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Stick insect (*Carausius morosus*; Phasmatodea: Lonchodidae) brain extract contains multiple fractions with allatostatic activity

GERD GÄDE[1], MATTHIAS W. LORENZ[2] and KLAUS-HUBERT HOFFMANN[2]

[1] Zoology Department, University of Cape Town, Rondebosch 7700, South Africa
[2] Institut für Tierökologie I, Universität Bayreuth, D-95440 Bayreuth, Germany

Juvenile hormone, allatostatins, purification, HPLC, stick insect, *Carausius morosus*, brain extract

Abstract. A first attempt to purify inhibitors of juvenile hormone biosynthesis from extracts of brains of the Indian stick insect, *Carausius morosus*, is reported here. A heterologous bioassay in the cricket, *Gryllus bimaculatus*, was used throughout the study. Separation of a prepurified extract using C-18 reversed-phase high performance liquid chromatography (RP-HPLC) resulted in a broad zone with biological activity. Upon re-chromatography of each of six active regions on a wide-pore C-18 column, the material separated in distinct peaks of biologically active fractions. Introducing a third purification step (C-8 column) resulted in a total of 18 absorbance peaks with allatostatic activity. It remains to be seen whether all these fractions contain authentic allatostatins which are active in stick insects, or whether other functions can be attributed to them.

INTRODUCTION

The corpora allata (CA) of insects are the site of biosynthesis of juvenile hormones (JHs) which are well-known to exert a crucial role in regulating post-embryonic larval development and adult reproduction (Tobe & Stay, 1985). Changes in the rate of JH synthesis are mainly brought about by peptides produced in the insect's brain. Such neuropeptides can be stimulatory in action (allatotropins) or inhibitory (allatostatins). Since it is hoped that such compounds may aid in the design of novel and safer insecticides (Keeley & Hayes, 1987; Couillaud & Peypelut, 1995) much effort has been made during the last decade to structurally characterise such JH synthesis-regulating substances. Whereas the primary structure of only one allatotropin is known (Kataoka et al., 1989), sequence data on a number of allatostatins which share a common C-terminal pentapeptide (Y/F-X-F-G-L/I-amide) from various species have been reported: the cockroaches *Diploptera punctata*, *Periplaneta americana* and *Blattella germanica* (Pratt et al., 1989, 1991; Woodhead et al., 1989, 1994; Weaver et al., 1994; Bellés et al., 1994), the blowfly *Calliphora vomitoria* (Duve et al., 1993, 1994, 1995; Duve & Thorpe, 1994), the cricket *Gryllus bimaculatus* (Lorenz et al., 1995a), the locust *Schistocerca gregaria* (Veelaert et al., 1996a, b) and the honeybee *Apis mellifera* (Kaatz, pers. comm.). However, in the blowfly, in the honeybee and in the locust, the allatostatins exert no allatoregulating activity but act as myoinhibitors of smooth muscles (Duve & Thorpe, 1994; Duve et al., 1994, 1995; Vanden Broeck et al., 1996; Veelaert et al., 1996a), indicating an additional function for this peptide family. The myoregulating activity of this peptide family has been also established in cockroaches (Lange et al., 1995).

In the cricket, *G. bimaculatus*, another group of neuropeptides with allatostatic activity having sequence similarity to myoinhibiting peptides (MIPs) of *Locusta migratoria* (Schoofs et al. 1991) and *Manduca sexta* (Blackburn et al., 1995) have been isolated and characterised (Lorenz et al. 1995b). The only identified allatostatin from butterflies shares no sequence similarity with any of the known allatoregulating peptides (Kramer et al., 1991).

The present study was initiated to isolate putative allatostatic compounds from the brain of the Indian stick insect *Carausius morosus*. It is thought that JH does not play a role in regulating vitellogenesis in this species. Already 60 years ago Pflugfelder (1937) had observed that specimens of *C. morosus* did lay eggs although they were allatectomised either in the last nymphal instar or early adult stage. Recently, this was corroborated by Bradley et al. (1995) by finding that primary induction of vitellogenin synthesis as well as post-induction vitellogenesis can occur in allatectomised *C. morosus*. However, these authors concluded from their experiments that a normal rate of vitellogenin uptake requires the presence of CA during the adult stage. Thus, adult stick insects may not produce JH at all or at a very low rate and, therefore, may not have any need to regulate JH titres via allatostatic neuropeptides. The question arises then: do stick insects contain allatostatic peptides in their brain and, if they do, what are the endogenous functions of such peptides? The answers to such questions may also shed some light on the general actions of some of the allatostatins isolated to date.

