*Acta Biologica Hungarica 59 (4), pp. 385–402 (2008)*
*DOI: 10.1556/ABiol.59.2008.4.1*

# BIOLOGICAL ACTIVITY AND IDENTIFICATION OF NEUROPEPTIDES IN THE NEUROSECRETORY COMPLEXES OF THE CABBAGE PEST INSECT, *MAMESTRA BRASSICAE* (NOCTUIDAE; LEPIDOPTERA)

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(Received: March 28, 2007; accepted: October 10, 2007)

The need for more environmentally sound strategies of plant protection has become a driving force in physiological entomology to combat insect pests more efficiently. Since neuropeptides regulate key biological processes, these "special agents" or their synthetic analogues, mimetics, agonists or antagonists may be useful tools. We examined brain-suboesophageal ganglia and corpora cardiaca-corpora allata complexes of the cabbage moth, *Mamestra brassicae,* in order to obtain clues about possible peptide candidates which may be appropriate for the biological control of this pest. With the aid of bioassays, reversed phase high performance liquid chromatography, and mass spectrometry, five neuropeptides were unequivocally identified and the presence of a further three were inferred solely by comparing mass spectra with known peptides. Only one neuropeptide with adipokinetic capability was identified in *M. brassicae.* Data from the established homologous bioassay indicated that the cabbage moths rely on a lipid-based metabolism which is aided by an adipokinetic hormone (viz. Manse-AKH) that had previously been isolated in many different lepidopterans. Other groups of neuropeptides identified in this study are: FLRFamides, corazonin, allatostatin and pheromonotropic peptide.

*Keywords: Mamestra brassicae* – neuropeptides of intermediary metabolism – myoactive neuropeptides – RP-HPLC – MALDI-ToF-MS – ESI-MS/MS

## INTRODUCTION

Neuropeptides regulate virtually all aspects of insect life: growth and development, reproduction, intermediary metabolism and homeostasis, as well as muscle movement [15, 17, 39], hence, they may be candidates for the development of alternative,

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safe and maybe even family-, genus- or species-specific control agents (e.g. [1, 16]). In order to employ neuropeptides as novel "insecticides", the first steps are to gather as much information as possible on the complement of neuropeptides present in the target pest species, their physiological effects and mode of action. This means that one has not only to identify a number of possible key neuropeptides that influence major actions in the species under study, but also to develop a set of biological assays to measure the endogenous action of the peptides.

The main objective of the present study is to identify neuropeptides that regulate the use of metabolic reserves, such as glycogen and triacylglycerides, and those that exert an influence on visceral muscles, thus having myotropic (i.e. myostimulatory or myoinhibitory) actions. The targeted species is the noctuid moth *Mamestra brassicae* which is a harmful pest insect species in Europe, including Hungary. *M. brassicae* produces 2–3 overlapping generations per year, feeds mainly on Brassicaceae and effects serious damage on different cabbage species in Hungary [27]. With respect to neuropeptide research, *M. brassicae* is almost terra incognita. To date, only a pheromonotropin (Mambr-PT) [9], the cloned gene for the pheromone biosynthesis activating neuropeptide (PBAN) and some other neuropeptides that have been deduced from the PBAN gene are known in *M. brassicae* [24], and limited studies are available with respect to their distribution and physiological function [23].

In other lepidopteran species, peptides such as allatostatin (AS; inhibits juvenile hormone, JH, biosynthesis in corpora allata, CA) and diuretic hormone (controls water balance and/or feeding activity) have been detected in the tobacco hornworm, *Manduca sexta*, while various PBANs and their modes of action were investigated in the silk moth, *Bombyx mori,* and corn earworm moth, *Helicoverpa zea*; prothoracicotropic hormone was studied in *B. mori* and *M. sexta* [14]. Information on neuropeptides regulating energy metabolism in lepidopterans is limited. An adipokinetic hormone (AKH) with a hyperlipaemic effect was first characterized in *M. sexta* (Manse-AKH) [41]; the same peptide sequence is found in *H. zea*, *B. mori* and *Vanessa cardui* [see 29]. Additionally, a decapeptide with a hypertrehalosaemic action was found in *H. zea* (Helze-HrTH) [25].

Recently, neuropeptide-producing organs from Lepidoptera were screened by mass spectrometric methods [2, 3, 4, 22]. The investigated species (*M. sexta* and *Galleria mellonella*) are those from which a fair number of neuropeptides had already been structurally characterized by conventional techniques. Our current research on *M. brassicae* uses biological assays to indicate the presence and activity of certain neuropeptides in brain and corpora cardiaca (CC), reversed phase high performance liquid chromatography (RP-HPLC) for peptide isolation from these tissues, and matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (ToF) and electrospray ionization (ESI) mass spectrometry (MS) for structural identification and peptide sequence determination.

# MATERIALS AND METHODS

## *Insects*

*M. brassicae* was reared on a semi-artificial diet at 23 ± 1 °C, ~70% R.H. and at 17 h light : 7 h dark regime as described by Nagy [35].

Adult male migratory locusts, *Locusta migratoria* (15–25 days old), and adult male American cockroaches, *Periplaneta americana* (undetermined age), were used in a heterologous test to monitor lipid and carbohydrate mobilization, respectively; locusts and cockroaches were reared as described previously [13].

To monitor myoactivity, a heterologous bioassay with adult Chilean giant cockroaches, *Blaberus craniifer* (undetermined age and both sexes), was developed; the stock colony was obtained from the Budapest Zoological Garden and maintained on a diet of standard dog food, fruits and water *ad libitum* at 30 ± 2 °C; ~50% R.H. and 12 h light : 12 h dark regime.

