

## Hypertrehalosaemic insect peptide periplanetin CC-2 and its analogues: Synthesis and biological evaluation

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### Insect adipokinetic peptides, hypertrehalosaemic peptides, *Tenebrio molitor*, new periplanetin CC-II analogues, Tem-HrTH peptide

**Abstract.** The effects of hypertrehalosaemic octapeptides (peptides from AKH family) on transcription and trehalosegenesis in the fat body of adult females of *Tenebrio molitor* L. were examined. A synthetic hypertrehalosaemic peptide from CC of *Periplaneta americana* periplanetin CC-2 (Pea-CAH-II) Glp-Leu-Thr-Phe-Thr-Pro-Asn-Trp-amide (1), its new synthetic analogues Glp-Leu-Thr-Phe-Thr-Pro-Asn-Phe-amide (2), Glp-Leu-Thr-Phe-Thr-Pro-Asn-D-Trp-amide (3), Glp-Leu-Thr-Phe-Thr-Pro-Asn-D-Phe- amide (4), and an octapeptide from *T. molitor* Glp-Leu-Asn-Phe-Ser-Pro-Asn-Trp-amide (Tem-HrTH) (5) were tested. Peptides 2, 3 and 5 suppress transcription in the fat body tissue measured as incorporation of [<sup>3</sup>H]-uridine into total RNA. The rate of RNA inhibition in the tissue depends on the peptide concentration. Periplanetin CC-2 (2) was inactive in this test. Endogenic *T. molitor* octapeptide (5) stimulates trehalose release from the fat body of females, whereas Pea-CAH-II analogues (2–4) do not increase trehalose release from this tissue.

### INTRODUCTION

Neuropeptides from the AKH/RPCH family, hypertrehalosaemic peptides included, originating in the CC of insects, show hypertrehalosaemic and hypolipaemic properties. These peptides stimulate trehalose and diglycerides release from insect fat body, e.g. AKH-I (from *Locusta migratoria*), periplanetin CC-2 (from *Periplaneta americana*) and Tem-HrTH (from *Tenebrio molitor*) (Konopińska et al., 1987, 1989; Rosiński & Gäde, 1988). Recently, it was established that neuropeptides from the AKH/RPCH family can inhibit the protein synthesis in the fat body of some insect species (Moshitzky & Applebaum, 1990; Carlisle & Loughton, 1986; Cusinato et al., 1991). Several processes are involved in the reproduction cycle in insects, such as vitellogenic protein synthesis, oocyte growth, maturation, and egg oviposition are under neuroendocrine regulation (Carlisle & Loughton, 1986; Moshitzky & Applebaum, 1990; Cusinato et al., 1991). The functional ability of insect ovaries depends indirectly on the fat body trophic activity and the hormonal control of a number of biochemical processes in this tissue from which large quantities of proteins are delivered to developing oocytes (Moshitzky & Applebaum, 1990). In *Tenebrio molitor*, the synthesis of vitellogenic proteins in the female fat bodies begins at the

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pharate pupal stage, (Mordue, 1965; Laverdure, 1972; Gerber, 1975). The characteristic female proteins stored in this tissue are released after adult ecdysis (Laverdure, 1972; Gerber, 1975). The inhibition of protein synthesis in the fat body of insects during oocyte maturation by AKH family peptides makes them useful candidate as insecticides for the control of reproduction in insects. The investigation of the physiological effects of endogenic peptides, such as periplanetin CC-2, Tem-HrTH, and synthetic analogues, can be helpful in the application of hypertrehalosaemic hormones for insect control. Continuing past investigations (Konopińska et al., 1987, 1989; Rosiński & Gäde, 1988) we performed further studies on the insect hypertrehalosaemic peptide, periplanetin CC-2, Glp-Leu-Thr-Phe-Thr-Pro-Asn-Trp-amide (1) (Pea-CAH-II) and its analogues including Glp-Leu-Thr-Phe-Thr-Pro-Asn-Phe-amide (2), Glp-Leu-Thr-Phe-Thr-Pro-Asn-D-Trp-amide (3), Glp-Leu-Thr-Phe-Thr-Pro-Asn-D-Phe-amide (4) and Tem-HrTH – Glp-Leu-Asn-Phe-Ser-Pro-Asn-Trp-amide (5), last of which originates in the corpora cardiaca of *T. molitor* (Gäde & Rosiński, 1990). The aim of this work was to study the effect of the peptides 1–5 above on RNA transcription and trehalogenesis in the fat body during oocyte maturation of female *T. molitor*. Also, investigation of the structure/function relationship was performed to estimate the role of the C-terminal Trp-residue of periplanetin CC-2 in its biological activity. The subjects of these investigations were Pea-CAH-II analogues (2–4), where the C-terminal Trp was replaced by other aromatic amino acids, such as Phe (2), D-Trp (3), or D-Phe (4).

