Belg. J. Zool. - Volume 129 (1999) - issue 1 - pages 105-112 - Brussels 1999

ISOLATION AND PARTIAL SEQUENCING OF A NOVEL MYOTROPIN FROM THE BRAIN OF THE DESERT LOCUST *SCHISTOCERCA GREGARIA* FORSK

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Abstract. A neuropeptide that stimulates the motility of the cockroach hindgut has been isolated from an extract of 7000 brain – corpus cardiacum – corpora allata – suboesophageal ganglion complexes of the desert locust *Schistocerca gregaria*. During HPLC purification, the myotropic activity of column fractions was monitored on the isolated hindgut of *Leucophaea maderae*. Due to the low amount of material, this myotropic peptide - designated as schistomyostimulin or Scg-MST – could only be partially sequenced : DSRW?GPK(?). Scg-MST shows no relevant sequence similarities with other peptides from vertebrate or invertebrate sources. It is the fifteenth myotropic peptide chromatographically isolated from *S. gregaria*.

Key words : insect, peptide, myotropin, grasshopper.

INTRODUCTION

Regulatory peptides mediate many physiological processes. In insects alone the number of different neuropeptides isolated and identified to date exceeds 200 (GÄDE, 1997). It is assumed that the number of different neuropeptides in each species may also exceed 200. In locusts the total number of neuropeptides identified to the present time is 58 (SCHOOFS *et al.*, 1997 ; VEELAERT *et al.*, 1998). Most of the locust peptides have been isolated on the basis of their stimulatory effect on insect visceral muscles and were designated myotropins. Some of the myotropins appear to be unique to insects or arthropods ; others seem to be members of peptide families spanning across phyla. Members of the myotropin peptide families have been associated with a variety of physiological activities such as myotropic activities, pheromonotropic activities, diuresis, diapause induction, and stimulation of cuticular melanization (for review see SCHOOFS *et al.*, 1993). Some of the members may be important neurotransmitters present in nerve endings innervating the locust oviduct, the salivary glands, the male accessory glands and the heart (SCHOOFS *et al.*, 1992) whereas others are stored in neurohaemal organs until release into the haemolymph (SCHOOFS *et al.*, 1992).

Two adipokinetic hormones (Scg-AKH-1 and -2) have been purified from *Schistocerca gregaria* (Table 1). Recently two schistomyotropins (Scg-MT-1 and -2), belonging to the -FXPRLamide family were purified from an extract of 7000 brains. Scg-MT-2 has a modified -FXXPRLamide C-terminus. The crustacean cardioactive peptide was also purified from brains of the desert locust (Scg-CCAP). This peptide has a corpus cardiacum-activating effect, being a releasing factor of adipokinetic hormone in locusts. SchistoFLRFamide (Scg-FLRFamide) and the Schistostatins (Scg-ASTs) are inhibitors of visceral muscle contractions.

TABLE 1

Myotropic and myoinhibiting peptides isolated from the desert locust Schistocerca gregaria (VEELAERT *et al.*, 1998)

Scg-AST-1	LCDFGVa	VANDEN BROECK *et al*, 1996
Scg-AST-2	AYTYVSEYKRLPVYNFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-211-18	LPVYNFGLa	VEELAERT *et al.*, 1996b; VANDEN BROECK *et al*, 1996
Scg-AST-3	ATGAASLYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-4	GPRTYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-5	GRLYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-6	ARPYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-7	AGPAPSRLYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-8	EGRMYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-9	PLYGGDRRFSFGLa	VANDEN BROECK *et al*, 1996
Scg-AST-10	APAEHRFSFGLa	VEELAERT *et al.*, 1996; VANDEN BROECK *et al*, 1996
Scg-AKH-1	pELNFTPNWGTa	STONE *et al.*, 1976
Scg-AKH-2	pELNFSTGWa	SIEGERT *et al.*, 1985
Scg-MT-1	GAAPAAQFSPRLa	VEELAERT *et al.*, 1997b
Scg-MT-2	TSSLFPHPRLa	VEELAERT *et al.*, 1997b
Scg-CCAP	PFCNAFTGCa	VEELAERT *et al.*, 1997a
Scg-FLRFamide	PDVDHVFLRFa	ROBB *et al.*, 1989

In this paper we describe the isolation and partial identification from *S. gregaria* of a novel myotropin that shows no relevant sequence similarity with any known vertebrate or invertebrate neuropeptide.

