

Isolation and Characterization of *Melanoplus sanguinipes* Adipokinetic Hormone: A New Member of the AKH/RPCH Family

Tina E. Taub-Montemayor,^{*,1} Klaus D. Linse,[†] and Mary Ann Rankin^{*}

^{*}Department of Zoology and [†]Protein Microanalysis Facility, University of Texas at Austin, Austin, Texas 78712-1064

Received September 5, 1997

A neuropeptide hormone isolated from corpora cardiaca of *Melanoplus sanguinipes* was purified by HPLC. The HPLC fractions were examined for adipokinetic activity with an *in vivo* bioassay. A single large UV absorbent peak was active in the mobilization of lipid while the other HPLC fractions showed no detectable activity. This large peak had a retention time and amino acid composition identical to synthetic Lom-AKH-I which was analyzed in a parallel manner. The primary sequence structure, pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH₂, was determined by automated gas-phase Edman degradation. The peptide was deblocked prior to sequencing using pyroglutamyl aminopeptidase and the sequence was confirmed with mass spectrometry. The C-terminus of the peptide was determined to be blocked, as indicated by the lack of digestion with carboxypeptidase A. The knowledge of the primary sequence of Mes-AKH allows the use of a commercially available synthetic peptide and its antibodies for use in future research with *Melanoplus sanguinipes*. © 1997 Academic Press

The primary structures of neuropeptides from the corpora cardiaca (CC) of several insect species have been elucidated. The first insect peptide identified was adipokinetic hormone I (AKH-I) in locusts and currently the total number of insect neuropeptide sequences exceeds 100. Over 30 of these peptides mobilize lipids and/or carbohydrates from the fat body and form

¹ Address correspondence to: Dr. T. E. Taub-Montemayor, Department of Zoology, University of Texas at Austin, Austin, Texas 78712-1064. E-mail: ttaub@mail.utexas.edu.

Abbreviations: AKH, adipokinetic hormone; CC, corpora cardiaca; HPLC, high performance liquid chromatography; Lom-AKH-I, adipokinetic hormone from *Locusta migratoria*; MALDI-TOF-MS, matrix assisted laser desorption ionization-time of flight-mass spectrometry; Mes-AKH, adipokinetic hormone from *Melanoplus sanguinipes*; RPCH, red pigment concentrating hormone; SP, synthetic peptide.

a family of structurally related neurohormones. This family of neuropeptides consists of the red pigment concentrating hormone (RPCH) from the sinus gland of prawns, the adipokinetic hormones from the CC of insects and the hypertrehalosaemic hormones involved in carbohydrate metabolism. Structure determination of members from the AKH/RPCH family established some common features of the peptides that account for their activity. All peptides express a conserved sequence motif that contains a blocked C- & N-terminal end. The active peptides are typically 8 to 10 amino acid residues in length and contain aromatic amino acids in at least two positions within their sequence. Glycine appears to be highly conserved at position 9. Furthermore, most peptides are uncharged under physiological conditions (1).

Recent studies have shown an adipokinetic effect in the hemolymph of *M. sanguinipes* following injection with the commercially available Lom-AKH-I and -II. As reported previously (2), Lom-AKH-I and -II have similar potencies in mobilizing lipids in the hemolymph of *M. sanguinipes*. However, Locusts respond to Lom-AKH-I at considerably lower levels than Lom-AKH-II (3). This result prompted the present study to identify and characterize all active components from the CC of *M. sanguinipes*. Only one fraction was found to have an adipokinetic effect in *in vivo* studies.

Our interest in the energy regulation of flight in the migratory grasshopper, *Melanoplus sanguinipes*, prompted us to examine the structure of the grasshopper Mes-AKH. In this paper, we report the isolation and characterization of the lipid-mobilizing peptide, AKH, from *M. sanguinipes*.

MATERIALS AND METHODS

Experimental animals. *M. sanguinipes* adults were used for this study and were reared as described previously (4). The adults were offspring reared in the laboratory from the matings of grasshoppers collected on the San Carlos Apache Indian Reservation in Arizona.

