Biochemistry and Molecular Biology

www.elsevier.com/locate/ibmb

A new member of the AKH/RPCH family that stimulates locomotory activity in the firebug, *Pyrrhocoris apterus* (Heteroptera)

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Received 25 March 1999; received in revised form 2 November 1999; accepted 1 February 2000

Abstract

A new member of the AKH/RPCH family was isolated and identified from the corpora cardiaca of the firebug *Pyrrhocoris apterus*. The peptide was isolated in a single step by reversed phase HPLC and the structure deduced from the multiple MS (MS^N) electrospray mass spectra and amino acid analysis as that of an octapeptide with the sequence pGlu–Leu–Asn–Phe–Thr–Pro–Asn–Trp–NH_2: this sequence was confirmed by synthesis. The synthetic peptide induced lipid mobilisation and stimulated locomotory activity in macropterous females. This peptide, designated as *Pyrrhocoris apterus* adipokinetic hormone (Pya-AKH), is the first identified adipokinetic hormone described in a representative species of the suborder Heteroptera.

Keywords: Adipokinetic hormone; AKH/RPCH; Neuropeptide; HPLC; Electrospray mass spectrometry; Lipid mobilisation; Locomotory activity; Bug

1. Introduction

The adipokinetic hormone/red pigment concentrating hormone family (AKH/RPCH family) is one of the best known groups of arthropod neuropeptides. This name comes from the first members of this family to be fully characterised, a chromatophorotropin (RPCH) from prawns (Fernlund and Josefsson, 1972), and an adipokinetic hormone (Lom-AKH) from *Locusta migratoria* (Stone et al., 1976). The AKHs are synthesised and released from the corpora cardiaca, but small quantities of AKH-I are also found in the brain of *L. migratoria* (Moshitzky et al., 1978; Bray et al., 1993). Flight is a primary stimulus for the release of the hormones into the haemolymph (Mayer and Candy, 1969) although little is known about the integration of flight activity with the neurosecretory system (Orchard, 1987; Goldsworthy, 1990). In locusts, where the hormones have been investigated intensively, the AKHs are known to be essential for long-term flight (Goldsworthy, 1983). The known actions of AKHs are, however, broader than their name implies. The peptides have been reported to act on the fat body to mobilise stores of lipid and carbohydrate, activate glycogen phosphorylase, accumulate cAMP (see Goldsworthy, 1983) and inhibit the synthesis of lipids (see Lee and Goldsworthy, 1996), proteins (Carlisle and Loughton, 1979), and RNA (Kodrík and Goldsworthy, 1995). Recently, it was reported that AKH also stimulates locomotory activity (Socha et al., 1999). AKHs are thus typical stress hormones: they stimulate catabolic reactions, making energy more available while inhibiting synthetic reactions.

AKHs are octa-, nona- or decapeptides with both termini blocked: the N-terminus by a pyroglutamate residue and the C-terminus by an amide. For a review of the characterization of other common structural features see Gäde (1997) and Gäde et al. (1997). More than 30 mem-

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0965-1748/00/$ - see front matter
PII: S0965-1748(00)00025-4

bers of the AKH/RPCH family have been identified but, in spite of this growing list of AKHs (Gäde, 1997; Gäde et al., 1997), no representatives of the suborder Heteroptera of the order Hemiptera have been characterised. Several years ago, three independent groups described a neuropeptide of the AKH/RPCH family isolated from cicadas (Gäde and Janssens, 1994; Raina et al., 1995; Veenstra and Hagedorn, 1995), from the suborder Homoptera, closely related to the Heteroptera. The lack of information on AKH peptides from Hemiptera is surprising, because this order is by far the most successful non-holometabolan order (Kristensen, 1981). The firebug, *Pyrrhocoris apterus* (L.), appeared to be an excellent insect for such study, because it has been used for many years as a convenient experimental model in the Institute of Entomology and there is a wealth of information about the biology of this species (for review see Socha, 1993).

P. apterus is a flightless heteropteran producing long-winged (macropterous) and short-winged (brachypterous) morphs. In central Europe, the bug lives at the foot of lime trees, whose seeds are the basic component of its food. Development from the egg to adult takes about 1 month under laboratory conditions (25–26°C and long-day, LD 18:6 h photoperiod). The larvae pass through five instars. Under constant laboratory conditions, development through four initial larval stadia takes approximately 10–14 days, while the final stadium lasts from 7 to 10 days. The adults live from 2 months to 1 year, depending on whether the individuals are maintained under conditions favouring reproductive activity or diapause (see Socha, 1993). Thus, this study was initiated to investigate AKH/RPCH peptides in the firebug *P. apterus*, a representative species of the suborder Heteroptera.

2. Materials and methods

2.1. Experimental animals

A laboratory stock culture of the firebug, *P. apterus*, was used in the present study. The origin and details on breeding of this culture are described elsewhere (Socha and Šula, 1996). Larvae and adults were kept in 0.5-l jars in mass culture (approximately 40 specimens per jar), and food and water were replenished twice a week. Freshly ecdysed adult females were transferred in groups of 10–20 specimens to small glass jars (250 ml) and kept under the same photoperiodic and temperature regimes in which they had developed. The bugs were supplied with linden seeds and water in small glass tubes plugged with cellulose wool.