## *Tissue extractions*

In the Budapest laboratory brain-suboesophageal ganglia (SOG) and CC-CA complexes were dissected under a lepidopteran Ringer solution [33] from 2–3 days old adult *M. brassicae* of both sexes. Batches of 50 organs were collected, homogenized by sonication in 300 µl of ice cold methanol : water : acetic acid (100 : 10 : 1) for brain-SOGs, or 80 : 20 : 0 for CC-CA, centrifuged for 3 min at 11,000 rpm and finally dried in a Speed-Vac. The samples were resuspended in 0.1% trifluoracetic acid (TFA) and filtered through a MILLEX® PVDF (0.45 µm × 13 mm). Fractionation of the crude extracts was performed on a Beckman HPLC system using a Bioszeparációs Technika (BST) Nucleosil 5 RP-$C_{18}$ column as described earlier [19]. One ml fractions were collected automatically, evaporated to dryness, and then resuspended in distilled water or Wright's Ringer solution (0.9 g/l NaCl, 4 g/l glucose, 0.2 g/l KCl, 0.2 g/l $CaCl_2$, 28.83 g/l HEPES, adjusted to pH = 6.9 by 1N NaOH) for functional characterization.

In Cape Town, other batches of 50 brain-SOG and CC-CA complexes were prepared for RP-HPLC as outlined previously [19]. The extracts were resuspended in 15% acetonitrile (ACN), loaded onto a Nucleosil C-18 column with guard column of the same material and run on a Gilson HPLC system; the setup is described elsewhere [11] and conditions for separation were provided previously [19].

## *Bioassays*

### *Adipokinetic and carbohydrate bioassays,* in vivo

The heterologous bioassay to monitor changes in the concentration of lipids (in locusts) and carbohydrates (in cockroaches) were performed as described previously [10], with haemolymph samples withdrawn from each individual before and after the injection of a test substance. As positive controls, crude CC extracts of *L. migratoria* or the synthetic cockroach peptide Peram-CAH-I (Peninsula Laboratories, Belmont, CA, USA) were used.

Homologous bioassays to monitor lipid and carbohydrate changes of *M. brassicae* were performed on 1–3 days old male moths. Because of the small size and fragility of the cabbage moth, it was not possible to take two haemolymph samples, therefore only one sample (1 µl) was withdrawn from individuals, thus, haemolymph was taken from one group of resting moths to obtain the unstimulated levels of metabolites; other groups of resting moths were injected with a test substance (crude extracts, or RP-HPLC fractions) kept for a further 90 min after which a sample was taken for respective measurements. As positive controls, the synthetic moth peptides, Manse-AKH and Helze-HrTH (Peninsula Laboratories) were injected, respectively. As with the heterologous assays, water was injected into one group of animals as negative control. Results were analyzed statistically using Student's *t*-test.

### *Myoactivity bioassay,* in vitro

Myoactivity was tested in a heterologous, *in vitro* assay, using the hindgut of *B. craniifer*. The hindgut was dissected from the cockroach, freed of adhering tissue and nervous connections and suspended in a small vertical chamber containing approximately 2 ml Wright's Ringer solution. The assay was performed according to [21]. An analogue recorder (Radelkis, Hungary) was used to monitor the spontaneous movement (amplitude, frequency and tonus) of the muscle via a sensitive transducer (supplied by a workshop of the Catholic University of Leuven, Belgium). As positive controls, the synthetic myotropic peptide of the migratory locust (Locmi-MT-II), and the pheromonotropic peptide of the armyworm, *Pseudaletia separata* (Psese-PT), (Bachem Feinchemikalien AG, Germany) were used in the range of $10^{-9} - 10^{-8}$ M (for sequences, see [14]). Crude extracts and HPLC-generated fractions were resuspended in 1 ml Wright's solution before they were added to the chamber. In this system, substances that induced a myoactive response (i.e. stimulatory or inhibitory) could be rinsed out with 2–3 rinses of Ringer after which the basal spontaneous movement of the gut was restored.

### *Mass spectrometry*

All MS-based experiments were performed at the University of Münster. For MALDI-MS, peptides were dissolved in 15% ACN and purified using ZipTips C18 (Millipore, Bedford, MA, USA). Peptide maps were generated with a TofSpec-2E instrument (Micromass, Manchester, UK) in reflectron mode. A saturated solution of α-cyano-4-hydroxycinnamic acid in ACN/0.1% TFA (1/2 v/v diluted 1/1 with the same solvent) was used as matrix, of which 0.5 µl were pre-spotted onto the target plate to provide a dry anchor; 0.5 µl each of peptide/crude extract and matrix were added to the plate and lightly mixed.

For UV analysis and peptide sequencing, nanoHPLC-MS/MS with a PepMap-$C_{18}$ column (Ultimate, LC-Packings, Amsterdam, The Netherlands; Esquire$_{3000}$, Bruker Daltonics, Bremen, Germany) was employed as described before [31].

## RESULTS AND DISCUSSION

### *Metabolites in* M. brassicae *haemolymph*

In the haemolymph of resting male specimens of *M. brassicae*, the lipid concentration (55.4 ± 11.9 mg ml$^{-1}$ lipids mean ± S.D.; n = 10) was approximately twice that of the carbohydrate concentration (28.9 ± 12.6 mg ml$^{-1}$; n = 5). The high concentration of lipids in the haemolymph of young adult *M. brassicae* is in keeping with data of young adults from *M. sexta* which use primarily lipids instead of carbohydrates during flight [42] and suggests that the prime energy source in *M. brassicae*, as in other Lepidoptera, may be lipids [15].

