Locust corpora cardiaca contain an inactive adipokinetic hormone

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Abstract A neuropeptide from the migratory locust, *Locusta migratoria*, has been identified as a novel member of the family of adipokinetic hormones (AKHs). The peptide is probably synthesised in the brain because it is the first AKH found in the storage lobe, whilst the three 'classic' *Locusta* AKHs are present in the glandular lobe of the corpora cardiaca. In locusts, the peptide has no biological activity usually associated with AKHs. There is only 36–56% sequence identity with the three Lom-AKHs, but 78% identity with the *Drosophila melanogaster* AKH, Drm-HrTH. The new peptide is active in the American cockroach, *Periplaneta americana*, and was provisionally named '*L. migratoria* hypertrehalosaemic hormone', Lom-HrTH; its biological role in locusts remains to be established. The high degree of identity with Drm-HrTH suggests that Lom-HrTH is an ancient molecule.

Key words: Brain; Hypertrehalosemia; Cockroach; *Drosophila*

1. Introduction

Adipokinetic hormones (AKHs) are short peptides of 8–10 amino acids which regulate various aspects of insect intermediary metabolism ([1]). AKHs induce increases of haemolymph lipid concentrations in locusts (adipokinetic effects) and increases of trehalose concentrations in cockroaches (hypertrehalosaemic effects. In locusts they are synthesised in the intrinsic cells of the glandular lobes (GL) of the corpora cardiaca (CC) [2]. All AKHs have common structural features: they are blocked at the N-terminus by a pyroglutamate residue and at the C-terminus by a carboxyamide, all known AKHs contain tryptophan in position 8 and phenylalanine or tyrosine in position 4.

The migratory locust is the only species with three different AKHs (Table 1), the closely related desert locust, *Schistocerca gregaria*, and *P. americana* contain two peptides. Other insect species such as *D. melanogaster* only contain one AKH. Peptides other than AKHs are also present in the CC, e.g. diuretic hormones and the locust insulin-related peptide which are both synthesised in the brain and released from the CC; in locusts these peptides are stored in and released from the nervous part of the CC, the storage lobe (SL).

The present study was initiated to analyse the spectrum of small molecular weight peptides in the locust CC by reversed-phase high-performance liquid chromatography (RP-HPLC). Known peptides can be identified by their retention times (e.g. AKHs and corazonin) or their molecular masses (mass spectrometry). Unknown peptides can then be characterised through sequencing and possibly predictions made as to their biological functions through sequence comparison with known invertebrate or vertebrate peptides.

2. Materials and methods

Whole locust corpora cardiaca, storage lobes, glandular lobes or brains were extracted with 100% methanol and chromatographed on a TSK G2000SW column at a flow rate of 1 ml 15% acetonitrile in 0.1% trifluoroacetic acid (TFA)/min. The low molecular weight fraction was run on an Aquapore RP-300 C_8 column with a gradient that started at 15% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min and increased at 1% acetonitrile/min. Molecular masses were determined with a Vestec benchtop laser time of flight spectrometer using bovine insulin as internal or external standard. Amino acid analyses were performed with an Applied Biosystems 420 analyser. Pulse-liquid sequencing was carried out using an Applied Biosystems Protein Sequencer 477A.

Concentrations of haemolymph lipids and carbohydrates were measured before injection of test materials and 90 min later as described earlier [3]. The percentage of the active form of fat body glycogen phosphorylase was determined in the direction of glycogen breakdown [4]. The increase of heart rate was measured in a semi-isolated preparation. The dorsal heart of an american cockroach was exposed in situ and contractions counted over three one minute periods and the values averaged. Peptides were then applied and the change in heart beat recorded.

2.1. Sequence identities

To compare AKH peptides of different lengths, e.g. the decamer peptide A (see below) and the octamer Drm-HrTH, only the first eight residues of the decamer were considered. In the case of Drm-HrTH six residues are identical with peptide A (score of 6). A score of 1 was added to the above value for the presence of the C-terminal carboxyamide and the total score expressed as the percentage of the number of features compared; for Drm-HrTH this is 78%.