2.2. Isolation/purification procedure

Corpora cardiaca (CC) often with corpora allata (CA) and a corresponding part of the brain were dissected from 3- to 5-day-old adults and placed in an ice-cold Eppendorf 1.5 ml centrifuge tube. For the purification of the peptide, tissues from several hundred insects were extracted in 80% methanol. Evaporated methanolic extract was re-dissolved and purified by single-step reverse phase high pressure liquid chromatography (RP HPLC; Merck-Hitachi D-6000 chromatography system) on a LiChrospher WP 300 RP-18 column (250 mm×4 mm, particles 5 μm, Merck), with a gradient from 25 to 100% B in 40 min (A=0.1% TFA in water; B=0.05% TFA in 40% acetonitrile) at a flow rate of 0.8 ml/min and UV detection at 215 nm. Biological activity in the fractions was tested in a bioassay (see below) and the active material (retention time 28.33 min) was used for the structure elucidation.

2.3. Amino acid analysis

The active fraction (about 500 pmol of the active substance) was hydrolysed in 6 M HCl at 110°C in sealed tubes for 18 h. The hydrolysate was evaporated to dryness and the amino acids determined as tertiary-butyldimethylsilyl derivatives by GC/MS (Šimek et al., 1994; Jegorov et al., 1997).

2.4. Molecular weight and structure elucidation

The molecular weight and amino acid sequence of the peptide were estimated by electrospray mass spectrometry performed on an LCQ mass spectrometer (Thermoquest, USA). The sample was introduced in CH_3CN–0.05% TFA (50:50) either via a syringe pump at 3 μl/min or via an RP-C18 150 mm×1 mm Symmetry C_8 column at 50 μl/min employing an LCQ electrospray ion source operated at 4.2 kV.

The amino acid sequence was deduced from the electrospray MS^N mass spectra obtained by the collision induced decomposition (CID) of the MH^+ ion and its product ions in a series of MS^N experiments that were performed with the ion trap mass spectrometer. The peptide was synthesised commercially by Genosys Biotechnologies Ltd. (Europe).

2.5. Bioassay

2.5.1. Sample treatment

Samples for bioassay (crude CC extract, HPLC fractions, synthetic peptide) were dissolved in 20% methanol in the Ringer saline. Theoretical stock solution concentration of the synthetic peptide was verified on a fluorometer (Spex Fluorolog) ($_{Ex}$280 nm, $_{Em}$340 nm) by comparison with known concentrations of a tryptophan

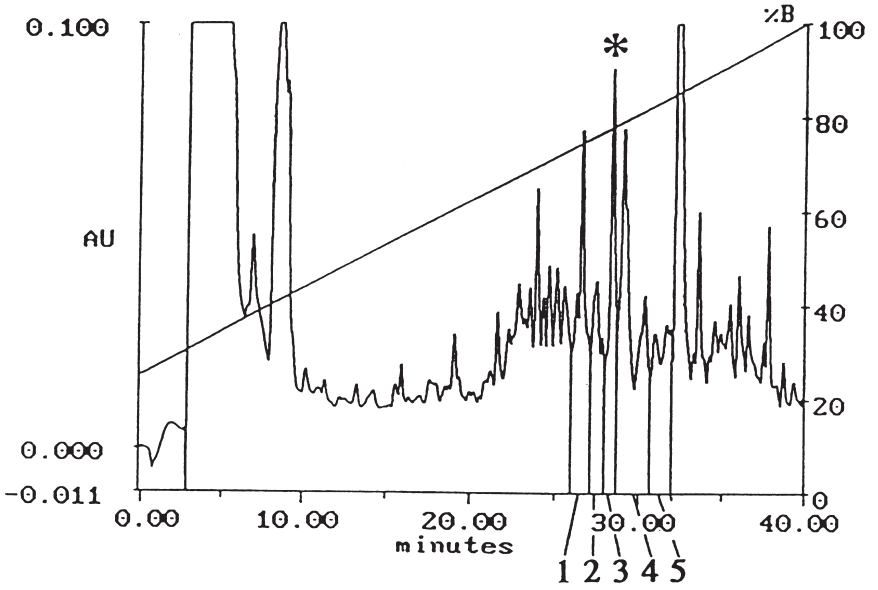

Fig. 1. The RP HPLC elution profile of an extract of 410 CC–CA complexes from *P. apterus* (for details see Section 2). Activity of the marked fractions was tested in the lipid mobilisation assay and is shown in Table 1. The Pya-AKH fraction is marked by an asterisk.

solution. The tested samples were injected into 10-day-old macropterous females—the reasons for this choice of age are explained elsewhere (Socha and Kodrík, 1998). Peptidic dilutions were prepared to give the desired content per 2 μl solution. This volume was injected using a syringe (5 or 10 μl; Hamilton Co., Reno, NV) through the metathoracic–abdominal intersegmental membrane into the thorax of the experimental bug. Control bugs were injected with 2 μl of 20% methanol in Ringer saline in the same way.