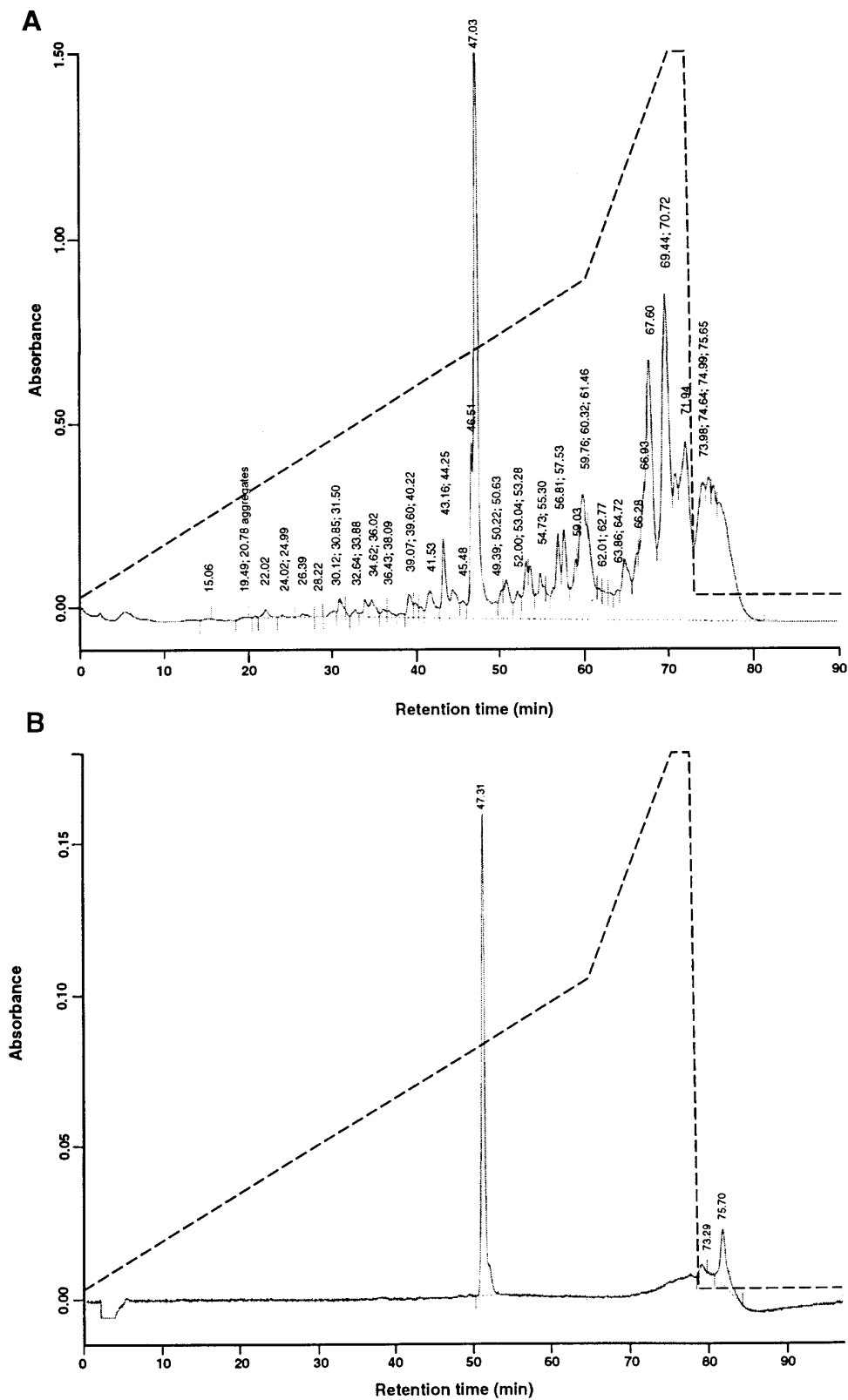


FIG. 1. Purification of Mes-AKH. (A) HPLC trace of CC extract: Peak eluting at rt 47.03 min contains the active compound as shown using *in vivo* AKH bioassay. (B) HPLC trace of control Lom-AKH-I: Peptide elutes at rt 47.31 min which is within the margin of error indicating that Mes-AKH and Lom-AKH-I may share the same characteristics.