### *Functional screening of neuropeptide material in neurohaemal and brain-SOG-complexes of* M. brassicae

### *Presence of metabolite-mobilizing material in selected neural complexes*

We tested crude extracts of the *M. brassicae* neural complexes first in commonly used heterologous assays (injection of the material into *L. migratoria* and into *P. americana* specimens) to monitor their effect on metabolite concentrations. The cabbage moth CC-CA complex (12 pairs) significantly raised the lipid concentration in locusts from 19.5 ± 2.7 mg ml$^{-1}$ before injection to 70.8 ± 11.0 mg ml$^{-1}$ 90 min post-injection (mean ± S.D.; n = 7; $p < 0.01$ in paired *t*-test), whereas control injections of distilled water had no significant effect (23.0 ± 14.6 mg ml$^{-1}$ at 0 min and 29.7 ± 17.8 mg ml$^{-1}$ at 90 min; parameters as above). The equivalent of 15 brain-SOG complex of *M. brassicae* failed to elicit a significant adipokinetic effect in locusts (data not shown), however, a maximal adipokinetic effect in the locust was achieved with the injection of 0.1 pair CC of a conspecific extract (data not shown).

When crude extract from CC-CA and brain-SOG (12 and 15 equivalents) were injected into *P. americana* cockroaches, which are known to use carbohydrates as their metabolic fuel, neither the CC-CA nor the brain-SOG complex of the cabbage moth had any hypertrehalosaemic effect (data not shown). In contrast, injection of the endogenous metabolic peptide hormone of *P. americana*, Peram-CAH-I at a dose of 10 pmol, significantly elevated the concentration of carbohydrates (data not shown).

From the two sets of experiments it is concluded that the CC-CA of *M. brassicae* but not the brain-SOG contain an adipokinetic substance and that large amounts have to be injected into locusts to achieve such an effect, whereas even large amounts of neurosecretory material of the cabbage moth was unable to raise the concentration of carbohydrates in the haemolymph of cockroaches. Such results are very reminiscent of the effect of synthetic Manse-AKH in locusts and cockroaches. Previous structure-activity studies have shown some specificity or selectivity of the locust receptor for AKHs: the $ED_{50}$ for Manse-AKH is 13.4 pmol, whereas it is 0.2 pmol for Locmi-AKH-I [12]. Moreover, the receptor of *P. americana* has been shown to have a different specificity and more than 50 pmol of Manse-AKH was needed for a measurable hypertrehalosaemic effect when injected into this cockroach [12].

A conspecific bioassay was established to confirm the presence of an adipokinetic factor in the CC-CA of *M. brassicae*. Resting cabbage moths were injected with test samples and metabolites in their haemolymph were analyzed, yielding consistent data (Table 1). Injection of *L. migratoria* CC (0.5 pairs) and 8 brain-SOG complexes from *M. brassicae* into cabbage moths, did not increase the circulating lipid concentration relative to the water-injected moths (non-significant 6% increase), whereas 8 pairs of *M. brassicae* CC-CA complex significantly raised haemolymph lipid levels (Table 1). These data support the results from the heterologous lipid bioassays and suggest that the CC-CA of *M. brassicae* contains an AKH peptide which is not present in the brain-SOG complex of the cabbage moth. The fact that hyperlipaemia was not elicited by the locust CC indicates that the putative *M. brassicae* AKH is structurally different to locust AKHs, resulting in poor receptor-ligand binding. When 15 pmol of synthetic Manse-AKH was injected, it had a significant adipokinetic effect in *M. brassicae* specimens: lipid level of $54.2 \pm 10.9$ mg $ml^{-1}$ in untreated moths, $60.8 \pm 13.8$ mg $ml^{-1}$ in water-injected moths and $78.8 \pm 10.2$ mg $ml^{-1}$ in Manse-AKH group ($n = 6$).

Conspecific carbohydrate bioassays demonstrated that circulating levels of this metabolite is more variable in cabbage moths. Despite this, it is clear that the injection of distilled water or 15 brain-SOG complexes had no significant effect on the carbohydrate concentration in *M. brassicae* (Table 1). A slight increase in carbohydrate levels was measured after the injection of 12 pairs of *M. brassicae* CC-CA, whereas the injection of 15 pmol Helze-HrTH elicited a significant hypertrehalosaemic effect (Table 1). Thus, carbohydrates in *M. brassicae* can be mobilized by small peptides and further suggests that, probably, very low amounts of a hypertrehalosaemic factor is synthesized and/or stored in the CC of *M. brassicae* relative to an AKH peptide, since 8 pairs of CC could elicit significant hyperlipaemia (Table 1).

*Table 1*
Biological activity of crude extracts of corpora cardiaca (CC) or brain-SOG complex of *M. brassicae* in *M. brassicae:* effect on haemolymph lipids and carbohydrates

| Acceptor insect | | *M. brassicae* Haemolymph lipids (mg ml$^{-1}$)[a] | |
|---|---|---|---|
| Treatment | n | 0 min | 90 min |
| No treatment | 4 | 56.6±14.0 | – |
| Control (10 µl distilled water) | 4 | – | 60.3±7.7 |
| *Locmi*-CC (0.5 gland equiv.) | 4 | – | 51.6±12.2 |
| *Mambr*-CC (8 gland equiv.) | 4 | – | 91.6±10.2[b] |
| *Mambr*-brain (8 brain equiv.) | 4 | | 60.5±18.5 |