Table 1
Effect of crude extract of corpora cardiaca and corpora allata complex (CC–CA) and RP HPLC purified fractions (see Fig. 1) of the same material on the change (mean±SD) in concentration of lipids in the haemolymph. Statistically significant differences at the 5% level are indicated by asterisks (experimental vs. control)

Sample	*n*	Increase in lipids (mg/ml)
CC–CA		
1 pair equivalents	8	12.20±2.36*
2 pair equivalents	6	15.24±3.68*
HPLC fractions (2 CC–CA pair equivalents)		
1.	5	2.46±3.22
2.	5	0.60±3.29
3.	6	15.75±3.98*
4.	5	1.68±3.11
5.	5	0.08±2.40
Control	5	−1.68±3.12

2.5.2. Lipid mobilisation assay

The lipid content of the haemolymph samples was determined by a method based on the sulpho-phosphovanillin test (Zöllner and Kirsch, 1962; modified by Holwerda et al., 1977 and by Van Marrewijk et al., 1984). The haemolymph samples were taken from the cut end of an antenna: a drop of haemolymph was leaked onto a piece of parafilm M and 0.5 μl taken up by micropipette (Eppendorf Varipipette 4810) and used for the determination of lipids. The samples were collected just before and 90 min after injection. Using the Microsoft Excel program, the optical densities at 546 nm in a spectrophotometer (UV 1601 Shimadzu) were converted to mg lipids per ml haemolymph with the aid of a calibration graph based on known amounts of oleic acid. Results were expressed as a mean of haemolymph lipid elevation for between 6 and 12 observations (difference of lipid levels before and after injection) ±SD. Standard deviations were shown only in one direction in the figure.

2.5.3. Locomotory activity

The computerized multichannel data acquisition system consisted of 30 monitoring units and an HP 6942A Multiprogrammer equipped with FET Scanner and A/D converter cards. The Multiprogrammer was connected to an IBM-compatible PC running a program written in HP Basic. Each female treated either by 10 pmol Pya-AKH or by saline (control) was immediately transferred to the monitoring unit and its locomotory activity recorded for 9 h under constant light at 26°C. The number of interruptions of an infrared beam, caused by movement of the bugs, was recorded by the computer and integrated over