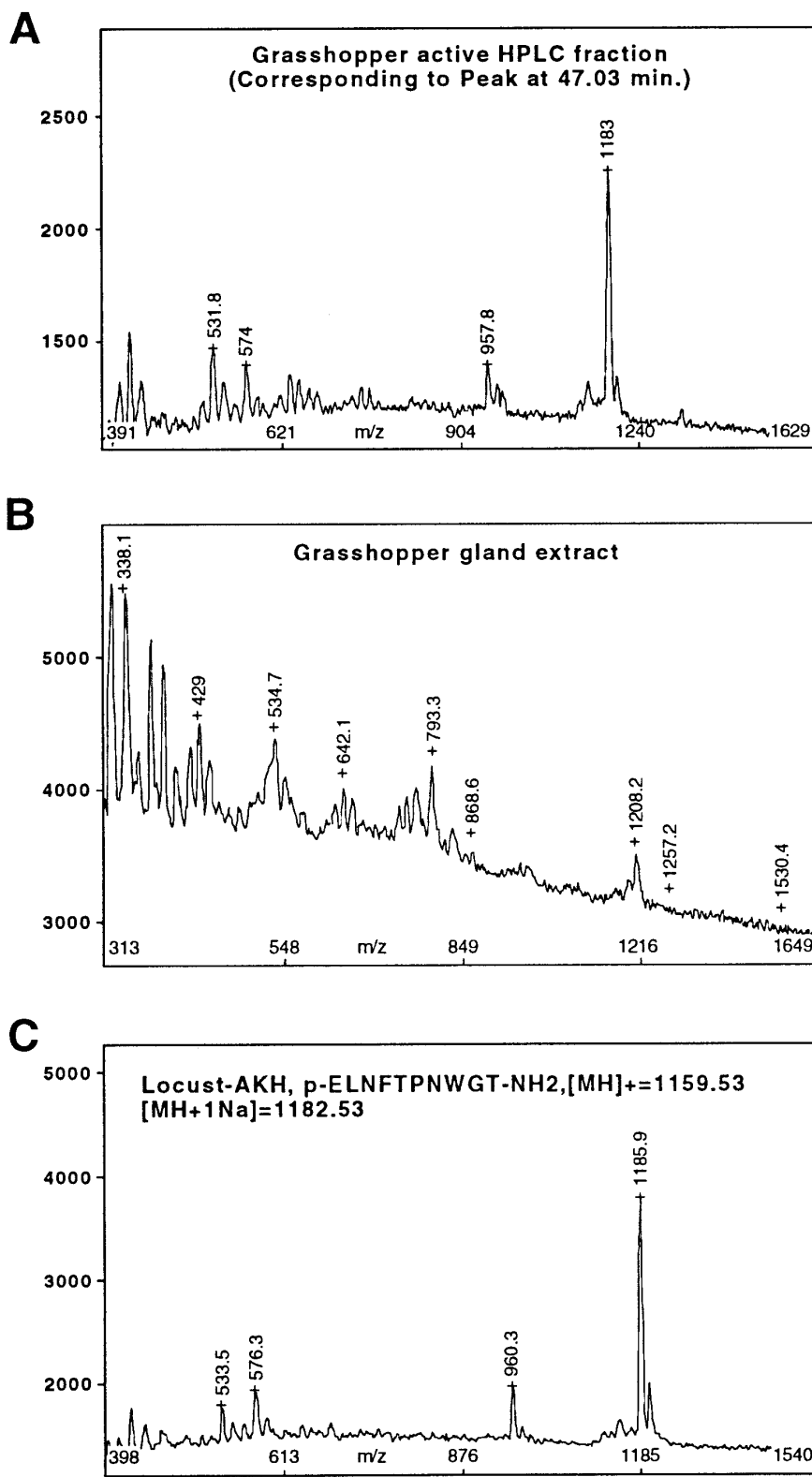
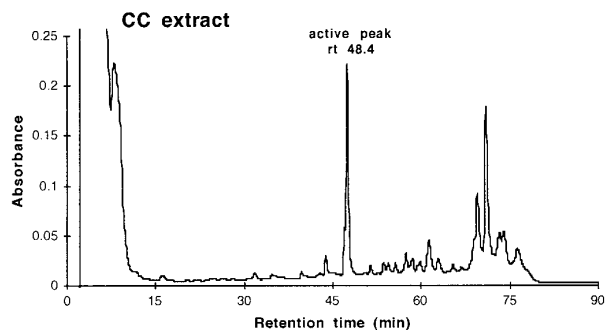


FIG. 2. Mass spectrometric analysis of the grasshopper active HPLC fraction, the gland extract and the Lom-AKH-I control peptide. A shows the MALDI-TOF-MS trace for peak 47.03 which can be compared with the MS trace of the whole grasshopper gland extract prior to purification (shown in B) and the MS trace of the control peptide shown in C.