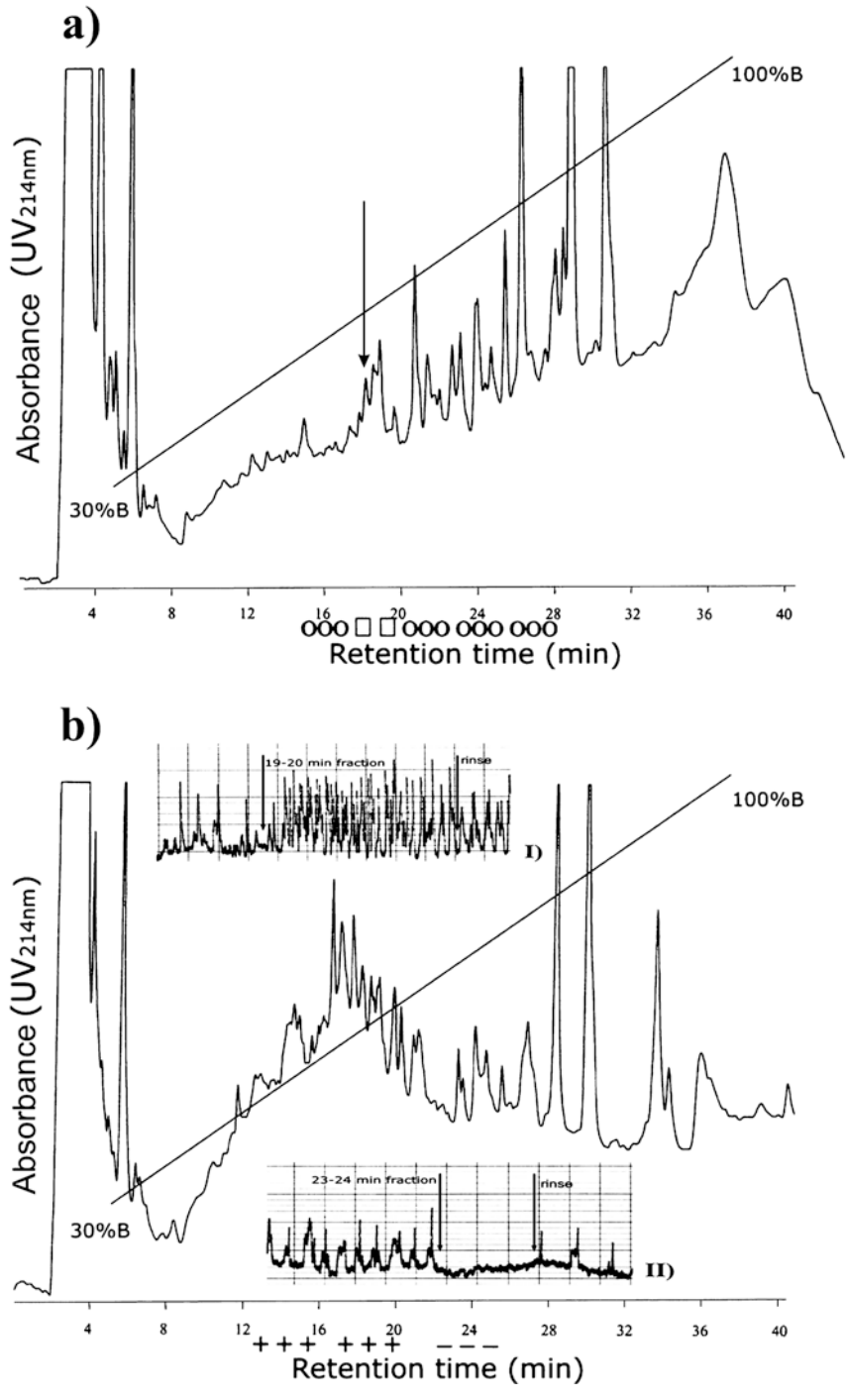
| Acceptor insect | | *M. brassicae* Haemolymph carbohydrates (mg ml$^{-1}$)[a] | |
|---|---|---|---|
| Treatment | n | 0 min | 90 min |
| No treatment | 5 | 28.9±12.6 | – |
| Control (10 µl distilled water) | 5 | – | 32.4±14.2 |
| *Helze*–HrTH (15 pmol) | 5 | – | 45.5±12.2[b] |
| *Mambr*-CC (12 gland equiv.) | 5 | – | 38.6±10.2 |
| *Mambr*-brain (8 brain equiv.) | 5 | | 26.5±13.5 |

[a] Values are given as mean ±S.D.
[b] Significantly different at $p \leq 0.05$ w.r.t. untreated group; Student's *t*-test.

In *H. zea*, two members of the AKH family have been elucidated and characterized: Manse-AKH, a nonapeptide (pQLTFTSSWGamide) that functions as an AKH in the corn earworm and Helze-HrTH, a decapeptide (pQLTFSSGWNamide) that has both an adipokinetic and a hypertrehalosaemic effect [25].

## *Isolation of metabolic and myoactive peptides by RP-HPLC and their identification by biological assays*

Biological assays with crude extracts of *M. brassicae* indicate the presence of an adipokinetic substance in the CC-CA complex (Table 1). To purify this putative AKH, an extract of 50 pairs of CC-CA was subjected to RP-HPLC and the eluant was monitored as shown in Fig. 1a. Only one fraction, eluting between 18 and 19 min demonstrated significant adipokinetic activity: circulating lipid concentration increased 3-fold in locusts (15 CC-CA equivalents were injected; n = 8); and 1.5-fold in *M. brassicae* (12 CC-CA equivalents were injected; n = 6), when compared with untreated moths. When synthetic Manse-AKH was chromatographed under identical conditions, it eluted around 18 min (see Fig. 1a); Helze-HrTH eluted one min earlier (results not shown). Further, when 15 pmol Manse-AKH was injected into resting


a)
100%B
30%B
Absorbance (UV214nm)
4
8
12
16
20
24
28
32
36
40
Retention time (min)
b)
19-20 min fraction
rinse
I)
100%B
30%B
23-24 min fraction
rinse
II)
Absorbance (UV214nm)
Retention time (min)

*M. brassicae*, it significantly increased the average lipid concentration (1.3–1.4 times) compared with water-injected and untreated moths, respectively. This information provides strong evidence that Manse-AKH, is very likely, the peptide contained in the CC-CA complex of *M. brassicae*.