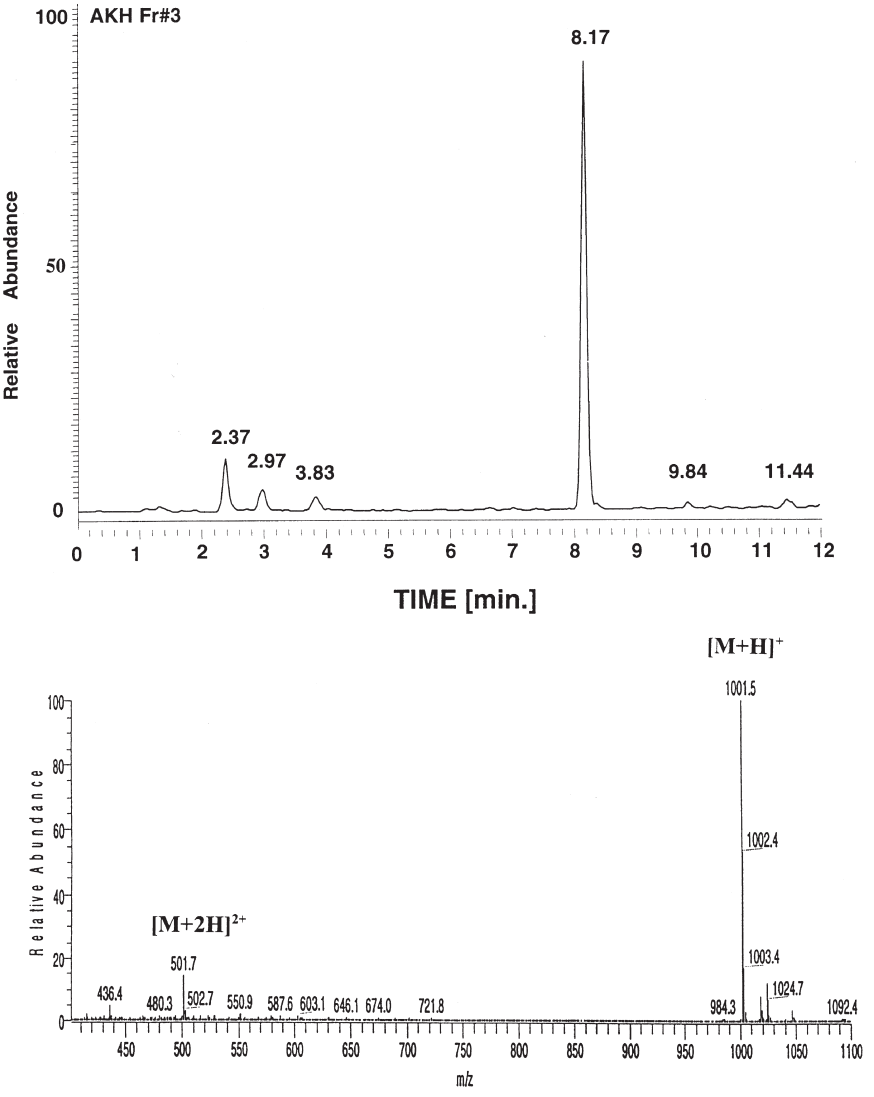

Fig. 2. The HPLC/ESI-MS analysis of the active material in fraction 3. Top panel: total ion chromatogram of *m*/*z*=400–1400. Gradient elution with CH_3CN–0.05% TFA from 20:80 to 80:20 in 10 min, flow rate 50 μl/min, column Symmetry C_8 (Waters, USA). For details, see Section 2. Bottom panel: ESI full scan mass spectrum of the base peak at RT=8.17 min with MH^+ 1001.5. The double-charged ion $(M+2H)^{2+}$ at *m*/*z*=501.7 and the $(M+Na)^+$ adduct at *m*/*z*=1023.6 further confirm the molecular weight of the target peptide.

periods of 1 h. Locomotory activity was measured in 46 bugs in both the experimental and control groups.

2.6. Data presentation and statistical analyses

The results of adipokinetic responses were plotted using the graphic program Prism (GraphPad Software, version 2.1, San Diego, CA, USA). The statistical analyses were performed with use of the program Instat (GraphPad Software, version 1.15, San Diego, CA, USA). For a similarity search of the Pya-AKH with other members of the AKH/RPCH family the DNASTAR (PROTEAN-Clustal method) software (DNASTAR, Inc., Lasergene, Madison, WI) was used.

An unstructured multivariate approach (Diggle, 1992) was applied as the method of statistical analysis for the locomotory activity data, because they consisted of two relatively short non-stationary time series. The data were first normalised by log transformation and the test statistic was calculated as

$$D = np\{\log(|\Sigma_0|) - \log(|\Sigma|)\}$$

where n denotes the total number of bugs, p is the number of observations per bug, Σ denotes the estimate of the variance matrix and Σ_0 the corresponding estimate using the overall sample means. The statistic D was then compared with critical values of chi-squared distribution using degrees of freedom $p(k-1)$, where k is the number of different groups. For more details on the calculation procedure see Diggle (1992). All matrix computations were performed by means of the Mx software package developed by Neale (1997).

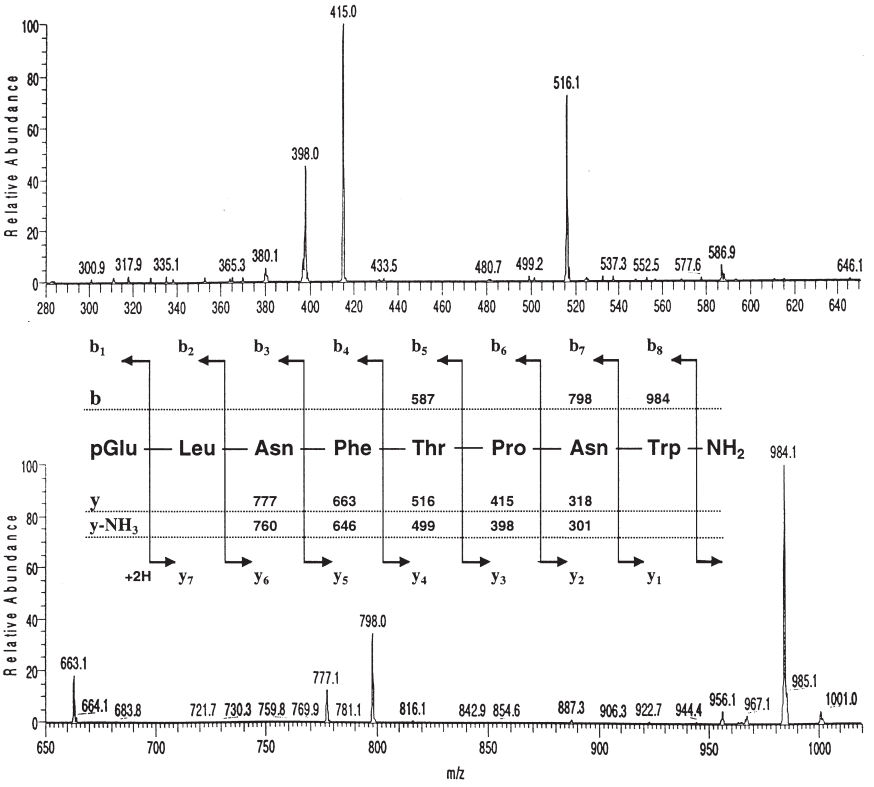

Fig. 3. CID ESI-MS^2 spectrum of the ion MH$^+$ (*m*/*z*=1001.5) from *P. apterus*. The Pya-AKH sequence insert shows theoretical calculated masses for b and y-type ions, which were observed in the MS^2 mass spectrum.