3. Results and discussion

Neuropeptides present in methanolic CC extracts from adult migratory locusts were separated by size-exclusion chromatography. The low molecular weight fraction revealed only five to seven substantial peaks after RP-HPLC; three of these peptides were the Lom-AKHs (Table 1). Two peaks, A and B, were regularly detected. Bisection of the CC revealed that both peaks reside in the SL (Fig. 1). Peptide A isolated from 100 *L. migratoria* CC contained the following amino acids: asparagine/aspartic acid (490 pmol Asx; 1 mol per mol peptide), glutamine/glutamic acid (595 pmol Glx; 1×), arginine (535 pmol Arg; 1×), threonine (545 pmol Thr; 1×), proline (620 pmol Pro; 1×), valine (480 pmol Val; 1×), phenylalanine (595 pmol Phe; 1×) and serine (915 pmol Ser; 2×); glycine (200 pmol Gly; 0×) and leucine (220 pmol Leu; 0×) were also detected the characterisation of peptide B will be described elsewhere). The presence of Glx indicated that the peptide may be N-terminally blocked by a pyroglutamyl residue as was found for all known AKHs [1]

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and a number of insect neuropeptides [5]. Mass spectrometry revealed a mass of $m/z = 1205.9$ (using bovine insulin as internal standard).

Peptide A was treated with pyroglutamate aminopeptidase [6] and a new peak with a mass of $m/z = 1098.6$ was identified after RP-HPLC, the parent molecule had a mass of $m/z = 1207.9$ (external standardisation). The difference ($\Delta = 109.3$) represents the mass of one pyroglutamyl residue (theoretical value 111.1; amino acid at the N-terminus, position 1 of the native peptide). The result of the sequence analysis of unblocked peptide A is shown in Table 1 (see also legend to Table 1). In addition to the residues found in amino acid analysis tryptophan was unequivocally identified in position 8. This amino acid is destroyed during acid hydrolysis and could not be detected in amino acid analysis. Glycine and leucine were deemed to be contaminants of the sample used for amino acid analysis (see above) because they were present at approximately half the amount of the other amino acids, they were not identified during the sequence analysis and the theoretical mass calculated according to the sequence matched the mass spectrometry data. Preliminary RP-HPLC, amino acid analysis and mass spectrometry data ($m/z = 1204.7$; bovine insulin as external standard) indicate that the identical peptide A is present in the desert locust, *Schistocerca gregaria*.

Peptide A was synthesised in its amidated form (Research Genetics Inc., Huntsville, AL, USA) because all known AKHs contain C-terminal carboxyamides. The RP-HPLC retention times of natural and synthetic peptides were identical and both peptides co-eluted when chromatographed simultaneously, indicating that both peptides are identical. Peptide A contains all the main structural features of AKH peptides [1] (see above) and is, thus, the fourth fully characterised AKH from *L. migratoria* and the third from *S. gregaria*. The peptide has 56% sequence identity with Lom-AKH-II and Scg-AKH-II, whilst there is only 44% identity with Lom-AKH-III and 36% with Lom-AKH-I. Natural peptide A showed no adipokinetic activity in locusts when tested at a dose of 2 SL equivalents (approximately 10 pmol); 5 pmol Lom-AKH-I per locust produced significant increases of 23.9 ± 3.7 mg lipid/ml ($n = 6$) within 90 min of injection. Peptide A has also 67% and 56% identity with the two *P. americana* AKHs, pea-CAH-I and pea-CAH-II (Table 1). When tested in cockroaches at a dose of 2 SL equivalents, peptide A induced a significant hypertrehalosaemic effect of 9.3 ± 3.7 mg trehalose/ml ($n = 6$); 5 pmol pea-CAH-II produced a slightly higher effect of 14.0 ± 3.9 mg/ml ($n = 6$). The highest degree of identity (78%) exists between peptide A and the AKH from the fruitfly *Drosophila melanogaster*, Drm-HrTH (Table 1).

Peptide A is the first fully characterised AKH from locust SL, the nervous part of the CC. The peptide was not detected in in vitro experiments which investigated the incorporation of radiolabelled tryptophan into Lom-AKH-I and Scg-AKH-II by isolated *S. gregaria* CC [2]. Thus, it appears that peptide A is probably synthesised in neurosecretory cells of the brain and transported to the SL via the nervi corporis cardiaci I and/or II, whilst the other locust AKHs are synthesised in the GL. A molecule with the same RP-HPLC retention time as peptide A and a mass of $m/z = 1204.5$ (internal standardisation) was detected in methanol extracts of *L. migratoria* brains.