The addition of the equivalent of 25 *M. brassicae* brain-SOG extract to the *in vitro* gut preparation, induced dramatic increase in the frequency, tonus and amplitude of the gut musculature. We attempted to isolate these active compounds from crude extracts by RP-HPLC as shown in Fig. 1b. All the generated fractions were tested for myoactivity on the hindgut of *B. craniifer*: six fractions had a stimulatory, i.e. myotropic effect and only three fractions had a suppressive (myoinhibitory) effect (Fig. 1b). Interestingly, the *M. brassicae* myoinhibitory peptides seem to be more hydrophobic than the myotropic ones. An example of a myostimulus in the *B. craniifer* hindgut bioassay is illustrated in Fig. 1b (inset I): after the addition of the 19–20 min RP-HPLC fraction (equivalent to 25 brain-SOG complexes), the frequency, amplitude of contraction, as well as the tonus of the cockroach hindgut muscle is elevated. A demonstration of a myoinhibitory effect is also provided (Fig. 1b, inset II): a visible drop in tonus, along with depressed amplitude, results from the addition of the 23–24 min fraction.

## *Screening and identification of putative neuropeptides in CC-CA and brain-SOG complexes of* M. brassicae *by mass spectrometry*

We utilized MALDI-ToF- and ESI-MS to further study and partially characterize peptides that are present in CC-CA and brain-SOG extracts of *M. brassicae* and were found to be biologically active in various bioassays. To date, the primary structure of a large number of small peptides from various lepidopteran species is known (for sequence databases see for example [14, 18, 36]). Certain peptides often have an identical sequence in insects from the same order [22] and, hence, one can screen

←————————

*Fig. 1.* RP-HPLC chromatogram ($UV_{214\ nm}$) of (a) a methanolic extract from 50 *M. brassicae* CC-CAs and (b) 50 *M. brassicae* brain-SOG complexes applied to a $C_{18}$-RP-HPLC column on a Beckman system. The column was developed with a linear gradient of 0.11% TFA in water (solvent A) and 0.1% TFA in 60% acetonitrile (solvent B) from 30% to 100% B in 30 min at a flow rate of 1 ml $min^{-1}$. (a) One min fractions (pooled from several runs) were tested for adipokinetic activity in *M. brassicae.* Significant lipid mobilization activity was achieved with the pooled 18–19 min fractions indicated with the □□□ symbols. Fractions indicated with ○○○ were inactive in the same bioassay system. Arrow indicates retention time of synthetic Manse-AKH in the same system. (b) One min fractions were tested in the *B. craniifer* hindgut myoactivity assay. Myotropic fractions are indicated by "+", whereas myoinhibitory fractions are shown by "–". Insets: examples of myoactivity patterns in *B. craniifer* hindgut bioassay caused by HPLC-generated fractions of *M. brassicae* brain-SOG complex. I. myotropic action of the 19–20 min fraction; II. myoinhibitory action of the 23–24 min fraction. Fractions, equivalent to 25 complexes were added to the assay chamber (2 ml volume in Wright's solution)

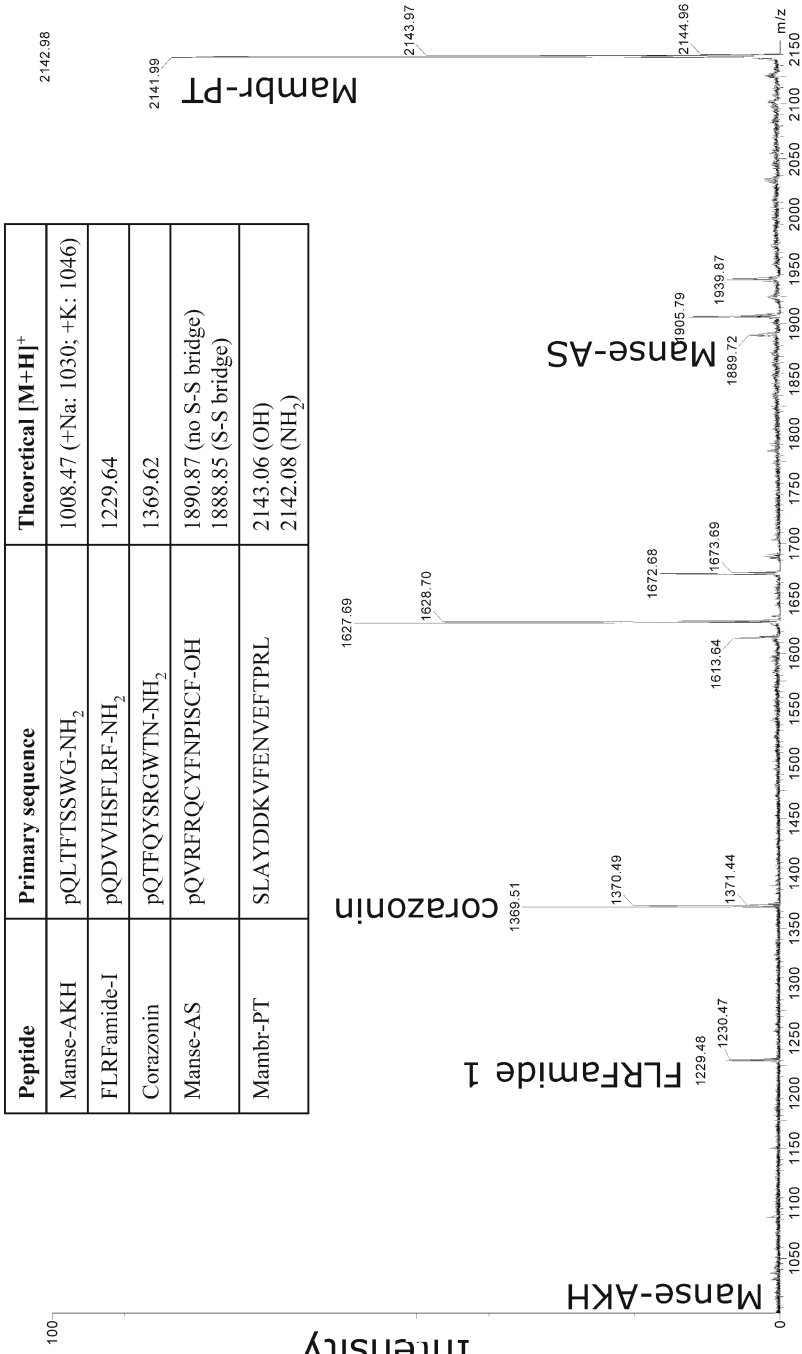



*Fig. 2.* MALDI-ToF-MS peptide spectrum of 50 *M. brassicae* CC-CA extract. Peptides were assigned by matching identical masses from insect databases and by sequencing information (see inset)