To find out what the possible functions of stick insect putative allatostatins are, the peptides have first to be identified. In this study we report on our preliminary results on the purification of stick insect brain allatostatic substances.

MATERIAL AND METHODS

Insects

Indian stick insects, *Carausius morosus* (Brunner) (Phasmatodea: Lonchodidae: Lonchodinae), were collected in the field around Cape Town or came from a laboratory colony. Animals were reared on ivy leaves under short day conditions (12 h light : 12 h dark) at ambient temperature (20–23°C). Mediterranean field crickets, *Gryllus bimaculatus* (de Geer) (Ensifera: Gryllidae), were reared as described previously (Lorenz et al., 1997).

Test for allatostatic activity

For testing the allatostatic activity of *C. morosus* brain extracts, the rapid partition assay (Feyereisen & Tobe, 1981), modified for cricket CA, was carried out as described earlier (Lorenz et al., 1997).

Tissue dissection, extraction and purification

Dissection: Brains were dissected from egg-laying stick insects (older than 20 days) and immediately transferred into ice-cold extraction medium (methanol : water : acetic acid, 100 : 10 : 1, v : v : v) and stored at -20°C.

Single CA from 3 day old virgin female crickets were used for the bioassays. All dissections were carried out in modified cricket ringer (86 mM NaCl, 5.4 mM KCl, 3 mM $CaCl_2$).

Extraction and purification: Brains were ultrasonicated in batches of 50, centrifuged for 10 min at 12,000 x g and 4°C. The supernatant was removed and the pellet re-extracted with fresh extraction medium. Supernatants of three repeated extractions were combined, dried in a vacuum centrifuge and stored at -20°C.

Prepurification on C18 SEP-PAK cartridges: The dried residues from the extraction step were redissolved in 1 ml 0.1% trifluoroacetic acid (TFA) in water, loaded onto the SEP-PAK cartridge and eluted with a stepwise gradient (5 ml each of 0.1% TFA in water, 0.1% TFA in 16% acetonitrile (CH_3CN), 0.1% TFA in 40% CH_3CN and 0.1% TFA in 100% CH_3CN). In total, 2,300 brains were processed in 10 SEP-

PAK runs. Aliquots of each fraction were tested on cricket CA for allatoregulating activity; we performed five replicates at a concentration of five brain equivalents each.

Purification by reversed phase liquid chromatography (RP-HPLC): Further purification of the brain material was carried out essentially as outlined in Lorenz et al. (1995b) with minor modifications.

First HPLC run: The 40% CH_3CN SEP-PAK fractions were processed in two separate runs (1,175 and 1,110 brain equivalents, respectively) on a 100 nm C-18 column eluted with a water/CH_3CN gradient and TFA as the ion-pairing reagent. Collected peak fractions between 16 and 60 min were tested on cricket CA (three replicates at a concentration of 30 brain equivalents each). Active fractions were subjected to the second HPLC run (equivalent to 2,185 brains) on a 300 nm C-18 column and eluted with a water/CH_3CN gradient and heptafluorobutyric acid as the ion-pairing reagent. Again, peaks were collected and aliquots of each fraction (three replicates at a concentration of 40 brain equivalents each) were used in the cricket bioassay. The most promising active peaks (that means peaks with the highest uv-absorbance and more than 50% inhibition of JH release) from this run were subjected to the third HPLC treatment (equivalent to 2,065 brains) on a C-8 column eluted with ammonium acetate in CH_3CN. Aliquots of the collected peaks were tested at three replicates and 50 brain equivalents each.

RESULTS AND DISCUSSION

A prerequisite for a joint project between laboratories in South Africa and Germany was to check the stability of crude samples which had to be sent for biological testing from Cape Town to Bayreuth. Once we had established that a crude, acidic methanol extract of brains from the stick insect exhibited allatostatic activity (5 equivalents used in five replicates; about 60% inhibition of JH release achieved; data not shown) after vacuum-drying and travelling for up to 14 days at ambient temperature, we could proceed to collect more than 2,000 brains. In the next pilot experiment we tested four SEP-PAK fractions (0%, 16%, 40% and 100% CH_3CN) at a concentration of 5 brain equivalents. Only the 40% CH_3CN fraction showed consistently allatostatic activity of about 60% inhibition of JH release (data not shown).