## MATERIAL AND METHODS

### Synthesis of peptides

Peptides 1–5 were synthesized by the solid-phase method. The C-terminal amino acid derivatives (Boc-Trp-OH, Boc-D-Trp-OH, Boc-Phe-OH and Boc-D-Phe) were connected to chloromethylated classical Merrifield resin containing 0.7–0.8 mmol of Cl/g resin by the standard cesium salt procedure. Protected amino acids were coupled to the resin with DCC in the presence of HOBt as a coupling reagent. DCM was used as a solvent. The following amino acid derivatives (Bachem, USA) were used: Z-Glp-OH, Boc-Thr(OBzl)-OH, Boc-Leu-OH, Boc-Asn-ONp, Boc-Phe-OH, Boc-D-Phe, Boc-Ser(Bzl)-OH, Boc-Pro-OH.

Boc-blocked  $\alpha$ -amino groups were deprotected with 30% TFA in DCM according to the general method. Neutralization was performed with 10% TEA in DCM. Protected heptapeptide amides were cleaved from the support resin with 20%  $\text{NH}_3$  in methanol and deprotected with TFA in DCM and then connected with Z-Glp-OH by liquid phase method in the presence of DCC. The final products were obtained by catalytic hydrogenation in the presence of 10% Pd/C. All peptides were desalted with Amberlit CG-4B and then purified on a Sephadex G-25 column, with 5% acetic acid as eluent, according to Konopińska et al. (1987). Final purification was carried out by preparative HPLC. Purity and homogeneity of the free peptides was established by analytical HPLC, amino acid analysis, TLC, and optical activity.

Physico-chemical data is summarized in Table 1.

Amino acid composition was performed on an AAA-1081 analyzer (Czech Republic). The optical activity of the compounds was determined on a Polamat (C. Zeiss, Jena, Germany) polarimeter.

Purity and homogeneity of all final products were checked by TLC on silica gel, amino acid analysis, and analytical HPLC.

Purification of free peptides was performed on preparative HPLC using a Beckman System Gold instrument with an ODS column (ODS 10  $\times$  250 mm), with detection at 220 nm.

### Insects

*Tenebrio molitor* (Coleoptera: Tenebrionidae) was maintained in the laboratory and reared as described by Rosiński et al. (1979). Pupae were sexed on the basis of differences in their genitals.

#### Fat body incubations with [<sup>3</sup>H]uridine and [<sup>14</sup>C]leucine

Adult females of *T. molitor* 3–5 days old were anesthetized with CO<sub>2</sub>. The fat bodies were dissected and incubated in 200 µl volume of saline based medium according to Rosiński & Gäde (1988) supplemented with 0.5% bovine serum albumine at 27°C for 3 h. The incorporation of [<sup>3</sup>H]uridine (5µCi/200 µl medium) into total RNA was estimated in 0.5N HClO<sub>4</sub> tissue extracts after removing unincorporated isotope by washing tissue precipitate in 0.3 N HClO<sub>4</sub>. The rate of uridine incorporation in the presence and absence of Tem-HrTH or analogues in the incubation medium was expressed in cpm per mg fat body protein. Protein concentration was measured according to Bradford (1976).

The rate of [<sup>14</sup>C]leucine incorporation into secreted or non-secreted proteins of the fat body was estimated for 12 h incubation in vitro in the medium described above. Radioactivity incorporated into proteins was determined according to the method of Mans & Novelli (1961). Results are presented in Table 1.

#### Fat body carbohydrate release in vitro

Fat bodies were dissected from 3–5 day-old females and incubated in vitro as outlined earlier (Rosiński & Gäde, 1988; Gäde & Rosiński, 1990). Carbohydrates released from the fat body into 1 ml of the incubation medium were measured using the phenol-sulphuric acid method of Dubois et al. (1956).