new mass data against the present sequence database and formulate a starting set of peptide sequences for further validation.

MALDI-ToF-MS of a desalted extract from the CC-CA complexes generated a number of ions between m/z 900 and m/z 2200, most of which corresponded to theoretical masses of known neuropeptides (Fig. 2). Many more ions between m/z 600 and 4200 were obtained in brain-SOG extracts (not shown); a few of which correspond to masses of known peptides.

## *Adipokinetic hormone (Manse-AKH)*

The presence of Manse-AKH in CCs of *M. brassicae* was indicated by a low-intensity ion of m/z 1030.6 (Fig. 2) which corresponds to the sodium ion. AKH peptides are well known for the preferential formation of sodiated and potassiated ions instead of protonated ions both in MALDI and ESI-MS [31]. In addition, mass signals for $[M + Na]^+$ and $[M + K]^+$ of Manse-AKH (m/z 1030 and 1046), as well as the sodiated and potassiated ions for a C-terminally Gly elongated Manse-AKH (m/z 1087 and 1103) were identified by MALDI-ToF-MS (Fig. 4a). Such elongated AKHs represent an incomplete cleavage of the precursor and have been observed in a few cases, e.g. Manse-AKH which was elongated by a Gly-Lys [3, 30], Locmi-AKH [6] and *Drosophila melanogaster* AKH [37].

Further evidence that *M. brassicae* CCs contain Manse-AKH was derived from HPLC separation of a CC extract in Cape Town monitoring tryptophan fluorescence in the eluant. Two peaks (numbered 7 and 8) with the highest absorbance eluted between 18 and 20 min (Fig. 3); peak number 8 had the same retention time as synthetic Manse-AKH under the same HPLC conditions. Furthermore, when an aliquot of peak 8 material was analyzed with ESI-MS, the total ion count revealed a distinct peak at m/z 1008.3 and the sequencing ESI spectrum (not shown) revealed distinct peaks for $[M + H]^+ = m/z$ 1008.3, $[M + Na]^+ = m/z$ 1030.3, $[M + K]^+ = m/z$ 1046.3, $[M + H - 17]^+ = m/z$ 991, and $[M + H + K]^{2+} = m/z$ 523.5. Taken together with data from the bioassays for metabolite-mobilization, there is clear evidence that Manse-AKH is, indeed, the hyperlipaemic hormone of *M. brassicae*. Manse-AKH is also the AKH found in other lepidopterans (see Introduction). There is no evidence that a second member of the AKH family is present in *M. brassicae*. Functionally, the action of Manse-AKH in *M. sexta* is to regulate carbohydrate metabolism in larvae and lipid mobilization in adults [42]. Similar actions are suggested for *M. brassicae*, at least in adults, based on the presented bioassay data. The potential of using such metabolic peptides for pest control management is outlined elsewhere [16].

## *Myoactive peptide (FLRFamide)*

The FLRFamide peptides are those that have the C-terminus ending with this peptide sequence. FLRFamide-1 (pQDVVHSFLRFamide) peptide was first isolated from

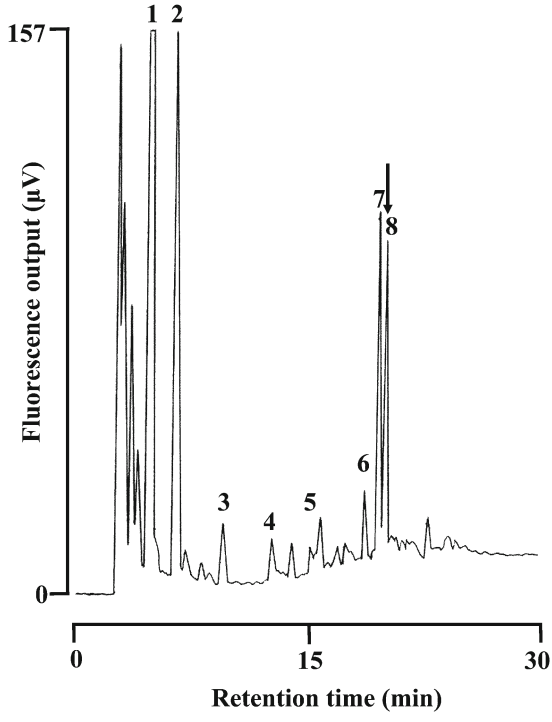



*Fig. 3.* RP-HPLC separation of a crude methanolic extract of 50 *M. brassicae* CC-CAs applied to a C18-RP-HPLC column on a Gilson system (see Materials and Methods for details). The column was developed under the same conditions as in Fig. 1. The eluant was monitored with a fluorescent detector at 276 nm (excitation) and 350 nm (emission). Prominent peaks (1–8) were manually collected, while minor peaks were collected as 2 min fractions. Aliquots of peak/fraction material were used for mass analyses and elucidation of primary peptide structures, where possible. Details of respective peak analyses are provided in Results and Discussion. Arrow shows retention time of synthetic Manse-AKH in the same system