MATERIAL AND METHODS

Animals, tissue extraction, and purification

S. gregaria was raised under laboratory conditions (ASHBY, 1972). Brains and adjacent retrocerebral complexes (7000) from 12-14-day adults were dissected and immediately placed in an ice-cold methanol/water/acetic acid (90:9:1) solution. The tissues were sonicated and centrifuged for 30 min (10,000 *g*; 4°C). Methanol was evaporated and the remaining aqueous residue was re-extracted with ethylacetate and n-hexane to remove the bulk of lipids. The organic solvent layer was decanted and the aqueous solution was dried in siliconized round-bottom flasks. Subsequently, it was dissolved in aqueous trifluoroacetic acid

(TFA) (0.1 %) and pre-purified on Megabond Elute C18 cartridges (10g/cartridge) (Varian, Harbor City, CA) that had been activated with $CH_3CN/H_2O/TFA$ (80:19.9:0.1) and then rinsed with 0.1 % TFA. The cartridges were eluted with 25 ml of 50 % and 80 % CH_3CN in 0.1 % TFA. Columns and operating conditions for high performance liquid chromatography (HPLC) on a Gilson HPLC system with variable wavelength detector (214 nm) were: (1.) Deltapak C18 column (25 x 100 mm) (Waters Associates, Milford, MA), solvent A: 0.1 % TFA in water; solvent B: 50 % CH_3CN in 0.1 % aqueous TFA. Column conditions: 100 % A for 8 min, followed by a linear gradient to 100 % B in 150 min; flow rate: 6 ml/min; detector range: 1 absorption unit full scale (Aufs); (2.) Protein C4 column (4.6 x 250 mm) (Vydac, Hesperia, CA), solvent A: 0.1 % TFA in water; solvent B: 50 % CH^3CN in 0.1 % aqueous TFA. Column conditions: 100 % A for 20 min, followed by a linear gradient to 100 % B in 50 min; flow rate: 1 ml/min; detector range: 0.5 Aufs; (3.) Phenyl spheri-5 column (4.6 x 250 mm) (Brownlee, Applied Biosystems, Foster City, CA), solvent A: 15 % CH_3CN in 0.1 % aqueous TFA; solvent B: 65 % CH_3CN in 0.1 % aqueous TFA. Column conditions : 100 % A for 20 min, followed by a linear gradient to 100 % B in 50 min; flow rate: 1.5 ml/min; detector range: 0.5 Aufs; (4.) Protein and Peptide C18 (4.6 x 250 mm) (Vydac, Hesperia, CA), solvent A: 10 % CH_3CN in 0.1 % aqueous TFA; solvent B: 40 % CH_3CN in 0.1 % aqueous TFA. Column conditions: 100 % A for 10 min, followed by a linear gradient to 100 % B in 60 min; flow rate: 1.5 ml/min; detector range: 0.5 Aufs; (5.) Microsorb-MV C18 (4.6 x 250 mm) (Rainin Instruments Co., Woburn, MA), solvent A: 15 % CH_3CN in 0.1 % aqueous TFA; solvent B: 50 % CH3CN in 0.1 % aqueous TFA. Column conditions: 100 % A for 20 min, followed by alinear gradient to 100 % B in 60 min, flow rate; 1ml/min; detector range: 0.2 Aufs.

Mass spectrometry and peptide sequencing

A sample containing 0.5-1 pmol/µl of the active peak was subjected to Maldi-TOF analysis (HILLENKAMP *et al.*, 1991). One µl was mixed with 1 µl of a 50 mM solution of α-cyano-4-hydroxycinnamic acid in CH_3CN:EtOH (50:50) containing 0.1 % TFA and applied on the multi-sample target. This mixture was air-dried and the target was then introduced in the instrument, a Micromass Tofspec (Micromass, Wythenshawe, UK) equipped with a N_2-laser (337 nm). The samples were measured either in the linear (acceleration voltage 24 kV) or in the reflectron mode (acceleration voltage 24 kV, reflectron voltage 28.6 kV). In either case, the laser energy was reduced until an optimal resolution and signal/noise ratio was obtained. The results of 20 to 50 shots were averaged to obtain the final spectrum. Automated amino acid sequencing was performed on a Beckman LF3600TC gas-phase protein sequencer (Beckman, Fullerton, CA) according to the Edman degradation principle.