#### Abbreviations

The symbols of the amino acids, peptides and their derivatives are in accordance with the 1983 Recommendation of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [*Eur. J. Biochem.* **13**: 9 (1984)]. AKH – adipokinetic hormone; Asn – L-asparagine; Boc – tert-butyloxycarbonyl; Bzl – benzyl; CC – corpora cardiaca; DCC – dicyclohexylcarbodiimide; DCM – dichloromethane; Glp – pyroglutamic acid; Gly – glycine; HOBt – 1-hydroxybenzotriazole; Hr – hypertrehalosaemic; Leu – L-leucine; Np – p-nitrophenyl; Phe – L-phenylalanine; Pro – L-proline; RPCH – red pigment concentration hormone; Ser – L-serine; TEA – triethylamine; TFA – trifluoroacetic acid; Thr – L-threonine; Tyr – L-tyrosine; Trp – L-tryptophan; Val – L-valine.

TABLE 1. Physico-chemical data of peptides (1–5).

Peptide	Yield (%)	[α] <sub>D</sub> <sup>20</sup> c = 0.1 methanol	Rt <sub>HPLC</sub> *	R <sub>f</sub> **			Amino acid analysis
				X	Y	Z	
Glp-Leu-Asn-Phe-Ser-Pr o-Asn-Trp-amide, Pea-CAH-II (1)	42	-31.6	39.87	0.73	0.85	0.56	Asn 1.05 Thr 2.30 Glp 1.09 Pro 1.1 Leu 1.08 Phe 1.20
Glp-Leu-Thr-Phe-Thr- Pro-Asn-Phe-amide, [Phe <sup>8</sup> ] Pea-CAH-II (2)	47	-43.3	33.66	0.72	0.83	0.57	Asn 0.92 Thr 1.85 Glp 0.89 Pro 0.90 Leu 0.91 Phe 1.83
Glp-Leu-Thr-Phe-Thr- Pro-Asn-D-Trp-amide, [D-Trp <sup>8</sup> ] Pea-CAH-II (3)	56	-51.4	32.45	0.77	0.86	0.47	Asn 0.98 Thr 1.98 Glp 1.03 Pro 0.94 Leu 1.01 Phe 0.96
Glp-Leu-Thr-Phe-Thr- Pro-Asn-D-Phe-amide, [D-Phe <sup>8</sup> ] Pea-CAH-II (4)	32	-17.7	37.45	0.77	0.85	0.60	Asn 1.02 Thr 2.10 Glp 0.87 Pro 0.81 Leu 0.97 Phe 2.20
Glp-Leu-Asn-Phe-Ser- Pro-Asn-Trp-amide, Tem-HrTH (5)	52	-36.4	37.89	0.81	0.77	0.23	Asn 2.12 Ser 1.09 Glp 1.03 Pro 0.99 Leu 1.01 Phe 1.05

\* HPLC on column ultrasphere ODS Beckman (250 mm × 4.6 mm, 223 nm) in gradient 0–100B (solvent B = 80% acetonitrile in water + 0.1% TFA) during 60 min.

\*\* TLC silica gel plates (Merck prod.) as eluents were used: X = n-butanol : acetic acid : ethyl acetate : water 1 : 1 : 1 : 1, Y = n-butanol : acetic acid : pyridine : water 30 : 20 : 6 : 24, Z = n-butanol : acetic acid : water 4 : 1 : 1.

## RESULTS AND CONCLUSIONS

In *T. molitor*, a rapid growth of ovaries can be observed starting 3–4 days after emergence of the adult female. The first gonotrophic cycle in virgin females proceeds until day 9 and does not repeat in further development (Michalik et al., 1996). The growth and development of oocytes is dependent on the deposition of a considerable amount of proteins, mainly vitellins, synthesized in the fat body. These proteins, released to haemolymph and transported to ovaries, are finally accumulated in the oocytes. All steps of vitellogenesis must be precisely controlled and synchronized. To maintain a normal rate of protein synthesis in the fat body, the neuroendocrine system (brain-CC-CA complex) and feeding stimuli are necessary. The incorporation of [<sup>14</sup>C]leucine into secreted, as well as non-secreted, proteins measured in fat body in vitro after insect decapitation at day 6 or after 6-day starvation was only 10% of the control (Fig. 1). These data show the importance of both feeding and the presence of neurohormones for efficient protein synthesis in the fat body.