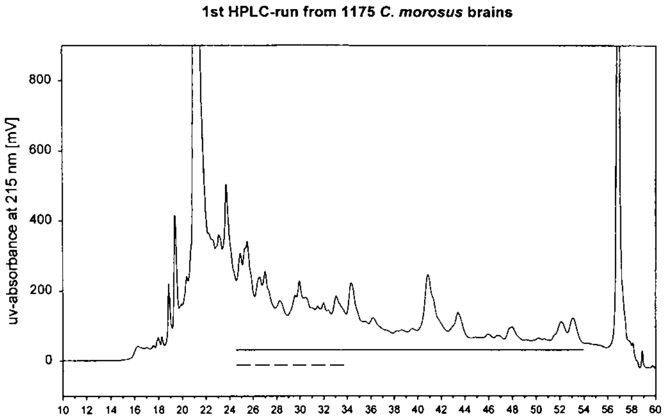


Fig. 1. Initial HPLC purification of a prepurified acidic methanol extract of 1,175 *C. morosus* brain equivalents. HPLC conditions were as follows: Column: LiChroCART 125-4 Superspher 100 RP-18 with guard column LiChroCART 4-4 LiChrospher 100 RP-18 (Merck). Solvent A: 0.115% trifluoroacetic acid (TFA) in water; solvent B: 0.1% TFA in acetonitrile (CH_3CN). Gradient: 0–5 min 0% B, 5–8 min 0% B – 20% B, 8–51 min 20% B – 33% B, followed by a wash at 100% B. Flow rate: 1 ml/min; uv-detection at 215 nm. The horizontal bar indicates fractions with allatostatic activity, the small bars indicate fractions that were chosen for further purification.

In another pilot experiment involving some 600 brain equivalents, a first HPLC separation of the 40% SEP-PAK fraction resulted in a large zone containing allatostatic activity (data not shown). We repeated this experiment with 1,175 brain equivalents (Fig. 1). Again, all peak fractions collected between 24.5 and 54 min inhibited JH release from cricket CA by at least 40% (see horizontal bar in Fig. 1). We were not entirely convinced that all these fractions indeed contained allatostatic activity; therefore, we had to further purify each material. If it were still active and separable into distinct peaks, then there was without doubt allatostatic material present.

We chose six corresponding fractions (see small bars in Fig. 1) combined from each of the two initial HPLC runs for further purification in a second HPLC step. These particular fractions were chosen because they strongly inhibited JH release and they had the highest uv-absorbance, indicating high amounts of bioactive material. Since these fractions eluted relatively early from the column, they should contain relatively short and polar peptides