Myotropic bioassay

The myotropic bioassay was performed as described by SCHOOFS *et al.* (1993). Hindguts of *Leucophaea maderae* were dissected under Ringer solution. Each hindgut was placed in an assay chamber filled with Ringer solution and attached to a transducer device, which allows recording of the frequency and of the amplitude of muscle contractions.

Ringer solution containing a purified material was applied to the bioassay chamber with the isolated hindgut of *L. maderae*.

RESULTS

Passage through two Megabond Elute columns was used to pre-purify the brain extract containing 7000 equivalents. The material eluting with 50% CH_3CN showed myotropic activity. During the HPLC purification process all fractions were tested in the myotropic assay. The fraction eluting at 66-72 min on the first HPLC column showed myotropic activity (Fig. 1A.). This fraction was further purified and myotropic activity eluted subsequently at 43 min on the second column (Fig. 1B), 38 min on the third column (Fig. 1C), 46 min on the fourth column (Fig. 1D) and at 45 min on the fifth column (Fig. 1E). After the fifth column purification step, the peptide showed apparent homogeneity. The mass spectrum is shown in Fig. 2. Only one molecular ion is present with mass of 968.3 Da.

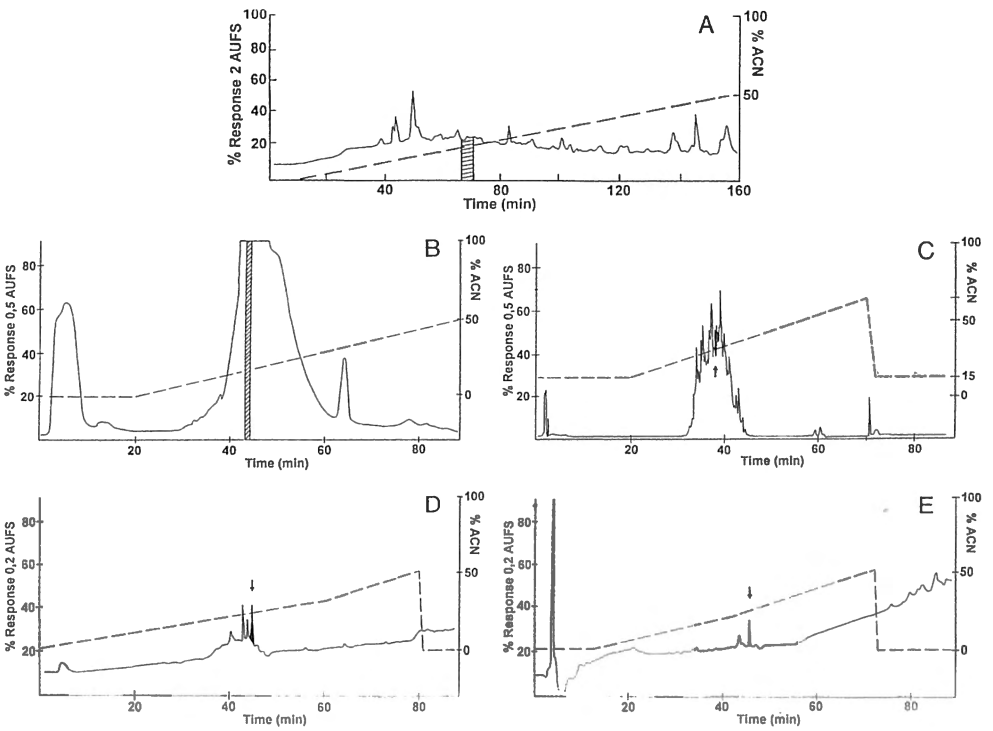

Fig. 1. – Purification of brain extract of *Schistocerca gregaria*. **A**: Fractionation on Deltapak C18 column. Fraction eluting at 66-72 min (shaded part) shows myotropic activity. **B**: Second fractionation on a Protein C4 column. The fraction eluting at 43 min contained the myotropic activity (shaded part). **C**: Third fractionation on a Phenyl spheri-5 column. Myotropic activity eluted at 38 min (Black peak and arrow). **D**: Fourth fractionation on a Protein and Peptide C18 column. Myotropic activity eluted at 46 min (Black peak and arrow). **E**: Final purification on a Microsorb-MV C18 column. Myotropic activity eluted at 45 min (black peak and arrow).