We also examined the possible influence of some hypertrehalosaemic peptides on protein synthesis in the fat body of adult virgin females. The results illustrating the incorporation of radioactive aminoacids into fat body proteins were inconsistent and difficult for interpretation, so we decided to characterize the above influence on the transcription level as it was observed in *Blaberus discoidalis* fat body by Lee & Keeley (1994) and in *Locusta migratoria* fat body by Kodrik & Goldsworthy (1995).

The effect of some synthetic peptides on transcription in the fat body of *Tenebrio* females was examined. Native *Tenebrio* octapeptide, Tem-HrTH, inhibits [<sup>3</sup>H]uridine incorporation into total RNA in a dose-dependent manner (Fig. 2). Almost 60% inhibition at  $5 \times 10^{-5}$  M concentration can be observed in the fat body from 3–5 days old adult females. A related peptide from *P. americana* cockroach, Pea-CAH-II, is inactive in the bioassay up to  $5 \times 10^{-4}$  M concentration (Table 2). It is structurally different from the Tem-HrTH octapeptide; it has Thr3 and Thr5 residues instead of Asn3 and Ser5 in its peptide sequence. However, the Pea-CAH-II analogues, in which the residue L-Trp was changed for D-Trp or Phe in position 8 of the peptide chain, showed an inhibition of RNA synthesis

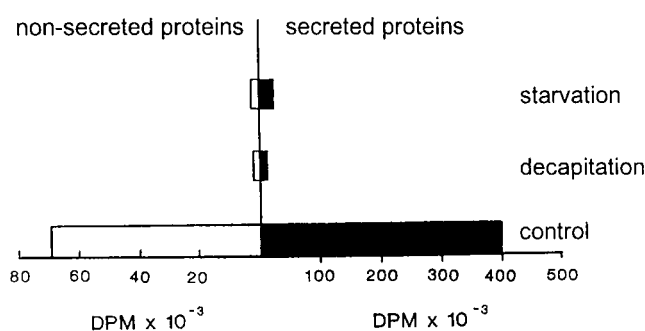


Fig. 1. The effects of decapitation and starvation on the rate of protein synthesis in the fat body of an adult virgin female of *T. molitor*. Fat bodies were dissected from 3–5 days old females and were incubated for 12 h at 27°C. Radioactivity in secreted proteins was determined in TCA precipitates. Radioactivity in non-secreted proteins was determined in KOH hydrolyzates after HClO<sub>4</sub> precipitation and washing. Values represent means for 5–6 assays.

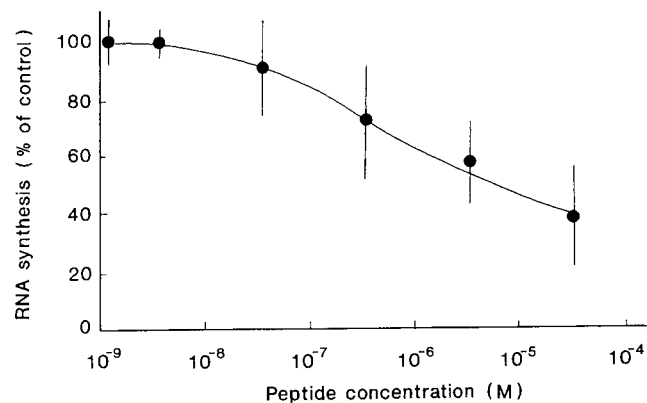


Fig. 2. The incorporation of [<sup>3</sup>H]uridine into RNA during the incubation of the fat body with different Tem-HrTH concentrations. Fat bodies were dissected from 3–5 days old virgin females and incubated for the period of 3 h. Values represent means ± SEM for 4–14 assays.

similar to that of *Tenebrio* peptide. However, replacement of the C-terminal L-Trp residue in the Pea-CAH-II molecule with phenylalanine in the D form results in the loss of ability of the analogue to influence transcription in the fat body.

TABLE 2. The incorporation of [<sup>3</sup>H]uridine into total RNA during the incubation of the female fat body with different concentration of Pea-CAH-II and its analogues.