A



B

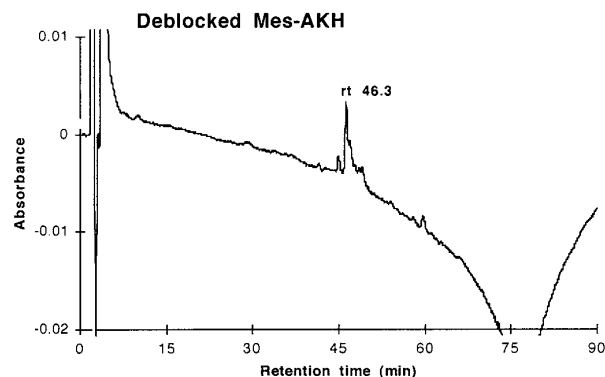


FIG. 3. N-terminal deblocking of Lom-AKH-I. Comparison of retention time shift for undigested synthetic Lom-AKH-I (A) & PGAP digested AKH (B). This reaction was done to establish the proper deblocking condition prior to deblocking the Mes-AKH. The fraction containing the deblocked Mes-AKH was spotted straight onto the peptide support disk and sequenced without further purification.

In vivo AKH bioassay. Adults between 10 and 14 days post eclosion were used for the analysis of hemolymph lipid levels. Test animals were initially bled from the base of the coxa of the right posterior leg prior to injection of the test material. Extruded hemolymph (1 μ l) was collected in a 5 μ l graduated micropipette. Intrahemocoelic injection of test materials or saline were made by piercing an intersegmental fold on the dorsal abdomen. Test materials (100 pmol) were dissolved in simple insect saline for a total volume of 1 μ l. A second hemolymph sample was taken 90 min. following injection of the hormone treatment. Hemolymph samples were tested for lipid content by the vanillin-reagent method (5).

Purification of AKH. CC complexes were dissected from 50 adult grasshoppers into 0.5 ml 10% acetic acid in H₂O and were ultrasonicated at 1.5 output for 3.5 min. then at 4.0 output for 0.5 min. The extract was centrifuged at 12 kg for 15 min. to collect the supernatant. The supernatant was stored at -70°C prior to analysis. Analytical and semipreparative reversed phase gradient HPLC were performed on the supernatant using a Beckman System Gold Gradient Instrument with 126 analytical pumps and equipped with a Ultrasphere 3 μ m ODS column, 2.0 \times 150 mm (Beckman Instruments, Fullerton, CA) or an ODS, 1.0 \times 150 mm (Separation Methods Technologies, Newark, Delaware). Gradients were developed using 0.1% TFA in water as solvent A and 0.08% TFA in 70% acetonitrile in water as solvent B. Detection was done at 214 nm. Flowrates were 180 to 210 μ l/min for the 2 mm column or 50 to 60 μ l/min for the 1 mm column. The HPLC system was controlled by a 466DX2/Si IBM

TABLE 1

Amino Acid Composition for Lom-AKH-I (Control Peptide) and for Mes-AKH

1A: Lom-AKH-I			1B: Mes-AKH		
AA	Theoretical	Found	AA	Predicted	Found
ASX	2	1.4	ASX	2	1.5
GLX	1	1	GLX	1	1
G	1	0.92	G	1	1.1
T	2	1.7	T	2	1.7
P	1	0.9	P	1	1.0
L	1	1.1	L	1	0.98
W	1	—	W	1	—
F	1	1.09	F	1	0.9

PS/Valuepoint PC. Fractions containing the peptide were collected and concentrated to a minimal volume in a Savant Speed Vac concentrator for further analysis.

MALDI-TOF-MS. MALDI mass data spectra were acquired with a LaserTec time-of-flight mass spectrometer fitted with a nitrogen laser (Vestec/Perseptive Biosystems, Houston, Texas). Acceleration voltage of 30 kV was employed unless otherwise specified. All samples were prepared in α -cyano-4-hydroxycinnamic acid. Typically, a 10 mg/ml solution of the matrix in 0.1% TFA/75% acetonitrile/water was used for sample preparation. For unknown samples, an aliquot was premixed with the matrix solution prior to spotting on to the probe tip for a final concentration of 1 to 10 pmol/ μ l. For a typical experiment, 3 to 5 μ l, in aliquots of 1.2 μ l were spotted on to the probe tip and allowed to dry at room temperature prior to placement in the acquisition chamber.

