Eur. J. Entomol. **96**: 275–278, 1999
ISSN 1210-5759

Myotropic neuropeptides from the retrocerebral complex of the stick insect, *Carausius morosus* (Phasmatodea: Lonchodidae)*

REINHARD PREDEL[1,**], ROLAND KELLNER[2,***] and GERD GÄDE[1]

[1]Zoology Department, University of Cape Town, Rondebosch 7701, South Africa
[2]Institut für Physiologische Chemie und Pathobiochemie, Duesbergweg 6, D-55099 Mainz, Germany

Key words. Phasmatodea, *Carausius morosus*, stick insect, neuropeptide, myotropin, neurohaemal organs, pyrokinin, CCAP, corazonin

Abstract. Myotropic neuropeptides were isolated from the retrocerebral complex of the stick insect, *Carausius morosus*, by using three HPLC steps. Bioactivity during purification was measured by heterologous bioassays monitoring the contractions of the hyperneural muscle and hindgut of the American cockroach. Additionally, fractions not active in these bioassays were tested in a homologous bioassay evoking contractions of the hindgut of *C. morosus*. Peptide sequence analysis and mass spectrometry yielded the following structures: Pro-Phe-Cys-Asn-Ala-Phe-Thr-Gly-Cys-NH_2 (CCAP), pGlu-Thr-Phe-Gln-Tyr-Ser-His-Gly-Trp-Thr-Asn-NH_2 (His^7-corazonin) and Asp-Glu-Gly-Gly-Thr-Gln-Tyr-Thr-Pro-Arg-Leu-NH_2 (Cam-PK-1). These neuropeptides are the first myotropins isolated from *C. morosus*. The most bioactive compound in the homologous bioassay, the *C. morosus*-hindgut assay, was CCAP.

INTRODUCTION

Myotropic neuropeptides of insects are substances which influence the activity of visceral muscles in an in vitro assay. Although most of these peptides also have other functions, the visceral muscle assay was initially used to detect such bioactive substances during purification from crude extracts. The myotropins of two groups of hemimetabolous insects have been thoroughly investigated: locusts (see Schoofs et al., 1993) and cockroaches (see Veenstra, 1989a,b; Holman et al., 1991a; Predel et al., 1995, 1997a,b, 1998a,b). To date, the following neuropeptide families are known from these insects to have stimulatory effects on visceral muscles: proctolin, insect kinins, sulfakinins, pyrokinins, tachykinin-like peptides and CCAP (not found in cockroaches yet) (for structures see Gäde, 1997). All of these peptides are active on the cockroach hindgut, the system which was used for the detection of the majority of insect myotropins (Holman et al., 1991b). The introduction of other visceral muscle assays, such as the heart and hyperneural muscle, led to the isolation of further myoactive substances which are not active on the hindgut. These include corazonin (Veenstra, 1989a) and the periviscerokinins (Predel et al., 1995, 1998a).

In the present paper we report the isolation of the first myotropic neuropeptides from the stick insect, *Carausius morosus*. For this purpose we used an extract of 900 corpora cardiaca/corpora allata (CC/CA) complexes that were initially prepared for the isolation of the glycosylated and non-glycosylated hypertrehalosaemic decapeptides residing in the CC of *C. morosus* (see Gäde et al., 1992). For comparative aspects, we used the same bioassays we had developed previously for the isolation of myotropins from the CC/CA of the American cockroach, *Periplaneta americana* (L.): the hyperneural muscle and hindgut of *P. americana*. Additionally, all fractions which were inactive in both assay systems, were tested on the hindgut of the stick insect.

MATERIAL AND METHODS

Insects

Stick insects, *Carausius morosus* (Brunner), were collected and reared in Cape Town/RSA as described previously (Gäde et al., 1997). Cockroaches, *Periplaneta americana* (L.), were reared under a 12 L : 12 D photoperiod at a constant temperature of about 28°C.

Visceral muscle bioassays

The assays using the isolated hyperneural muscle and hindgut of the American cockroach as well as the hindgut of the stick insect were performed as previously described by Predel et al. (1994). Briefly, the isolated visceral muscles were fixed in a vertical chamber, containing only 100 µl saline in this study, and continuously rinsed from below. Contractions were recorded photoelectrically by an isotonic transducer.