Peptide A has a number of unique structural features. The four previously known locust AKHs contain leucine and asparagine in positions 2 and 3 (Table 1), whilst the combination valine-threonine shows similarity with pea-CAH-I and pea-CAH-II. Peptide A is also the first charged locust AKH (aspartic acid) and the first AKH to contain arginine (see below). The C-terminal serine-proline segment (residues 9 and 10) has never been observed in an AKH before, however, such segments are present in positions 5 and 6 of e.g. Drm-HrTH and pea-CAH-I (Table 1) and they always follow an aromatic amino acid, either phenylalanine or tryptophan. Peptide A is the only known AKH nona- or decamer which does not contain a glycine in position 9 and, thus, may not have resulted from a C-terminal extension of an AKH octamer. Proline residues, present in position 6 of e.g. Lom-AKH-I and Lom-AKH-III, introduce β-bends [7]. Proline present in position 10 does not have the same consequences for the overall structure of the peptide or peptide-receptor interaction.

The use of synthetic peptide A confirmed the results of the above bioassays: even a dose of 500 pmol (equivalent to ap-

Table 1
Sequences of insect neuropeptides

Peptide [mass]	Sequence											Identity
Lom-AKH-I (1158.5)		**pGlu**	Leu	Asn	**Phe**	Thr	Pro	Asn	**Trp**	Gly	Thr**NH$_2$**	**36%**
Lom-AKH-II (903.4)		**pGlu**	Leu	Asn	**Phe**	**Ser**	Ala	Gly	TrpNH$_2$			**56%**
Scg-AKH-II (933.4)		**pGlu**	Leu	Asn	**Phe**	**Ser**	Thr	Gly	**TrpNH$_2$**			**56%**
Lom-AKH-III (1073.2)		**pGlu**	Leu	Asn	**Phe**	Thr	Pro	Trp	**TrpNH$_2$**			**44%**
Peptide A/Lom-HrTH (1204.3)		**pGlu**	**Val**	**Thr**	**Phe**	**Ser**	**Arg**	**Asp**	**Trp**	**Ser**	**ProNH$_2$**	
Drm-HrTH (975.1)		**pGlu**	Leu	**Thr**	**Phe**	**Ser**	Pro	**Asp**	**TrpNH$_2$**			**78%**
Pea-CAH-I (972.4)		**pGlu**	**Val**	Asn	**Phe**	**Ser**	Pro	Asn	**TrpNH$_2$**			**67%**
Pea-CAH-II (987.5)		**pGlu**	Leu	**Thr**	**Phe**	Thr	Pro	Asn	**TrpNH$_2$**			**56%**
Pea-corazonin (1369.5)	**pGlu**	Thr	Phe	Gln	Tyr	**Ser**	**Arg**	Gly	**Trp**	Thr	Asn**NH$_2$**	**42%**
Scg-corazonin (1350.5)	**pGlu**	Thr	Phe	Gln	Tyr	**Ser**	His	Gly	**Trp**	Thr	Asn**NH$_2$**	**33%**

Sequences of locust AKHs and peptide A. Sequencing of unblocked peptide A revealed the following result: cycle 1 = 281 pmol Val; cycle 2 = 241 pmol Thr; cycle 3 = 245 pmol Phe; cycle 4 = 73 pmol Ser; cycle 5 = 49 pmol Arg; cycle 6 = 66 pmol Asp; cycle 7 = 33 pmol Trp; cycle 8 = 31 pmol Ser; cycle 9 = 9 pmol Pro; cycle 10 = void. Peptides from other insect species are given for comparison; molecular masses are provided in parentheses. Amino acids identical with those in peptide A are given in bold type face, underlined are closely related residues (as far as structures and codons are concerned) in identical positions (Asn and Asp, Thr and Ser, Phe and Tyr). References for all relevant AKH sequences can be found in [1].