This may represent (a) a protonated peptide ion $(M+H)^+$. In that case the presence of a minor peak at 984.6 may represent an oxidized form of the peptide. Alternatively (b) the 984.6 peak may be indicative of a potassium adduct, in which case the 968.3 Da compound may represent the sodium adduct $(M+Na)^+$. This will also mean that the $(M+H)^+$ ion is not seen in the MALDI spectrum. This phenomenon, however, has previously been observed with insect neuropeptides (VERHAERT & DE LOOF, 1998 ; VERHAERT *et al.*, 1998). The molecular mass of the peptide will be 967.3 in case a and 945.3 in case b.

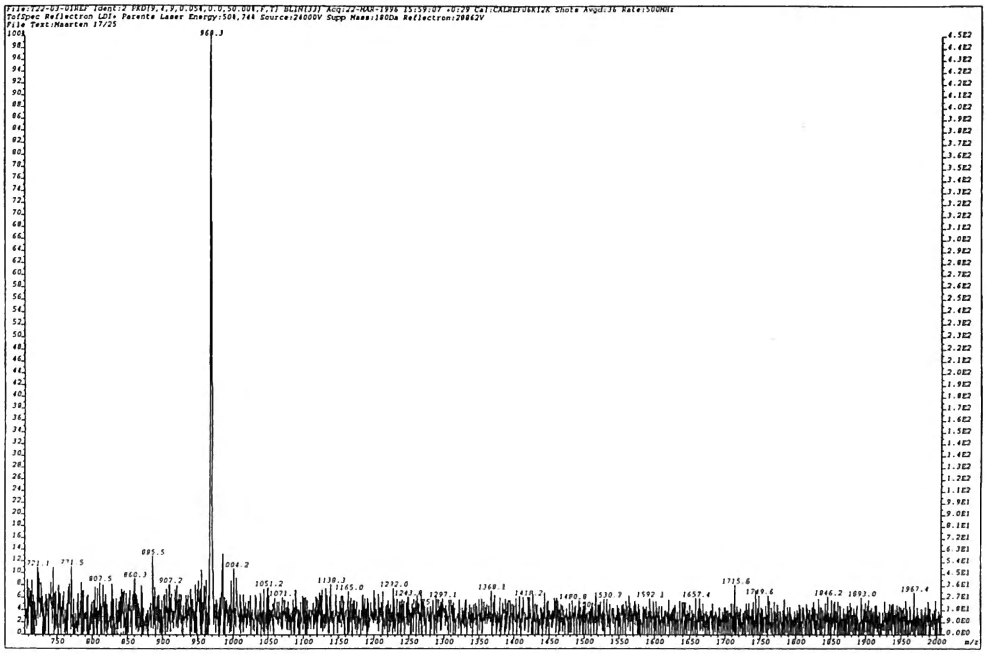

Fig. 2. – MALDI-TOF mass spec spectrum of Scg-MST showing a single ion species of m/z 968.3 Da.

Fig. 3 shows the effect of 20 brain equivalents of the purified peptide. Its activity is comparable to the effect of the schistomyotropins and the locustamyotropins on the hindgut of *L. maderae*. The peptide was sequenced by Edman degradation and a partial sequence was obtained : DRSW?GPK?. The peptide is called schistomyostimulin (Scg-MST).