Extraction and purification of the peptides

The CC/CA of about 900 stick insects were dissected into 80% methanol and stored at −50°C in Eppendorf tubes. Glands were extracted as described previously (Gäde et al., 1992); run in 8 batches on RP-HPLC, the two AKH/RPCH-family decapeptides were collected and the rest was dried and used for this study. Four batches containing each about 225 CC/CA were

* This paper is based on a lecture presented at the 6th European Congress of Entomology held in České Budějovice, Czech Republic, August 1998.

** Present and corresponding address: Institut für Allgemeine Zoologie und Tierphysiologie, Friedrich-Schiller-Universität, Erbertstraße 1, D-07743 Jena, Germany; e-mail: b6prre@pan.zoo.uni-jena.de.

***Present address: Biomed Fo/GBT, Merck KGaA, D-64271 Darmstadt, Germany.

sonicated in 200 µl of methanol/water/trifluoroacetic acid (90/9/1, v/v/v) solution. After centrifugation, the pellets were subjected to two further extraction cycles. 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 module 802, a model 811 mixing chamber (all Gilson, Inc.) and a LKB 2151 variable wavelength detector set at 214 nm. All separations were carried out on RP-C_{18} columns (250 mm × 4.6 mm, 100 Å, 5 µm). The operating conditions for the HPLC-columns were as follows: Step 1: Alphasil 5C_{18} (HPLC-Technology Ltd, Cheshire, UK). Solvent A: 0.11% trifluoroacetic acid (TFA) in water. Solvent B: 60% acetonitrile, containing 0.1% TFA. Conditions: 10–40% B over 15 min, then 40–50% B over 15 min, finally 50–90% B over 20 min. Flow rate: 0.7 ml/min. Step 2: Alphasil 5C_{18} Solvent A: 0.11 % heptafluorobutyric acid (HFBA) in water. Solvent B: 60% acetonitrile, containing 0.1% HFBA. Conditions: 25–70% B over 60 min. Flow rate: 1 ml/min. Step 3: Nucleosil 100 C_{18} (Bischoff Analysentechnik GmbH, Leonberg, Germany). Solvent A: 0.055% TFA in water. Solvent B: 60% acetonitrile, containing 0.05% TFA. Conditions: 20–60% B over 40 min. Flow rate: 1 ml/min. After the first HPLC run the corresponding biologically active fractions were pooled and the combined material used for the next purification step.

Enzymatic deblocking

About 20 pmol of native substance was dissolved in 25 µl buffer, containing 100 mM Na_2HPO_4, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), glycerol (5% v/v) and 0.5 units pyroglutamate aminopeptidase (Sigma). Following incubation for 20 min (37°C), 25 µl 0.05% TFA in acetonitrile/water (20/80, v/v) was added to stop the reaction. Subsequently the sample was fractionated under conditions described for HPLC-step 3 (see above).

Amino acid sequence analysis

The active peaks from the final HPLC-separations were loaded onto a polybrene coated glass fibre filter and sequenced by automated Edman degradation using a model 477A sequenator connected to model 120A on-line PTH-analyzer (Applied Biosystems, Weiterstadt, Germany). Sequencing and PTH analysis were carried out with standard programmes.

Mass spectrometry

Mass analyses were performed with a Kratos Kompact MALDI II Pulsed Extraction instrument (Shimadzu, Duisburg, Germany). The matrix was a methanolic solution of alpha-cyano-4-hydroxycinnamic acid. All observed spectra represented the average masses in the positive mode.

RESULTS

An extract of 900 CC/CA was originally chromatographed to purify the two hypertrehalosaemic peptides from *C. morosus* (see Gäde et al., 1992). All fractions, not containing hypertrehalosaemic peptides, were pooled and dried. This extract was re-chromatographed in 4 batches on the Alphasil 5C_{18} column with TFA as organic modifier (Fig. 1). The resulting fractions between 17 and 40 min were collected by UV peaks, aliquots representing 10 CC/CA equivalents were subsequently tested in the cockroach hyperneural muscle and hindgut assays. Additionally, fractions without bioactivity on both systems were tested in the *Carausius*-hindgut assay. Four fractions, labelled 2, 5, 6 and 12 in Fig. 1, showed strong myoactivity and were selected for further purification. The chosen