3. Results and discussion

RP HPLC analysis of the methanolic CC–CA extract from *P. apterus* generated a number of UV-absorbing peaks (Fig. 1) which were tested in the lipid mobilisation assay. The results obtained from fractions in the region where AKHs are expected to elute are shown in Table 1. None of the other fractions were active (data not shown). The highest adipokinetic activity was in fraction 3, where the response of the assay bugs was comparable with that obtained to crude extracts of CC–CA. Further RP HPLC separations (data not shown) indicated high purity of the material, and HPLC/ESI-MS analysis revealed a base peak with (M+H)$^+$ 1001.5 Da (Fig. 2) indicating a molecular weight of 1000.5 Da for the peptide. Quantitative amino acid analysis showed stoichiometric ratios of leucine (1), threonine (1), proline (1), phenylalanine (1), aspartic acid (2) and glutamic acid (1) in the acid hydrolysate. Treatment of the peptide with pyroglutamate aminopeptidase (Boehringer Mannheim) and subsequent HPLC/ESI-MS analysis revealed that its N-terminus is blocked by a pyroglutamic acid residue. The amino acid sequence was deduced from the multiple tandem (MS^N) mass spectra obtained by a consecutive isolation, storage and collisionally induced dissociation (CID) of the (M+H)$^+$ ion in the ion trap (and resulting in the MS^2 spectra), followed by an analogous process with selected product ions (resulting in a set of the MS^3, MS^4, etc. spectra). The MS^2 (or MS/MS) spectrum of the peptide from *Pyrrhocoris* and the sequence product ions are shown in Fig. 3.

The intensive (MH–NH$_3$)$^+$ product ion, *m*/*z* 984.3, and for the adipokinetic peptides characteristic y-type and b-type product ions (Raina et al., 1995) show diagnostic fragmentations at nearly every peptide bond except those between pGlu–Leu. They clearly indicate a C-terminus amido block and a partial peptide sequence (amino acid positions 3–8 in the peptide). The structure was further deduced from a set of the collected MS^N spectra. Thus, the correct assignment was confirmed by a CID experiment with an ion, *m*/*z* 984.3, giving MS^3 mass spectra with a series of diagnostic (y-NH$_3$) and b-type ions and, as expected, with an absence of the y-type ions (Fig. 4).

Structurally important ions from the set of MS^N spectra are summarised in Table 2. The power of MS^N scans is exemplified in Fig. 5 on the selective MS^4 and MS^3 decomposition of the b$_5$ (*m*/*z* 587) and y$_3$ (*m*/*z* 415) product ions arising from the N- and C-terminus peptide sequence fission, respectively.

Finally, a complete primary sequence was established from information about the N-terminus pyroglutamic amino residue, the amino acid composition and the mass spectral data, and was assigned as an octapeptide pGlu–Leu–Asn–Phe–Thr–Pro–Asn–Trp–NH$_2$ belonging to the AKH/RPCH family. The structure was confirmed by synthesis and MS^N experiments on the native and the

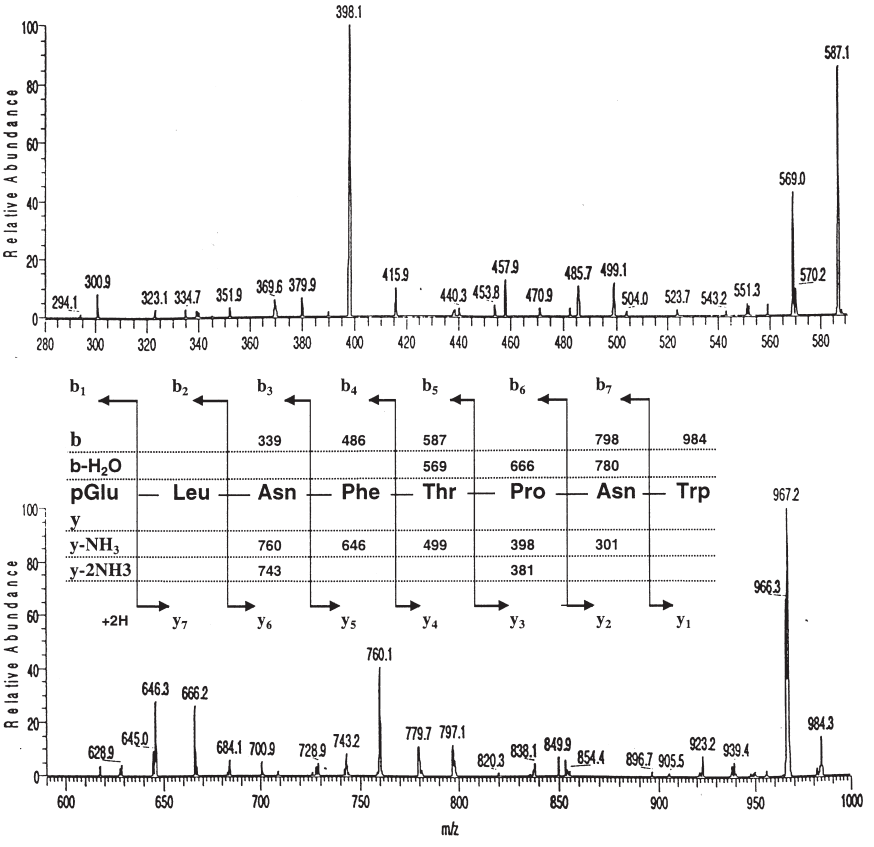

Fig. 4. CID ESI-MS^3 spectrum of the product ion *m*/*z*=984.3 obtained from consecutive CID of the MH$^+$ (*m*/*z*=1001.5). The Pya-AKH sequence insert shows theoretical calculated masses for b and y-type ions, which were observed in the MS^3 mass spectrum.

synthesized peptides. Both compounds showed identical chromatographic (the synthetic peptide co-eluted with the natural one) and mass spectral properties.