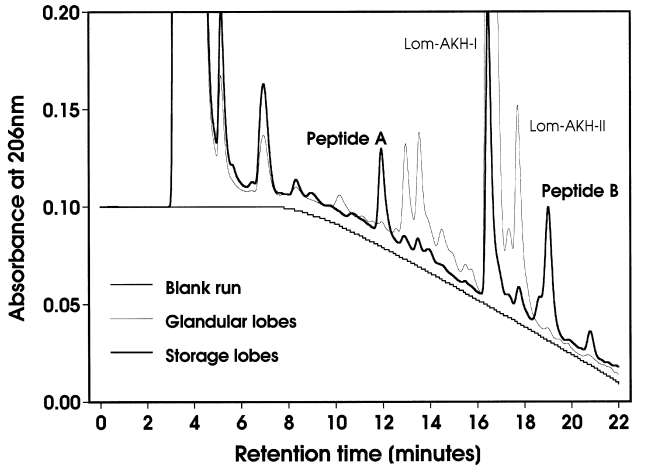

Fig. 1. Chromatogram of SL and GL extracts from *L. migratoria*. Methanol extracts from 25 *L. migratoria* SL and GL were individually chromatographed. Peptide A eluted after 12.0 min and peptide B after 19.2 min. Lom-AKH-III eluted outside the shown time frame.

proximately 100 *L. migratoria* SL) did not produce an adipokinetic effect in locusts (Fig. 2). Drm-HrTH, however, induces adipokinetic effects in locusts with an ED_{50} of 39.5 pmol [8]. When synthetic peptide A was tested in *P. americana* significant hypertrehalosaemic effects were found; the ED_{50} of approximately 25 pmol is higher than the value for pea-CAH-I (1 pmol or Drm-HrTH (7.3 pmol) [9]. The maximal hypertrehalosaemic effect of peptide A is approximately 50% lower than the value for pea-CAH-II. Peptide A has no hypertrehalosaemic activity in locusts (data not shown). The presence of arginine or the absence of proline from position 6 together with the Ser-ProNH_2 segment may explain the unusual performance of peptide A in adipokinetic and hypertrehalosaemic bioassays. Only a detailed structure/activity study can clarify which feature leads to a gain or loss of activity.

Peptide A did not stimulate the conversion of inactive to active fat body glycogen phosphorylase in locusts. 15 min after the injection of 500 pmol peptide A into adult locusts the percentage of active phosphorylase remained unchanged (Fig. 3); 50 pmol Lom-AKH-I induced an increase from 10% in controls to approximately 50%. In *P. americana*, 50 pmol peptide A showed a significant increase from 18 to 38% ($P = 0.0007$). 500 pmol caused the same activation as 50 pmol pea-CAH-II, i.e. 65% active form was found. Peptide A interacts with a receptor(s) on the cockroach fat body, but either does not stimulate with the locust fat body or interacts a receptor(s) which is(are) not coupled to glycogen phosphorylase. It seems unlikely that peptide A binds to the AKH receptor(s) on the locust fat body but it cannot be ruled out that it acts as an antagonist. The target tissue for Lom-HrTH in locusts must contain a receptor(s) which resembles the pea-CAH receptor(s) of the cockroach fat body more closely than the Lom-AKH receptor on the locust fat body and, thus, the pea-CAHs from cockroaches may bind but not the Lom-AKHs.

The cardioactive neuropeptide pea-corazonin (Table 1), present in CC [10] and the central nervous system (CNS) of *P. americana* [11] and *D. melanogaster* [12], also shows structural similarity with peptide A. At concentrations of 0.2×10^{-6} and 2×10^{-6} M peptide A had no effect on locust and cockroach heart beat whilst pea-corazonin and pea-CAH-I (neurohormone D) increased the heart rate in cockroaches by approximately 40%. Peptide A has a higher degree of identity with pea-corazonin (42%) than with Scg-corazonin (33%) from *S. gregaria*; the structure of Lom-corazonin is unknown. It is particularly interesting that Scg-corazonin does not contain arginine. It seems justified to align peptide A and pea-corazonin as shown in Table 1: the former peptide contains the segment Ser-Arg-Asp-Trp (residues 5–8) whilst the latter contains Ser-Arg-Gly-Trp (residues 6–9). Additionally, the C-terminal carboxyamides align as well as tyrosine and threonine (positions 5 and 10 of pea-corazonin) with phenylalanine and serine (positions 4 and 9 of peptide A). Peptide A may be considered as an intermediate between the Lom-AKHs and