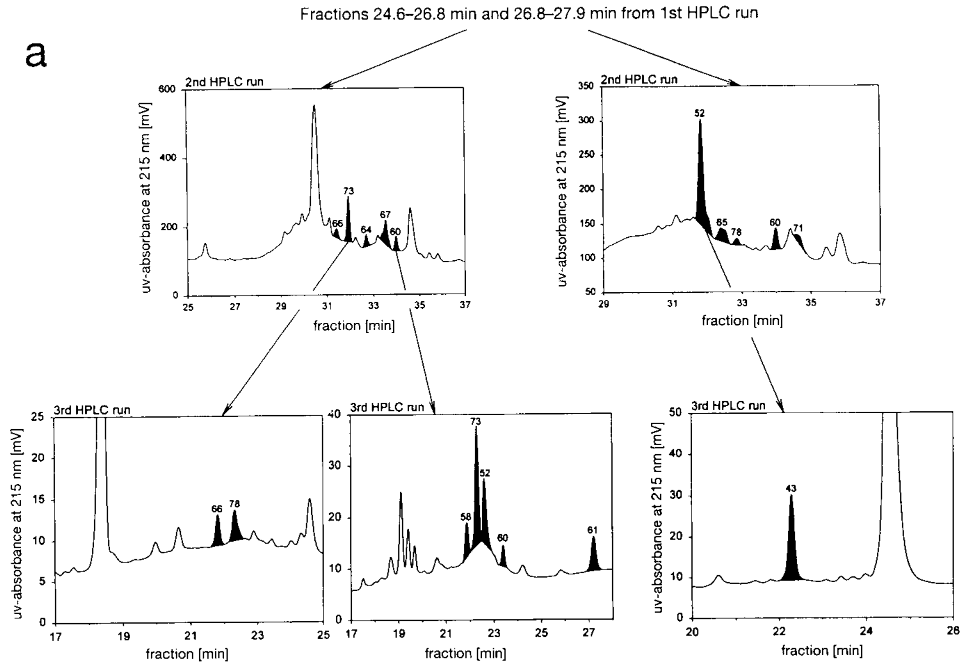
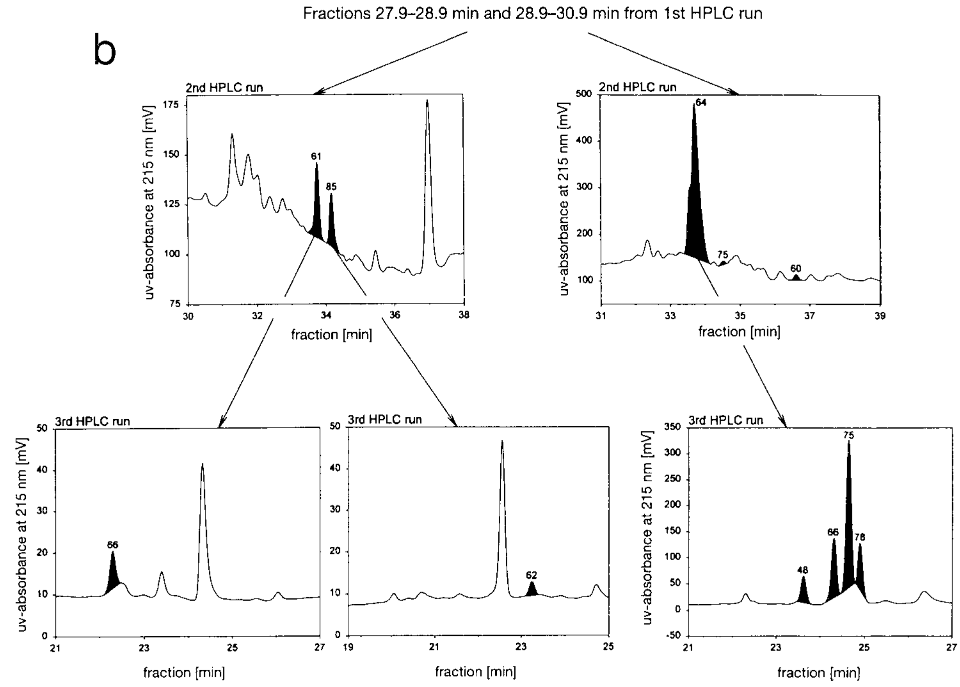
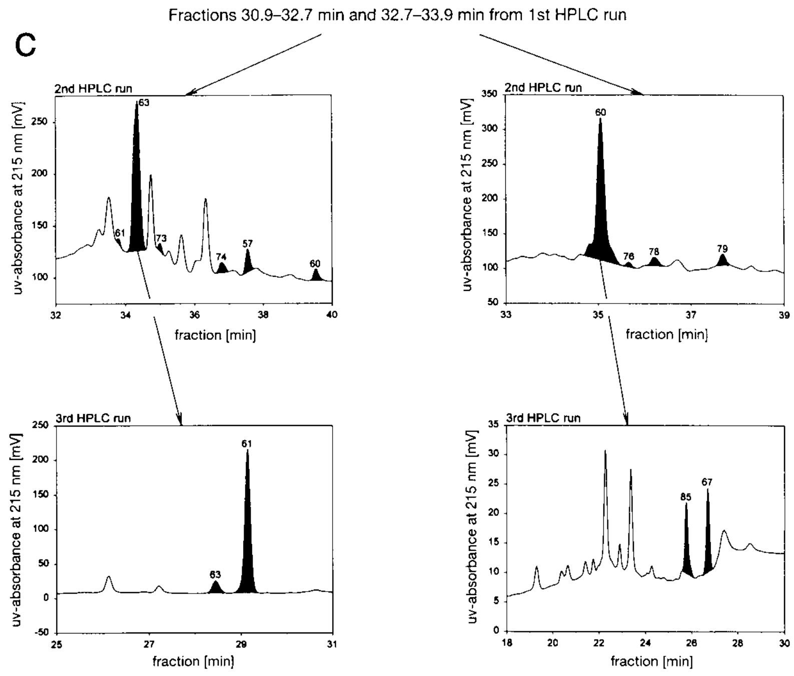