From its structure and the activity in the lipid mobilisation assay, the peptide is a new member of the AKH/RPCH family—*P. apterus* AKH. To our knowledge this is the first member of the family identified within the suborder Heteroptera. The peptide has been designated Pya-AKH.

A similarity search using DNASTAR software revealed maximal identity with Lom-AKH-I and Phm-AKH (Table 3). This is not surprising because the Pya-AKH sequence is identical to the first eight amino acids of these two decapeptides. Further, the analysis showed 87.5% similarity with five octapeptides (which means an exchange of one amino acid) (Emp-AKH, Lom-AKH-III, Miv-CC, Pea-CAH-II and Tem-HrTH), and with four (or five) decapeptides (which means an exchange of one amino acid within the first eight amino acids of the molecule and the addition of two more amino acids at the C-terminus) (Cam-HrTH-I, II, Phl-CC, Del-CC and Rom-CC; see review by Gäde et al., 1997). Surprisingly, there is relatively low similarity (62.5%) with the cicada peptides Plc-HrTH-I and II (Gäde and Janssens, 1994; Veenstra and Hagedorn, 1995; Raina et al., 1995), despite the close phylogenetic relationship between Homoptera and Heteroptera.

The synthetic peptide induces a dose-dependent response in the lipid mobilisation assay (Fig. 6). The peptide has an estimated ED_{50} of 2.35 pmol, with the maximal response requiring at least 10 pmol. Surprisingly, higher doses appeared to be less effective: this is not a characteristic of the response to AKHs that has been reported previously, but seems to be a characteristic of this bug's lipid mobilisation, because high doses of Lom-AKH-I also exert an attenuated effect (see Fig. 6).

In a previous study using Lom-AKH-I in *P. apterus* (Socha and Kodrík, 1998), this decapeptide had about twice the potency and 50% greater efficacy than the Pya-AKH reported here. We have no explanation for this apparent anomaly except to suggest that the mobilisation of lipids may not be the major physiological role of this peptide in the firebug and there may be other adipokinetic factors in the firebug that remain to be identified. It is perhaps significant that the maximal increases in lipid levels in the haemolymph are similar for crude extracts of retrocerebral complexes and the purified active peptide and Lom-AKH-I (Table 1 and Fig. 6). These maximal responses are greater than that seen for Pya-AKH. On the other hand, the existence of other

Table 2
Summary of the diagnostic product ions arising from the CID ESI-MS^N N- and C-terminal cleavage of the amide (peptide) bond which were employed for structure elucidation. The data were obtained from a set of the MS^N mass spectra of the Pya-AKH by electrospray ion trap mass spectrometry. The rationalised sequence ions are labelled using nomenclature discussed elsewhere (Biemann, 1988)

m/z observed	b-type ions	MS^N CID on (*m/z*)	*m/z* observed	y-type ions	MS^N CID on (*m/z*)
1001	$(\mathbf{M+H})^+$				
984	$\mathbf{b_8}$	MS^2			
967	b_8-NH_3				
798	$\mathbf{b_7}$	MS^2			
			777	$\mathbf{y_6}$	MS^2
			760	y_6-NH_3	MS^3 (984)
684	$\mathbf{b_6}$	MS^3 (984)			
			663	$\mathbf{y_5}$	MS^2
			646	y_5-NH_3	MS^3 (984)
587	$\mathbf{b_5}$	MS^2			
569	b_5-H_2O	MS^3 (798)			
			516	$\mathbf{y_4}$	MS^2
			499	y_4-NH_3	MS^3 (984)
486	$\mathbf{b_4}$	MS^3 (984)			
			415	$\mathbf{y_3}$	MS^2
			398	y_3-NH_3	MS^3 (984)
			381	y_3-$2NH_3$	MS^4 (398)
363	$[T+F+N+H]^+$	MS^4 (587)			
339	$\mathbf{b_3}$	MS^3 (984)			
			318	$\mathbf{y_2}$	MS^2
313	$[T+P+N+H]^+$	MS^3 (798)			
			301	y_2-NH_3	MS^3 (984)
249	$[T+F]^+$	MS^4 (587)			
225	$\mathbf{b_2}$	MS^4 (587)			
			223	$[P+N+CO–NH_3]^+$	MS^4 (398)
			212	$[P+N+H]^+$	MS^3 (415)
			204	$[W+NH_2+H]^+$	MS^3 (415)
			184	$[P+N–CO+H]^+$	MS^3 (415)
			167	$[P+N–CONH_2+H]^+$	MS^3 (415)

adipokinetic peptides in *P. apterus* CC seems to be unlikely, because each fraction obtained after the HPLC analysis was tested in the lipid mobilisation assay and only one was active. This particular fraction was analysed again in another HPLC run under different separating conditions and no heterogeneity of the material was revealed. Moreover, chemical analysis of the peak showed one clearly readable signal. For these reasons we exclude the possibility of the presence of other peptides with adipokinetic activity.