nerve cord of *M. sexta* [28] and its sequence is similar to a so-called leucomyosuppressin (LMS; pQDVDHVFLRFamide) which was first isolated from the cockroach, *L. maderae,* inhibiting the spontaneous contraction of the hindgut [20]. A FLRFamide in *M. brassicae* CC, was indicated by an ion (Fig. 2) and its sequence was verified by ESI-MS/MS to be FLRFamide-1 (Fig. 4b). It is possible that this may be one of the peptides of *M. brassicae* that demonstrated myoinhibitory activity in bioassays in the current study. A LMS peptide was shown to be abundantly present in the CC-CA complexes of the honey bee, *Apis mellifera* [5], while FLRFamide-1, was found in the CC-CA complexes of adult *M. sexta* [3], in larval brain preparations of the tomato moth, *L. oleracea* [2] and in larval brain-retrocerebral-SOG complex of the greater wax moth, *G. mellonella* [22].

## *Cardioactive peptide (Corazonin)*

Corazonin (pQTFQYSRGWTNamide) was first isolated and characterized in insects as a cardioactive peptide from the CC of *P. americana* [40]. Later it was shown to induce the dark colouration in certain insects, cause reduction in the rate of silk spinning in *B. mori* during larval-pupal metamorphosis, and to be involved in the initiation of events in the ecdysis sequence of *M. sexta* [see, 38]. This multifunctional peptide is synthesized in the lateral neurosecretory cells of the brain and in neurons of the ventral nerve cord and stored in the CC [36]. In our study, an ion at m/z 1369.5 was detected in CC extracts of *M. brassicae* (Fig. 2) and in RP-HPLC fraction 5 (Fig. 3); this mass is indicative of corazonin which has been sequenced several times in insects (see, for example [3, 22, 38]). Since corazonin is known to exert so many varied and vital functions in insects, it may be a good candidate for further exploration as insecticide; the drawback, however, is that the primary sequence is far too conserved and is, hence, not specific for pest insects only.

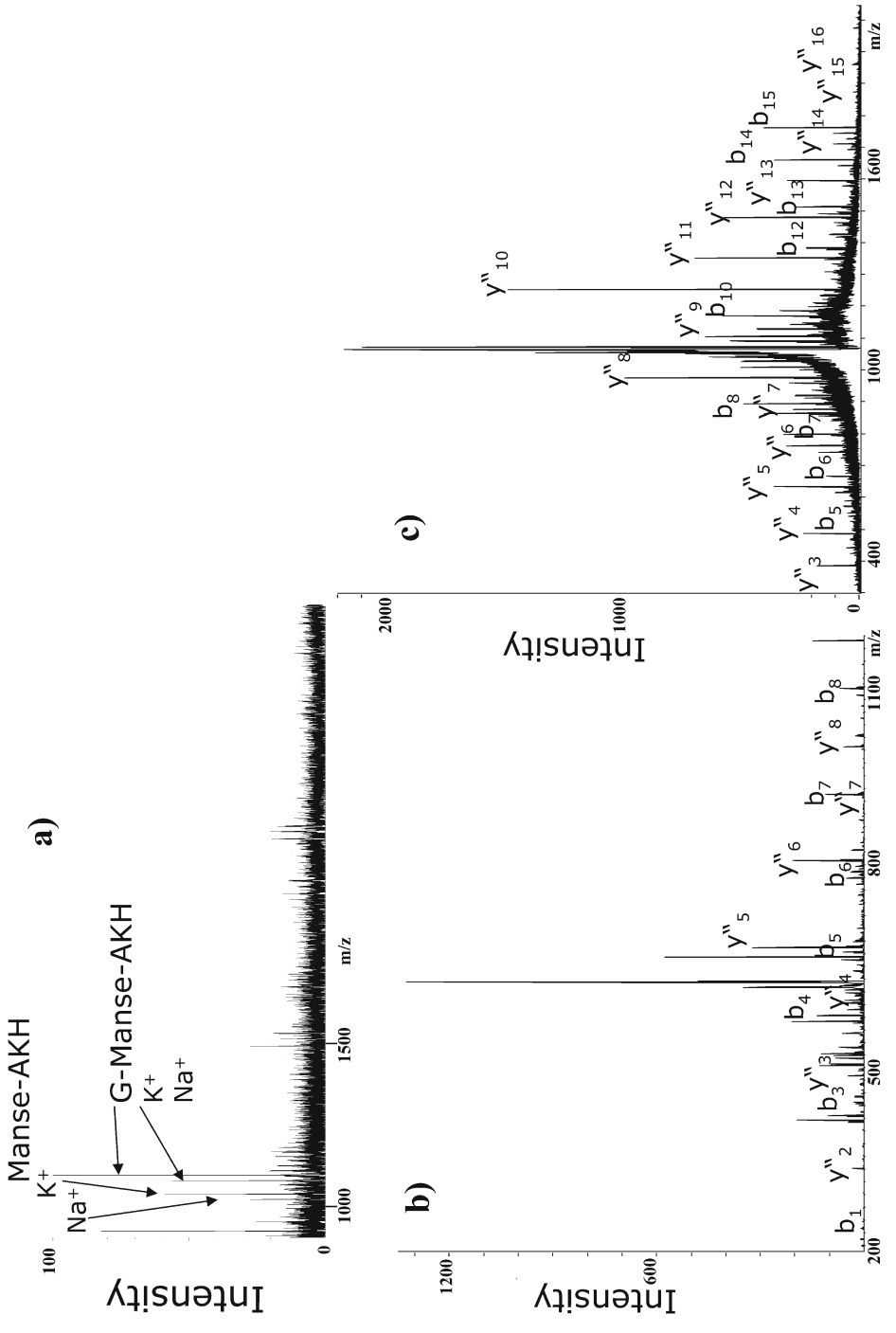
## *Allatostatin*

The peptide Manse-AS (pQVRFRQCYFNPISCF-OH) was first identified as an AS from whole heads of pharate adults of *M. sexta* [32] and later shown, by using immunochemical techniques, to be present in other insects including *P. unipuncta* [26] and found to be localized in the lateral neurosecretory cells of the brain of larval *M. sexta* and *L. oleracea* [2]. Manse-AS has also been identified in CC of adult *M. sexta*, in larval brain of *M. sexta* and *L. oleracea* [3, 4], but interestingly, it was not detected in *G. mellonella* [22].