Enzymatic deblocking of N-terminus. Lyophilized *Pfu* pyroglutamic aminopeptidase (E.C. 3.4.19.3) 10 mU (TAKARA Biomedicals via PanVera Corp., Madison, WI) was reconstituted with 50 μ l buffer (pH 7.0) containing 250 mM sodium phosphate with 50 mM DTT and 5 mM EDTA. The enzyme solution was stored in 5 μ l aliquots at -20°C. Enzyme activity was monitored by digestion of synthetic Lom-AKH-I (200 pmol). The shift in the HPLC pattern between digested and undigested Lom-AKH-I was used to confirm enzymatic activity. Purified AKH from *M. sanguinipes* (200 pmol) was incubated with 5 μ l (1mU) enzyme solution at 37°C for 20 hr. in the dark.

Enzymatic digest of C-terminus of unblocked and blocked peptides. A synthetic octapeptide, ILEPVHGV, was incubated with carboxypeptidase A (E.C. 3.4.17.1, Sigma) for 5 min. at 25°C to yield the heptapeptide. This control peptide does not contain a blocked C-

TABLE 2

Sequence Data for Lom-AKH-I (Control Peptide) and Mes-AKH

Position	2	3	4	5	6	7	8	9	10
(A) Lom-AKH-I									
AA	L	N	F	T	P	N	W	G	T _{-NH₂}
Yields	68	56	38	8	8	4	1.5	1.1	(0.6)
(B) Mes-AKH									
AA	L	N	F	T	P	N	W	G	T _{-NH₂}
Yields	28	14	13	3	5	4.8	1.6	1.6	(1.1)

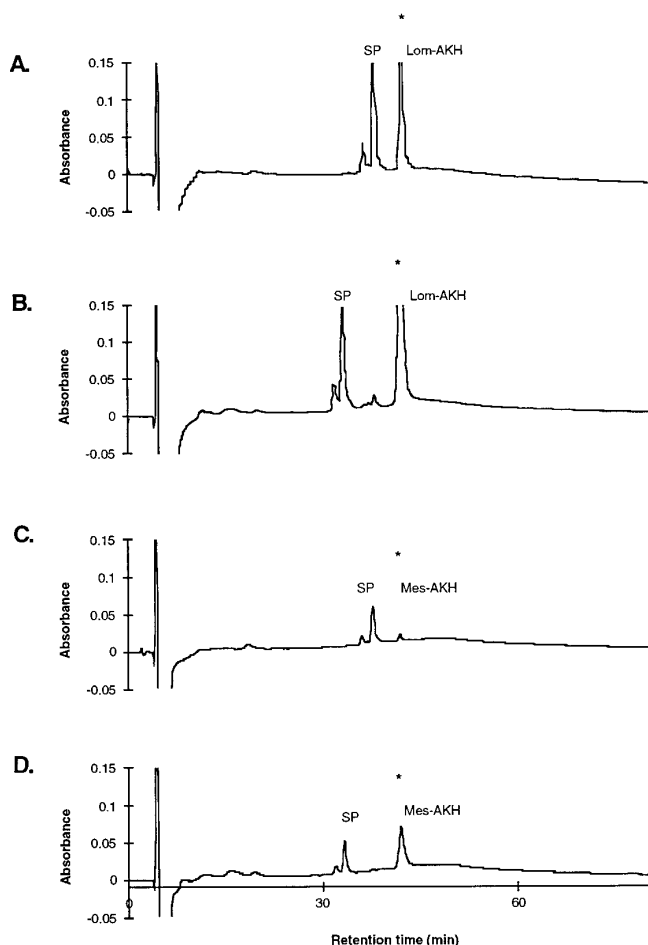


FIG. 4. Identification of amide blocked C-terminus. Digest of Lom-AKH-I & Mes-AKH using carboxypeptidase A. Lom-AKH-I is known to contain an amide blocked C-terminal and was used together with a synthetic peptide (SP), as an internal standard, to show that the enzyme was active and that it did not deblock the protected C-terminal end. (A) HPLC trace of Lom-AKH-I and SP mix prior to C-terminal digest. (B) HPLC trace of Lom-AKH-I and SP mix after C-terminal digest. Note: Peak representing Lom-AKH-I (*) does not show a shift in rt indicating that no digest occurred. Peaks representing SP do shift indicating that enzyme is active. (C) HPLC trace of Mes-AKH and SP mix prior to C-terminal digest. (D) HPLC trace of Mes-AKH and SP mix after C-terminal digest. Note: Peak representing Mes-AKH (*) does not show a shift in rt indicating that no digest occurred. Peaks representing SP do shift indicating that enzyme is active.

terminus, therefore the enzyme activity can be monitored using the peptide as an internal control. The active HPLC fraction from the CC extract was incubated with 1 unit carboxypeptidase A under the same conditions as the control peptide. Analytical reversed phase HPLC was done as described above.