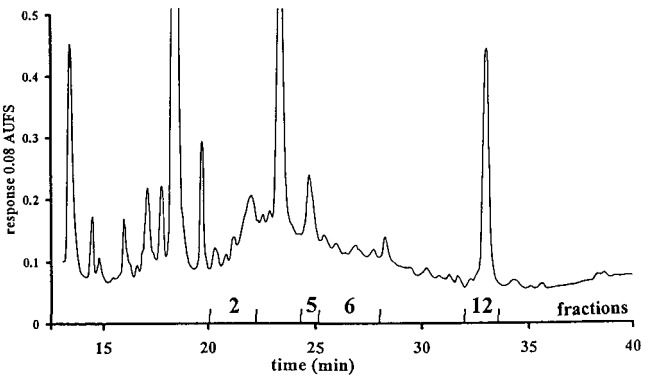

Fig. 1. Initial purification of an extract of 900 CC/CA of *C. morosus* (4 batches with 225 organs, respectively) on a RP-C_{18} Alphasil column with 0.1% TFA as organic modifier. Peaks were collected manually and aliquots were tested in the different muscle bioassays. Active fractions are numbered. AUFS, absorption units full scale, at 214 nm.

fractions were separated on the Alphasil 5C_{18} column with HFBA as ion-pairing reagent. A number of fractions which stimulated visceral muscle activity were detected (not shown). Only those fractions which were strongly bioactive in one of the bioassays and contained sufficient material for structural analysis were purified to homogeneity on a Nucleosil column. This final purification step was developed with a lower concentration of ion-pairing reagent (TFA) in the mobile phase than in step 1 and resulted in four biological active fractions.

The most abundant myotropin, resulting from purification of fraction 5 (HPLC-step 1, see Fig. 1), was only active on the hyperneural muscle. An aliquot of this substance (about 20 pmol) was incubated with pyroglutamate aminopeptidase. Rechromatography thereafter resulted in a significantly different retention time (more hydrophilic), indicating the loss of pyroglutamate. Subsequently, most of the remaining sample (about 1 nmol after three HPLC-steps) was deblocked and subjected to sequence analysis which gave the primary structural data of (pGlu)-Thr-Phe-Gln-Tyr-Ser-His-Gly-Trp-Thr-Asn. This was interpreted as being the structure of His^7-corazonin (Veenstra, 1991). Mass spectrometric analysis of the remaining material confirmed the sequencing data obtained for this peptide (Fig. 2).

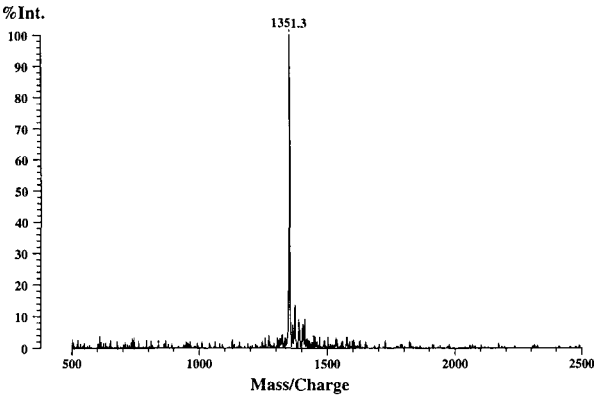

Fig. 2. MALDI-TOF mass spectrum of the natural purified His^7-corazonin (averaged mass).

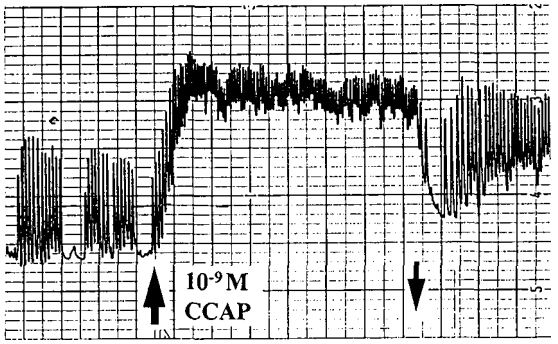

Fig. 3. Response of a hindgut preparation of *C. morosus* to synthetic CCAP (10^{-9} M). The application lasted about six minutes. Native CCAP was the only highly effective compound (in this homologous bioassay) which has been found in an extract from the retrocerebral complex of the stick insect.