Name of peptide	Peptide concentration (M)				
	5 × 10 <sup>-8</sup>	5 × 10 <sup>-7</sup>	5 × 10 <sup>-6</sup>	5 × 10 <sup>-5</sup>	5 × 10 <sup>-4</sup>
Pea-CAH-II (1)	–	69 ± 14	66 ± 25	78 ± 19	81 ± 13
[Phe <sup>8</sup> ]-Pea-CAH-II (2)	–	98 ± 13	80 ± 21	–	–
[D-Trp <sup>8</sup> ]-Pea-CAH-II (3)	–	76 ± 25	77 ± 12	60 ± 23	40 ± 18
[D-Phe <sup>8</sup> ]-Pea-CAH-II (4)	80 ± 24	68 ± 27	79 ± 30	–	–

Data represents % control synthesis. Fat bodies were dissected from 3–5 days old virgin females and were incubated in vitro for the period of 3 h. The values shown are the means ± SEM for 4–14 assays. Significant values are marked with asterisks (test t-Students).

Endogenic *Tenebrio* peptide also stimulates the release of trehalose from the fat body of females in vitro, whereas Pea-CAH-II octapeptide is weak trehalosaemic agonist (Fig. 3).

It was shown in several papers that three conservative amino acid residues in the AKH/RPCH peptides, Glp<sup>1</sup>, Phe<sup>4</sup>, and Trp<sup>8</sup> are particularly important to maintain their biological activity (Ford et al., 1988; Gäde & Hayes, 1990; Hayes et al., 1990). The replacement of the amino acid in position 8 in the Bld-HrTH decapeptide with L-leucine or L-serine causes the loss of hypertrehalosaemic activity of such analogues in cockroaches, *Blaberus discoidalis* (Hayes et al., 1990) and *P. americana* (Gäde & Hayes, 1990). Three analogues of Pea-CAH-II were inactive in the bioassay. Wheeler et al. (1990) suggested that the biological activity of several AKH/RPCH peptides was connected with an existence of β-turn between amino acid residues 5–8 of the chain, including Pea-CAH-II

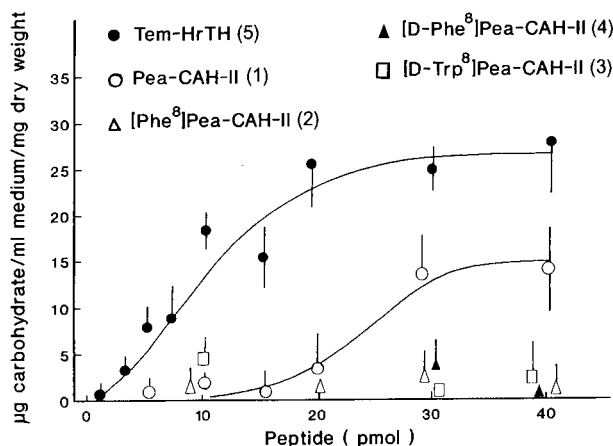


Fig. 3. The release of carbohydrate in response to Tem-HrTH, Pea-CAH-II and Pea-CAH analogues by the in vitro fat body from 3 days old females. The tissue was incubated in 1 ml medium at 30°C. The amount of tissue was determined by dry weight measurement. Values are given as means of 5–6 determinations  $\pm$  SEM.

octapeptide. It seems that in the Pea-CAH-II peptide the heterocyclic indole ring of Trp<sup>8</sup> can interact with the Phe<sup>4</sup> aromatic ring to stabilize the conformation of the molecule or perhaps coordinate the two rings to interact with a receptor aromatic amino acid residues. The data obtained in this work indicate that all three types of structural modifications introduced at the C-terminal of the Pea-CAH-II chain are particularly critical for the trehalosegenic activity of analogues in *Tenebrio*. However, the same structural changes have qualitatively different meanings with regard to the analogues influence on RNA synthesis in fat body. The trehalosegenically inactive [D-Trp<sup>8</sup>]-Pea-CAH-II analogue, where native Trp was substituted by its D-enantiomer in the Pea-CAH-II chain, has the ability of inhibition of the RNA synthesis ( $60 \pm 23$  and  $77 \pm 12\%$  of the control, for the peptide in concentrations of  $5 \times 10^{-5}$  and  $5 \times 10^{-6}$  moles, respectively). Above substitution in peptide molecule shows an opposite effect to native Pea-CAH-II (Konopińska et al., 1987, 1989).

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