Amino acid analysis. The amino acid composition of the peptides was determined by amino acid analysis with an automated PITC based amino acid hydrolyzer/analyzer (420H, ABI) system as previously described (6).

Sequence analysis. The deblocked AKH was sequenced by Edman based gas phase degradation using a LF3000 gas phase sequencer (Beckman Instruments). Fractions containing peptides in solution

were applied to a peptide support disk according to the manufacturer's specifications (Beckman Instruments, Fullerton, CA) followed by sequence analysis.

Sequence searches. Blast searches were performed using available protein databases through the National Library of Medicine Genbank databases, using the search algorithm according to Altschul, *et al.* (7).

RESULTS AND DISCUSSION

The isolation procedure reported revealed a single peptide with adipokinetic activity in *M. sanguinipes*. Amino acid analysis performed on an aliquot of the active fraction of the CC extract revealed that the peptide is present in the CC of *M. sanguinipes* at amounts equivalent to approximately 80 pmol/gland pair (average based on our examination of 50 CC glands containing an amount of 2 nmol AKH following extraction). This suggests the possibility of evaluating individual gland pairs in future experiments by using an analytical HPLC system and nanopore reversed phase ODS columns with an ID of 1mm or smaller.

Characterization of the purified peptide using analytical HPLC, amino acid analysis and mass spectrometry was performed to determine its chromatographic behavior (see Figure 1B and compare with chromatographic trace of whole CC extract, 1A), composition and mass. MALDI-TOF-MS analysis confirmed the presence of the peptide in the active HPLC fraction (see Figure 1 and 2A), in the crude extract (Figure 2B) and in the control peptide solution (Figure 2C). Note that the peptide is detected in the mass trace as a set of two peaks, reflecting the presence of a peptide-sodium 1:1 adduct and a peptide-sodium 1:2 adduct. The 1:1 species is predominant in Figure 2A & 2C, whereas the 1:2 species dominates the mass/charge trace in the extract (Figure 2B). These results indicated that Mes-AKH may be identical to Lom-AKH-I which contains a pGlu at the N-terminus. To confirm our suspicion we performed

TABLE 3

Primary Structures of Peptides of the AKH/RPCH Family for Orthoptera (Adapted From 1)

Code name	Species	Sequence
Lom-AKH-I	<i>Locusta migratoria</i>	pQLNFTPNWGTamide
	<i>Schistocerca gregaria</i>	
	<i>Melanoplus sanguinipes</i>	
Rom-CC	<i>Romalea microptera</i>	pQVNFTPNWGTamide
Grb-AKH	<i>Gryllus bimaculatus</i>	pQVNFSTGWamide
	<i>Acheta domesticus</i>	
	<i>Gryllodes sigillatus</i>	
	<i>Romalea microptera</i>	
Lom-AKH-II	<i>Locusta migratoria</i>	pQLNFSAGWamide
Seg-AKH-II	<i>Schistocerca gregaria</i>	pQLNFSTGWamide
	<i>Schistocerca nitans</i>	
	<i>Anabrus simplex</i>	
Lom-AKH-III	<i>Locusta migratoria</i>	pQLNFTPWWamide

further analytical experiments to conclusively determine the total primary structure of the peptide.