The other bioactive substances were purified in amounts much less than His^7-corazonin (between 40–100 pmol after three HPLC-steps). Mass spectral analysis yielded the following $[M+H]^+$ ions: 901.4, 956.8 and 1236.0. Substances with masses [M] of about 900.4 Da and 1235.0 Da were active in all biossays used. Whereas the substance with a mass of 955.8 Da did not stimulate the hyperneural muscle, it was the only one with a high potency on the *Carausius* hindgut (see Fig. 3).

Incubation of the 900.4 Da and 955.8 Da substances with pyroglutamate aminopeptidase and subsequent chromatography revealed that the latter substance was not N-terminally blocked by pGlu and that the former substance was completely destroyed in the procedure, an indication of the occurrence of an unspecific cleavage site of the enzyme. Thereafter, the remainder of the peptides were subjected to sequence analysis. The peptide with a molecular weight of about 900.4 Da probably contained pGlu since it gave no signals in the sequencing cycles. The peptide with a molecular weight of 955.8 Da was sequenced as Pro-Phe-Xxx-Asn-Ala-Phe-Thr-Gly-Xxx, a structure identical to the crustacean cardioactive peptide (CCAP) (Stangier et al., 1987) when replacing the Xxx-positions with Cys which is not detectable during Edman degradation. Sequencing of the 1235.0 Da peptide yielded the following structure: Asp-Glu-Gly-Gly-Thr-Gln-Tyr-Thr-Pro-Arg-Leu. It is a new member of the pyrokinin-family, code-named Cam-PK-1; all members of this peptide-family contain a N-terminal amidation which is in agreement with the mass data we obtained for this peptide (see above).

DISCUSSION

We have successfully isolated and identified the first myotropic neuropeptides from the stick insect, *Carausius morosus*. The occurrence of these peptides in the retrocerebral complex suggests a role as hormones. The most abundant myotropin identified was His^7-corazonin, earlier described from *Schistocerca gregaria* (Veenstra, 1991). The yields obtained from 900 CC/CA were comparable with those from 800 CC/CA of the American cockroach, *Periplaneta americana*, as revealed during the purification of pyrokinins from this cockroach species (Predel et al., 1997a). Corazonin-peptides appear to be widely distributed in insects, always occurring in a single isoform (Veenstra, 1989a, 1991, 1994). Their conservative structure with merely one known amino acid replacement (Arg or His at position 7) suggests that not only the C-terminus is essential for biological activity, as is the case for most of the other insect myotropic peptides (for review see Gäde, 1997). It has to be noted, however, that no biological function is yet known for corazonins, with the exception of their putative role in regulation of visceral muscles of *P. americana*. Even other species of cockroaches showed no sensitivity to corazonin when their hyperneural muscle was used as bioassay (Predel et al., 1994). So, the common function of this peptide remains unclear*.

Other myotropic substances were present in amounts ten-fold less than those of His^7-corazonin. Among these peptides, CCAP was the most active compound in a homologous assay, the hindgut assay of *C. morosus*. This peptide, originally found in crustaceans (Stangier et al., 1987), has been isolated from different insects and the myoactivity is well documented (Stangier et al., 1989; Cheung et al., 1992; Furuya et al., 1993; Lehman et al., 1993). Moreover, CCAP seems to be involved in the ecdysis behavior, at least in moths (Gammie & Truman, 1997). In locusts, it was recently found that CCAP could also have functions within the retrocerebral complex itself. Here, a function as adipokinetic hormone release-inducing factor is discussed (Veelaert et al., 1997; Flanigan & Gäde, 1999).

The third peptide which was identified belongs to the pyrokinin-family (Holman et al., 1986). Cam-PK-1 is the second pyrokinin-isoform in insects with a replacement of Phe by Tyr in the active core. It was shown earlier that such a replacement is not critical for myoactivity (Nachman et al., 1986; Predel et al., 1999). A fourth, highly myoactive, substance with a molecular weight of about 900.4 Da was not successfully sequenced during this study. It is likely that this substance also belongs to the pyrokinins because of its bioactivity in all visceral muscle bioassays used. This can be deduced from previous experiments which clearly showed that the hyperneural muscle of *P. americana* is not sensitive to peptides of the kinin- and sulfakinin-families (Predel et al., 1997a,b). These are additional myotropic peptides known to be stored in the retrocerebral complex of insects (see Gäde, 1997).