Fig. 2a–c. Further purification of six corresponding fractions (as indicated in Fig. 1), combined from each of the two initial HPLC runs. Upper panel: 2nd HPLC run of 2,185 brain equivalents; lower panel: 3rd HPLC run of 2065 brain equivalents. Some of the active fractions from the 2nd HPLC run were further purified in the 3rd HPLC run as indicated by arrows. Conditions were as follows: 2nd HPLC run: Column: Capcell Pak C18 SG 300, 250 x 4.6 mm with guard column (same material, Shiseido). Solvent A: 0.13% heptafluorobutyric acid (HFBA) in water, solvent B: 0.13% HFBA in CH_3CN. Gradient: 0–2 min 5% B, 2–52 min 5% B – 60% B, followed by a wash at 100% B. Flow rate: 1 ml/min; uv-detection at 215 nm. 3rd HPLC run: Column: Capcell Pak C8 SG 300, 150 x 4.6 mm with guard column (same material, Shiseido). Solvent A: 20 mM ammonium acetate in water (pH 7.0), solvent B: 20 mM ammonium acetate in 80% CH_3CN. Gradient: 0–40 min 6.3% B – 62.5% B followed by a wash at 100% B. Flow rate: 1 ml/min; uv-detection at 215 nm. Numbers on top of each peak indicate percentage inhibition of JH release.


b
Fractions 27.9–28.9 min and 28.9–30.9 min from 1st HPLC run
2nd HPLC run
2nd HPLC run
3rd HPLC run
3rd HPLC run
3rd HPLC run
uv-absorbance at 215 nm [mV]
fraction [min]
61
85
64
75
60
66
62
48
66
75
78


c
Fractions 30.9–32.7 min and 32.7–33.9 min from 1st HPLC run
2nd HPLC run
2nd HPLC run
3rd HPLC run
3rd HPLC run
uv-absorbance at 215 nm [mV]
fraction [min]
63
61
73
74
57
60
60
76
78
79
63
61
85
67

which are easier to handle: for example, loss of material due to unspecific binding to the walls of the reaction tubes used for collection should be lower in the chosen polar fractions than in the more apolar ones.

The resulting chromatograms (Fig. 2a–c) show clearly that each of the originally active fractions can be separated into various peaks of which two or more (marked in black) exhibit allatostatic activity. Since inhibition of JH release ranged from 52 to 85% (as indicated on top of each peak) we believe that the responsible material contains true allatostatic material. Moreover, when material from active peaks which had the highest uv-absorbance (as indicated by arrows in Fig. 2a–c) were separately re-chromatographed under different HPLC conditions, each of these peak fractions was separated into various new absorbance peaks of which one to five contained biological activity (see Fig. 2a–c, lower panel). In total, 18 absorbance peaks with remarkable allatostatic activity in crickets could be identified. One has to bear in mind that even more active fractions are anticipated, since the currently isolated ones are only representative of the more polar material purified in the first HPLC run (see Fig. 1); the more apolar material (from retention times 33.9 to 54.0 min, see Fig. 1) will undoubtedly yield additional biologically active fractions.

It is quite surprising to find so many active fractions in an insect which supposedly does not need to control its JH titre during adult life. Conversely, up to 14 allatostatins have been structurally identified from cloned cDNA sequences in cockroaches (Donly et al., 1993; Stay et al. 1994) and 10 allatostatins in *S. gregaria* (Vanden Broeck et al., 1996), suggesting that high numbers of allatostatins can be present in a single species. This may be true for *C. morosus* as well. The questions arise: (a) are all these fractions allatostatic peptides of the Y/F-X-F-G-L/I-amide or MIP family, and (b) are any of these fractions active in the stick insect itself? The answer to the first question is hard to get, because from the amount of material available, there was an insufficient amount left of some of the peaks to attempt sequencing. Some fractions, however, contain sufficient material and we hope to achieve primary sequence elucidation. As to the conspecific action of the material, we have performed some pilot experiments in setting up an in vitro assay to measure JH synthesis in CA of *C. morosus*. To date we know that the CA of young adults (0 to 4 day old) and of young 6th instar larvae (0 to 3 day old) apparently do not synthesise any detectable JH, whereas in older adults (about 14 days and older) 9 out of 25 CC-CA-complexes do synthesise JH but in rather low amounts (around 0.5 pmol JH/h/insect) (unpublished results). If it proves to be correct that the stick insect contains authentic allatostatins, then it would be a good idea to look for functions other than JH regulation for these peptides. They might act as myoregulators or inhibit vitellogenin production in the fat body, as found in *B. germanica* (Martín et al., 1996). Recent findings of allatostatins in blood cells of *D. punctata* may be indicative of additional functions of these molecules (B. Stay, pers. comm.). A neuromodulator action cannot be excluded (Stay et al., 1992). At least, as a working hypothesis it is not envisaged that such peptides in the stick insect are made, but not used and, therefore, a clear function has to be found for such a high number of neuropeptides.

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