The pGlu has a cyclic structure that renders the Lom-AKH-I a molecule not suitable for Edman based gas phase sequencing. Direct sequencing of the purified peptide resulted in no visible sequence information, thereby confirming that the peptide was blocked at the N-terminal end. Next, we proceeded to deblock the peptide to allow for sequence analysis. The enzyme PGAP was used for the deblocking reaction. In order to demonstrate activity of the enzyme solution, we used Lom-AKH-I as a control. Figure 3 shows the result of the deblocking reaction performed on the active fraction of the unknown peptide. The analysis of both the blocked and the deblocked peptide was done with analytical gradient reversed phase HPLC, as described in Material and Methods. Activity of the enzyme was exhibited by the shift in retention time of the deblocked peptide versus the blocked peptide. The enzyme specifically cleaves the N-terminal pGlu releasing a shorter peptide (N-1) that exhibits a shorter retention time than the blocked peptide. Our first attempts to deblock the peptide with PGAP from a calf liver source (Boehringer Mannheim) were not successful, however the pGlu was readily removed using the pfu-PGAP from *Pyrococcus furiosus*. After establishing our digestion conditions, we were also able to deblock the purified Mes-AKH peptide. This resulted in a retention time shift of the peptide analyzed under the above conditions, as expected. These results allowed us to conclude that Mes-AKH is N-terminally blocked by a pyroglutamate residue, as predicted from earlier studies on the family of RPCH/AKH peptides (see review, 1). Using this information, we were able to deblock the purified peptide and determine the amino acid sequence of the remaining residues. Composition of the peptides and sequencing results are shown in Tables 1 and 2.

The similarities between Lom-AKH-I and Mes-AKH, supported by analytical HPLC, MALDI-TOF-MS, amino acid composition and sequencing results, suggested that Mes-AKH also contained a C-terminal amide group. To test this, Mes-AKH was incubated with carboxypeptidase A, an enzyme reported to digest peptides sequential from the C-terminus. We therefore expected a threonine at the C-terminus that would be cleaved if not blocked with the amide group. The synthetic peptide, ILEPVHGV (SP), used as an internal standard and Mes-AKH were examined on HPLC before and after treatment with the carboxypeptidase A solution. In a separate experiment, Lom-AKH-I was also examined by HPLC following incubation with the same enzyme solution and SP. In both experiments the retention time for SP shifted following digestion with the enzyme solution from 19 to 16 minutes while the retention time for Mes-AKH and Lom-AKH-I remained

unchanged. This demonstrated that the active carboxypeptidase was able to cleave the unblocked internal standard and that the two AKH peptides were blocked. Results are shown in Figure 4. The increase in peak size in the Mes-AKH peptide in the HPLC run following addition of the enzyme solution is likely due to failure of the peptide to dissolve completely prior to the removal of the initial aliquot for HPLC examination.

The primary sequence data of AKH from *M. sanguinipes* adds a third member to the Lom-AKH-I group of *Locusta migratoria* and *Schistocerca gregaria*. A comparison of all reported AKH peptide sequences of orthopteran species are shown in Table 3. In general the AKH/RPCH family of peptides is highly conserved (see review of all known primary sequences to date, 1). However, there are insufficient data to make any significant conclusions about evolutionary relationships based on the sequence data currently available. We performed Blast searches of both the sequence of the mature peptide and the preprohormone sequences. The search done for the mature peptide produced 58 hits and the search done for the prepropeptide produced 16 hits. Results of both searches confirmed the highly conserved nature, but also showed that this gene is present in distantly related species, such as *C. elegans*, as well as in soybean and pea seeds. Search results for a prepropeptide AKH precursor showed a significantly lower percent of homology. This may reflect the fact that the mature peptide may have to be highly conserved to allow functioning in different organisms, whereas the prepropeptide may not require such a highly conserved primary structure for function.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Michelle Gadush and Sandie Smith of the Peptide Microanalysis Facility at the University of Texas at Austin. We are grateful to Jack W. Kent, Jr. for his critical reading of this manuscript. This study was supported by USDA Grant 91-37302-6572 to MAR.

REFERENCES

- Gäde, G. (1996) *Z. Naturforsch [C]*. **51**(9-10), 607-617.
- Kent, J. W., Jr., Teng, Y.-M., Deshpande, D. and Rankin, M. A. (1997) *Physiol. Entomol.* **22**, in press.
- Goldsworthy, G. J., Mallison, K., Wheeler, C. H. (1986) *J. Insect Physiol.* **32**, 95-101.
- McAnelly, M. L., & Rankin, M. A. (1986) *Biol. Bull.* **170**, 368-377.
- Stone, J. V., & Mordue, W. (1980) in *Neurohormonal techniques in insects* (Miller, T. A., Ed.), pp. 31-80, Springer-Verlag, New York.
- Linse, K. D., Smith, S., and Gadush, M. (1997) in *Techniques in Protein Chemistry VIII* (Marshak, D. R., Ed.), pp. 197-206, Academic Press, San Diego, CA.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403-410.