It is interesting and revealing to compare the present results on the presence of myotropic neuropeptides in the retrocerebral complex of the stick insect with previous results generated for the American cockroach using similar bioassays (see Predel et al., 1997a,b, 1999, in prep.). Only a few myotropic neuropeptides were found in the corpus cardiacum of the stick insect, but at least 15 in the American cockroach. No member of the kinin and sulfakinin family has been detected in the stick insect CC, whereas

* In a recent paper (Tawlik et al., 1999) His^7-corazonin was described as dark-color-inducing neuropeptide in locusts.

these are the most abundant myotropic neuropeptides (together with corazonin) in the American cockroach CC.

ACKNOWLEDGEMENTS. We thank M.-P. Janssens for his help in collecting stick insects and dissecting CC/CA complexes. Partial financial support came from Volkswagen Foundation (I, 73024; to GG and RK) and Foundation for Research Development (South Africa, to GG). R. Predel was supported with a post-doctoral fellowship of the German Academic Exchange Service (D-94/20522).

REFERENCES

CHEUNG C.C., LOI P.K., SYLWESTER A.W., LEE T.D. & TUBLITZ N.J. 1992: Primary structure of a cardioactive neuropeptide from the Tobacco Hawkmoth, Manduca sexta. *FEBS Letters* **313**: 165–168.

FLANIGAN J.E. & GÄDE G. 1999: On the release of the three locust (Locusta migratoria) adipokinetic hormones: effect of crustacean cardioactive peptide and inhibition by sugars. *Z. Naturforsch.* (in press).

FURUYA K., LIAO S., REYNOLDS S.E., OTA R.B., HACKETT M. & SCHOOLEY D.A. 1993: Isolation and identification of a cardioactive peptide from Tenebrio molitor and Spodoptera eridania. *Biol. Chem. Hoppe-Seyler* **374**: 1065–1074.

GÄDE G. 1997: The explosion of structural information of insect neuropeptides. In Herz W., Kirby G.W., Moore R.W., Steglich W. & Tamm Ch. (eds): *Progress in the Chemistry of Organic Natural Products. Vol. 71.* Springer, Wien, New York, pp. 1–128.

GÄDE G., KELLNER R., RINEHART K.L. & PROEFKE M. 1992: A tryptophan-substituted member of the AKH/RPCH family isolated from a stick insect corpus cardiacum. *Biochem. Biophys. Res. Commun.* **189**: 1303–1309.

GÄDE G., LORENZ M.W. & HOFFMANN K.-H. 1997: Stick insect (Carausius morosus; Phasmatodea: Lonchodidae) brain extract contains multiple fractions with allatostatic activity. *Eur. J. Entomol.* **94**: 361–368.

GAMMIE S.C. & TRUMAN J.W. 1997: Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, Manduca sexta. *J. Neurosci.* **17**: 4389–4397.

HOLMAN G.M., COOK B.J. & NACHMAN R.J. 1986: Primary structure and synthesis of a blocked myotropic neuropeptide isolated from the cockroach, Leucophaea maderae. *Comp. Biochem. Physiol. (C)* **85**: 219–224.

HOLMAN G.M., NACHMAN R.J., WRIGHT M.S., SCHOOFS L., HAYES T.K. & DE LOOF A. 1991a: Insect myotropic peptides: isolation, structural characterization, and biological activities. *Am. Chem. Soc. Symp. Ser.* **453**: 40–50.

HOLMAN G.M., NACHMAN R.J., SCHOOFS L., HAYES T.K., WRIGHT M.S. & DE LOOF A. 1991b: The Leucophaea maderae hindgut preparation – a rapid and sensitive bioassay tool for the isolation of insect myotropins of other insect species. *Insect Biochem.* **21**: 107–112.

LEHMAN H.K., MURGIUC C.M., MILLER T.A., LEE T.D. & HILDEBRAND J.G. 1993: Crustacean cardioactive peptide in the sphinx moth, Manduca sexta. *Peptides* **14**: 735–741.