In the present study, a peak was detected at m/z 1888.7 in CC extract of *M. brassicae* (Fig. 2) and in the HPLC-generated fraction 5 (Fig. 3); this mass corresponds to Manse-AS containing a disulfide bridge (see inset to Fig. 2). Classically, ASs are peptides that inhibit JH biosynthesis in the CA *in vitro*, although this is not always the case [36]. From a functional aspect, Manse-AS is very important: it is the only AS of lepidopterans which demonstrates a clear inhibition of JH synthesis *in vitro* [4]. As we will see below (section 3.6), other lepidopteran ASs are also suggested to be present in *M. brassicae* but none of those are known to be active in inhibiting JH synthesis.

## *Putative pheromonotropic neuropeptide (β-SG-NP)*

A peptide at m/z 2142.0 was observed in high abundance in *M. brassicae* CC-CA extract (Fig. 2). ESI MS/MS sequencing of peak 4 (see Fig. 3) resulted in well represented N- and C-terminal ion series which allowed the assignement as β-suboesophageal neuropeptide (β-SG-NP), i.e. Mambr-PT (SwissProt O45027, amino acids 67–87) (Fig. 4c). However, the mass data can only be correctly interpreted, when the


a)
Manse-AKH
G-Manse-AKH
K+
Na+
K+
Na+
Intensity
m/z
b)
c)

C-terminus is amidated (see inset in Fig. 2). The peptide sequence was previously deduced by molecular cloning of full PBAN-encoding cDNA from *M. brassicae* [24]. The peptide is part of a pre-prohormone that encodes for several other peptides, such as diapause hormone, PBAN, α-SG-NP, β-SG-NP and γ-SG-NP. Thus, finding β-SG-NP in the CC-CA of the cabbage moth implies that the other peptides encoded on the pre-prohormone are very likely also expressed in *M. brassicae*.

The purification and primary sequence identification of PT peptides in the cabbage moth had also been attempted by conventional isolation and Edman degradation sequencing methodology, resulting in a slightly different (see underlined residues) sequence (SLAYVQKVFENVEFVPRLamide) [9]. A peptide with this sequence was not found in the present study and it may be possible that the observed sequence discrepancy arose due to erroneous Edman sequencing interpretation. Biologically, PBAN is especially important because it stimulates the biosynthesis of pheromones which, in turn, are imperative for attracting conspecific males [17]. PBAN is thought to be one of the most hopeful candidates as control agent against pest insects. So far, most research in this line has centered on analogues of PBAN that either result in serious disruption of pheromone production, or those (pseudopeptide analogues) that are better able to penetrate the insect cuticle and/or are more resistant to peptidases than the parent molecule [1, 16, 34].

## *Other putative neuropeptides from the CC-CA complex*

In HPLC peak 1 (Fig. 3) a signal was detected at m/z 1182.2; this agrees with the mass of a FMRFamide-2 (DPKQDFMRFamide, mw 1181.57) found in *D. melanogaster* which has myostimulatory action [37]. In fraction 3 (Fig. 3), besides Manse-AKH, a mass of 934.4 was also detected; the latter could correspond to a so-called A-type AS (characterized by the C-terminus FGL/I/Vamide). The mass may represent helicostatin-1 (SPHYDFGLamide, mw 933.43); this A-type AS was isolated in the moth *H. armigera* and was also identified by MS in *M. sexta* and *L. oleracea* [4]. The A-type ASs are known to have no true allatostatic activity in Lepidoptera but rather inhibit the spontaneous muscle activity of the foregut [8].

←

*Fig. 4.* (a) MALDI-ToF-MS and (b, c) ESI-MS/MS spectra. (a) A crude methanolic extract of CC-CA from *M. brassicae* was analyzed. Signals for the pairs 1030/1046 and 1087/1103 correspond to the sodiated and potassiated forms of Manse-AKH and a Gly-extended Manse-AKH, respectively. (b) Peak material at m/z 615.4 in the crude extract of CC-CA was analyzed by ESI-MS/MS. Assignment of the fragment ions correspond to that for FLRF amide-1. (c) Peak material at m/z 1071.5 in the crude extract of CC-CA was analyzed by ESI-MS/MS. Assignment of the fragment ions correspond to that for PBAN β-SGN

## *Putative peptides identified in* M. brassicae *brain-SOG complex*

In the crude extract of the brain-SOG of the cabbage moth, a large number of signals were observed which were only of partial interest for this study. We assigned Manse-AS and [Arg$^7$]-corazonin (see above) and a partial sequence for short neuropeptide F-1 (sNPF-1$^{4–11}$) of *D. melanogaster* (SPSLRLRFamide, mw: 973.58 [37]). The sNPFs stimulate ovarian development and/or oocyte growth [7].

ACKNOWLEDGEMENTS

The authors thank G. Kovács and Gy. Vajdics (Budapest) for maintaining the insect colonies, Dr. G. Fekete (Budapest) for help in performing various bioassays and A.-M. Mehlich and D. Ackermann (Münster) for technical assistance regarding MS. For funding, we acknowledge projects from the NRF Hungary-RSA agreement (DAK-1/2000) and OTKA/Hungary (T 32229) as well as project grants to GG from NRF (Gun No. 2053806) and University of Cape Town (URC funding to GG and HGM).

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