Intriguingly, the octapeptide sequence that we have now identified as Pya-AKH has been synthesised previously and assayed for lipid mobilisation in locusts: it was also found to be less active than the [Gly8a–Thr8b]-C-terminally extended (deca)peptide analogue of Pya-AKH (=Lom-AKH-I) in locust assays performed both in vivo and in vitro (Lee and Goldsworthy, 1996). It will be of interest to explore further the structure–activity relationships of the receptors for AKHs in the firebug.

The synthetic Pya-AKH also stimulated locomotory activity. Time serial analysis revealed highly significant (D=149.41, df=9, $P<0.0001$) differences between locomotory activities of Pya-AKH-treated and control firebugs when expressed as an activity ratio (Fig. 7). The most apparent stimulation was observed 3 h after the treatment when the activity of hormonally treated firebugs was twice that of the controls. We reported earlier that Lom-AKH-I stimulates locomotory activity in *P. apterus* females (Socha et al., 1999). In the present study we demonstrate the ability of Pya-AKH to stimulate locomotory activity as well. The ratio of locomotory activity between the AKH-treated and control females was found to be very similar for both tested hormones, i.e. 1.8 for Lom-AKH-I (Socha et al., 1999) and 2.1 for Pya-AKH (present paper). Nevertheless, some minor differences were recorded: maximal locomotory activity in the bugs treated by Lom-AKH-I was reached within 2 h, while in those treated by Pya-AKH this was achieved 3 h after injection. More rapid stimulation of locomotion in Lom-AKH-I-treated bugs might be explained by the higher potency and efficacy of this peptide in the lipid mobilisation assay compared with Pya-AKH. This suggestion is also supported by the finding that the time course of the stimulatory effects of AKH on locomotory activity correlates positively with the observed effects of the hormone in elevating the levels of lipids in the

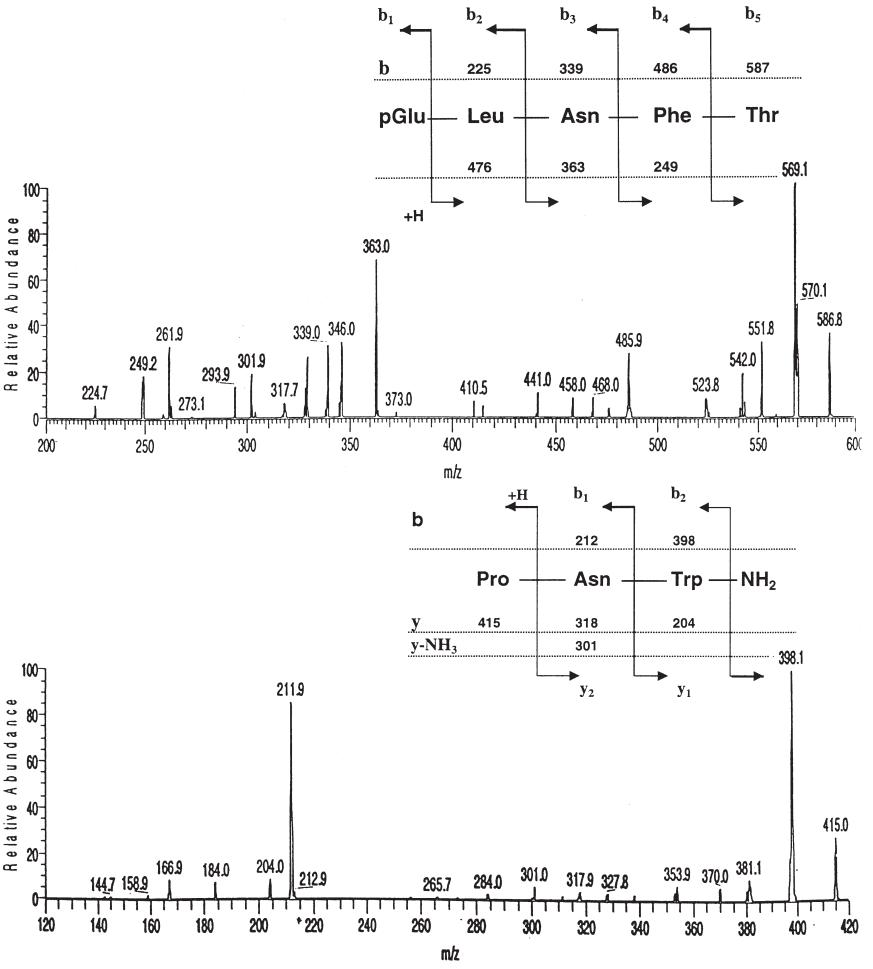

Fig. 5. ESI-MS^N spectra of the N-terminus b_5 (m/z=587) and C-terminus y_3 (m/z=415) product ions. Top panel: MS^4 spectrum obtained by the isolation/CID of m/z=1001.5 (MH^+)→984.3 (MH^+–NH_3)→587.2 (b_5)→. Bottom panel: CID MS^3 spectrum of m/z=1001.5 (MH^+)→415.2 (y_3)→. For diagnostic y- and b-type ions, see Table 2.