NACHMAN R.J., HOLMAN G.M. & COOK B.J. 1986: Active fragments and analogs of the insect neuropeptide leucopyrokinin: structure-function studies. *Biochem. Biophys. Res. Commun.* **137**: 936–942.

PREDEL R., AGRICOLA H., LINDE D., WOLLWEBER L., VEENSTRA J.A. & PENZLIN H. 1994: The insect neuropeptide corazonin: physiological and immunocytochemical studies in Blattariae. *Zoology* **98**: 35–49.

PREDEL R., LINDE D., RAPUS J., VETTERMANN S. & PENZLIN H. 1995: Periviscerokinin: a novel myotropic neuropeptide from the perisympathetic organs of the American cockroach. *Peptides* **16**: 61–66.

PREDEL R., KELLNER R., KAUFMANN R., PENZLIN H. & GÄDE G. 1997a: Isolation and structural elucidation of two pyrokinins from the retrocerebral complex of the American cockroach. *Peptides* **18**: 473–478.

PREDEL R., KELLNER R., RAPUS J., PENZLIN H. & GÄDE G. 1997b: Isolation and structural elucidation of eight kinins from the retrocerebral complex of the American cockroach. *Regul. Pept.* **71**: 199–205.

PREDEL R., RAPUS J., ECKERT M., HOLMAN G.M., NACHMAN R.J., WANG Y. & PENZLIN H. 1998a: Isolation of periviscerokinin-2 from the abdominal perisympathetic organs of the American cockroach, Periplaneta americana. *Peptides* **19**: 801–809.

PREDEL R., KELLNER R., NACHMAN R.J., RAPUS J. & GÄDE G. 1999: Differential distribution of pyrokinin-isoforms in cerebral and abdominal neurohemal organs of the American Cockroach. *Insect Biochem. Mol. Biol.* **29**: 139–144.

SCHOOFS L., VANDEN BROECK J. & DE LOOF A. 1993: The myotropic peptides of Locusta migratoria – structures, distribution, functions and receptors. *Insect Biochem. Mol. Biol.* **23**: 859–881.

STANGIER J., HILBICH C., BEYREUTHER K. & KELLER R. 1987: Unusual cardioactive peptide (CCAP) from pericardial organs of the shore crab, Carcinus maenas. *Proc. Natn. Acad. Sci. USA* **84**: 575–580.

STANGIER J., HILBICH C. & KELLER R. 1989: Occurrence of crustacean cardioactive peptide (CCAP) in the nervous system of an insect, Locusta migratoria. *J. Comp. Physiol. (B)* **159**: 5–11.

TAWLIK A.I., TANAKA S., DE LOOF A., SCHOOFS L., BAGGERMAN G., WAELKENS E., DERUA R., MILNER Y., YERUSHALMI Y. & PENER P.P. 1999: Identification of the gregarization-associated dark-pigmentotropin in locusts trough an albino mutant. *Proc. Natn. Acad. Sci. USA* **96**: 7083–7087.

VEELAERT D., PASSIER P., DEVREESE B., VANDEN BROECK J., VAN BEEUMEN J., VULLINGS H.G., DIEDEREN J.H., SCHOOFS L. & DE LOOF A. 1997: Isolation and characterization of an adipokinetic hormone release-inducing factor in locusts: the crustacean cardioactive peptide. *Endocrinology* **138**: 138–142.

VEENSTRA J.A. 1989a: Isolation and structure of corazonin, a cardioactive peptide from the American cockroach. *FEBS Letters* **250**: 231–234.

VEENSTRA J.A. 1989b: Isolation and structure of two gastrin/CCK-like neuropeptides from the American cockroach homologous to the leucosulfakinins. *Neuropeptides* **14**: 145–149.

VEENSTRA J.A. 1991: Presence of corazonin in three insect species, and isolation and identification of [His7] corazonin from Schistocerca americana. *Peptides* **12**: 1285–1289.

VEENSTRA J.A. 1994: Isolation and structure of the Drosophila corazonin gene. *Biochem. Biophys. Res. Commun.* **204**: 292–296.

Received January 4, 1999; accepted April 12, 1999