DISCUSSION

Five HPLC runs were needed to isolate a myotropic peptide from the nervous system of the desert locust, *S. gregaria*. After final purification, 20 brain equivalents still showed myotropic activity on the hindgut of *L. maderae*. Maldi-TOF spectrometry revealed an ionic mass of 968.3 Da presumptive $(M+H)^+$ if not $(M+Na)^+$. Due to the low amount of purified material only a partial sequence was obtained : DSRW?GPK(?) (? : missing amino

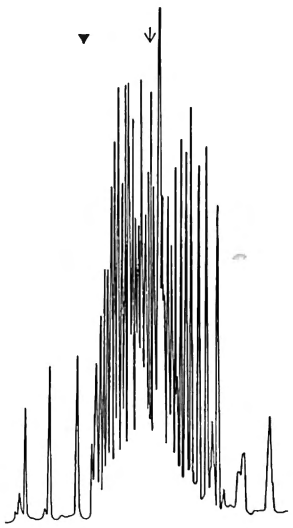

Fig. 3. – Myotropic assay on the hindgut of *Leucophaea maderae*. Response of the hindgut to 20 equivalents of purified Scg-MST. Arrow-head, application of Scg-MST; arrow, rinse with saline; 1cm horizontal axis is 1min.

acids). This partial sequence has to our knowledge no relevant similarities with any known amino acid sequence. The mass 967.3 or 945.3 Da should enable us to calculate the missing amino acid(s) of myostimulin. Unfortunately no obvious amino acid combination fits this mass. This could be explained if (one of) the missing amino acid(s) were modified which would agree with the observation that no relevant signal was observed at the respective blank cycle(s) during Edman degradation reactions.

Of all known insect regulatory peptides, myotropins are by far the largest group. In *S. gregaria*, two myotropic peptides, the schistomyotropins (Scg-MT-1 and -2) have been identified previously. They are closely related to the locustamyotropins (Lom-MT-1 till -4) and the locustapyrokinins (Lom-PK-1 and -2) (SCHOOFS *et al.*, 1997). All those peptides belong to the -FXPRLamide peptide family (X being Ser, Val, Thr, Gly), which occurs widely among insects. This pentameric carboxyterminal sequence is also found in the pheromone-biosynthesis-activating peptide of *Heliothis zea* (Hez-PBAN: RAINA *et al.*, 1989) and *Bombyx mori* (Bom-PBAN I and II: KITAMURA *et al.*, 1989,1990) as well as in the melanization and reddish-coloration hormone of *Pseudaletia separata* (Pss-MRCH: MATSUMOTO *et al.*, 1992). The same C-terminal -FXPRLamide motif is also found in the diapause hormone of *B. mori* (Bom-DH: IMAI *et al.*, 1991). Two adipokinetic hormones, Scg-AKH-1 and Scg-AKH-2 were isolated from *S. gregaria* (STONE *et al.*, 1976). These hormones induce the mobilization of lipids from the fat body (ORCHARD & LANGE, 1983) and induce the synthesis of flight-specific lipophorins (VAN DER HORST *et al.*, 1979). Lom-AKH-$1^{14\text{-}10}$, most probably a degradation product of Lom-AKH-1, is a stimulator of visceral muscle contractions (SCHOOFS *et al.*, 1993).

By screening chromatographically-purified fractions in a bioassay to measure adipokinetic hormone release, a peptidergic, adipokinetic, hormone-releasing factor (Scg-CCAP) was isolated from 7000 brains of the dessert locust *S. gregaria*. Scg-CCAP stimulates the release of adipokinetic hormone in a dose-dependent manner in *S. gregaria* (VEELAERT *et al.*, 1997a). The peptide was first isolated from the shore crab, *Carcinus maenas*, as a cardioactive peptide, CCAP (STANGIER *et al.*, 1987). Later, CCAP was isolated from *Locusta migratoria* by affinity chromatography (STANGIER *et al.*, 1989).

The locusts *L. migratoria* and *S. gregaria* are the insect species from which the largest number of myotropins (including Scg-MST) have been isolated and sequenced (SCHOOFS *et al.*, 1997 ; VEELAERT *et al.*, 1998).

ACKNOWLEDGEMENTS

We are grateful to Mr. Roger Jonckers for insect rearing, J. Vanden Broeck, Marijke Christiaens and J. Puttemans for technical assistance. D. Veelaert is a recipient of a post-doctoral fellowship of the K.U. Leuven Research Council. This research is part of E.U. FAIR CT96-1648, FWO G.0264.96 and F.K.F.O. 2.0173.94. The authors wish to thank the Fund for Scientific Research of Flanders (F.W.O.) for protein sequencing equipment.

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