Table 3
Primary structures of peptides of the adipokinetic hormone/red pigment-concentrating hormone family showing high similarity (100% and 87.5%) with Pya-AKH. For details see the text

Peptide	Species	Sequence
Lom-AKH-I[a]	*Locusta migratoria*	**pQ** L N **F** T **P** N **W** G Tamide
Phm-AKH[a]	*Phymateus morbillosus*	**pQ** L N **F** T **P** N **W** G Samide
Emp-AKH[a]	*Empusa pennata*	**pQ** V N **F** T **P** N **W**amide
Lom-AKH-III[a]	*Locusta migratoria*	**pQ** L N **F** T **P** W **W**amide
Miv-CC[a]	*Microhodotermes viator*	**pQ** I N **F** T **P** N **W**amide
Pea-CAH-II[a]	*Periplaneta americana*	**pQ** L T **F** T **P** N **W**amide
Tem-HrTH[a]	*Tenebrio molitor*	**pQ** L N **F** S **P** N **W**amide
Cam-HrTH-I, II[a]	*Carausius morosus*	**pQ** L T **F** T **P** N **W** G Tamide
Phl-CC[a]	*Phymateus leprosus*	**pQ** L T **F** T **P** N **W** G Samide
Del-CC[a]	*Decapotoma lunata*	**pQ** L N **F** S **P** N **W** G Namide
Rom-CC[a]	*Romalea microptera*	**pQ** V N **F** T **P** N **W** G Tamide
Pya-AKH	*Pyrrhocoris apterus*	**pQ** L N **F** T **P** N **W**amide

[a] Data taken from Gäde et al. (1997).

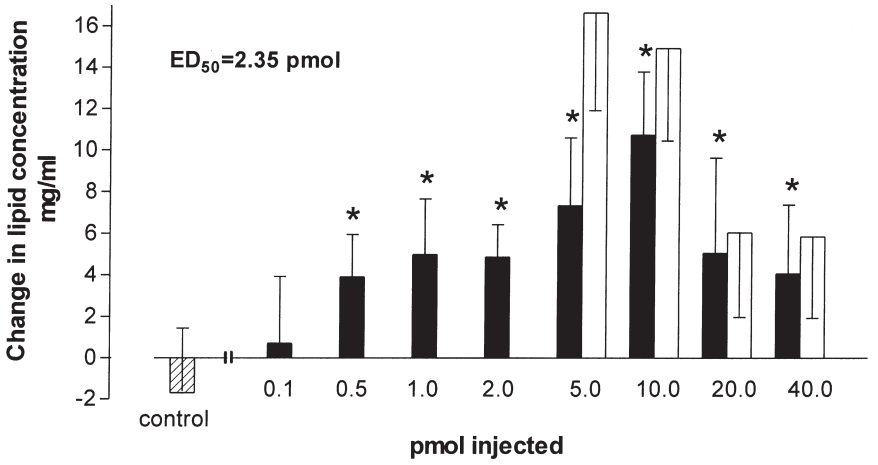

Fig. 6. The effect of increasing doses of Pya-AKH (■) and high doses of Lom-AKH-I (□) on the elevation of haemolymph lipid level in *Pyrrhocoris* females. Control bugs were injected with saline. Statistically significant differences at the 5% level (experimental vs. control) are indicated by asterisks.

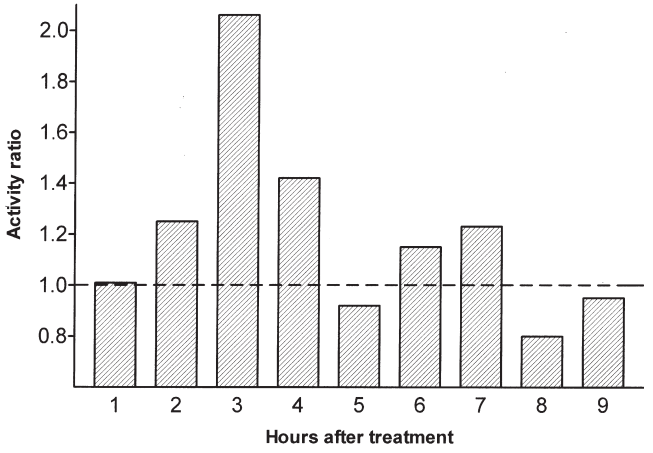

Fig. 7. The ratio of locomotory activity (mean number of infrared beam crosses per female per hour) between *P. apterus* females injected with 10 pmol Pya-AKH and controls that received only the solvent.

haemolymph (unpublished data). All these observations indicate that the AKH effect on locomotory activity represents a physiologically significant phenomenon. The mechanism of its action is not known yet, but we have hypothesised that either metabolic and/or neuromodulatory pathways could be involved (Socha et al., 1999).

Acknowledgements

This study was supported by grant No. A6007804 from the Grant Agency of the Czech Academy of Sciences (DK) and by a grant from The Royal Society (GJG and DK). The authors thank Mrs J. Irová, D. Rienesslová and J. Jabůrková for technical assistance.

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