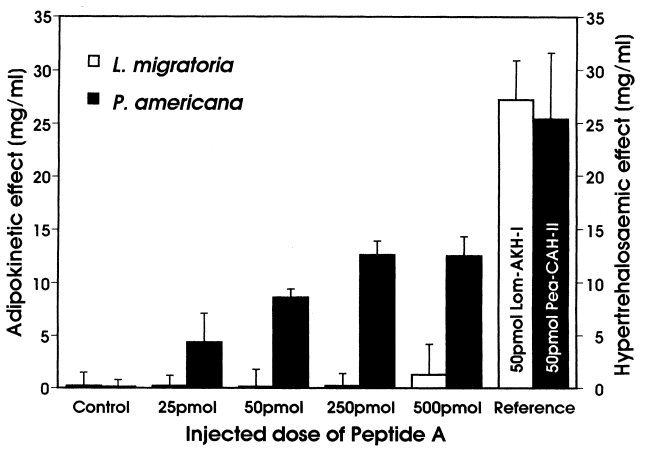

Fig. 2. Adipokinetic and hypertrehalosaemic effects of synthetic peptide A. Peptide A was injected with 10 μl of filter-sterilised saline; 50 pmol Lom-AKH-I and 50 pmol pea-CAH-II were also tested. Changes in lipid concentrations in locusts are shown in the left-hand column of each pair and the axis on the left-hand side of the figure, changes in total carbohydrate concentrations in cockroaches are shown in the right-hand column and the axis on the right-hand side. Columns represent averages of $n = 6$ observations ± S.D.

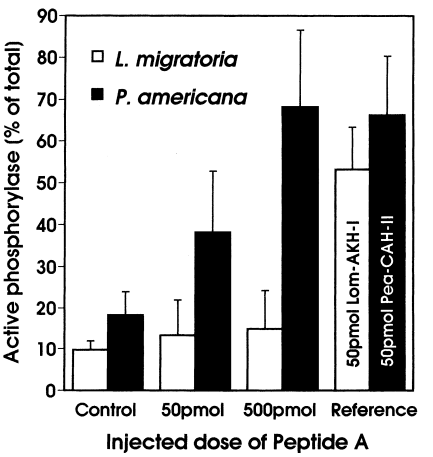

Fig. 3. Effects of synthetic peptide A on fat body glycogen phosphorylase in adult *L. migratoria* and *P. americana*. Phosphorylase activation was measured 15 min after injection of peptides. Values are given as the percentage of the active form of total glycogen phosphorylase activity present in each sample. As reference 50 pmol Lom-AKH-I was injected into locusts and 50 pmol pea-CAH-II into cockroaches. Columns represent averages of $n = 6$–8 observations ± S.D.

corazonin since it has all the hallmarks of AKHs and sequence similarity with corazonin but is not biologically active in either bioassay. Veenstra [10] concluded that the presence of arginine in pea-corazonin excludes it from membership of the AKH family, but the discovery of arginine in peptide A as well as the very similar organisation of the precursors of corazonin [12], Drm-HrTH [13] and the Lom-AKHs [14] argue in favour of common evolution.

Peptide A clearly belongs to the AKH family, but the peptide is inactive in appropriate locust bioassays and is provisionally named *L. migratoria* hypertrehalosaemic hormone, Lom-HrTH, because it induces increased haemolymph trehalose levels in cockroaches. An appropriate name will be assigned when the physiological role in locusts is established. Since Lom-HrTH is present in a neurohaemal tissue it can be assumed that it is released into the haemolymph to fulfil a hormonal role. The peptide, however, may not be involved in the hormonal regulation of energy metabolism, behaviour and/or development. Lom-HrTH does not appear to be a myotropic peptide because it was not detected by Schoofs and co-workers during their exhaustive studies [5]. The peptide's presence in the brain may indicate neurotransmitter and/or neuromodulator function as was found for the crustacean red pigment-concentrating hormone [15], pea-CAH-I from cockroaches [16] and Mas-AKH from the tobacco hornworm, *Manduca sexta* [17]. AKH-like immunoreactivity is present throughout the CNS of insects [18,19] but no AKH from the insect CNS has been isolated and fully characterised. The discovery of Lom-HrTH may indicate that AKHs are indeed generally present in the insect CNS. The sequence similarities between Lom-HrTH, Drm-HrTH and pea-corazonin are particularly interesting since the insect species involved belong to the phylogenetically old hemimetabolous orthoptera (locusts) and the phylogenetically recent holometabolous diptera (*D. melanogaster*) which suggests that Lom-HrTH is an ancient peptide.

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