Amino Acid Composition of Cockroach Hypertrehalosaemic Hormones

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Hypertrehalosaemic hormones I and II from the corpus cardiacum of the American cockroach (*Periplaneta americana*) were separated by reversed-phase high-performance liquid chromatography using a Nucleosil C_{18} column with a trifluoroacetic acid/acetonitrile gradient. The eluent was monitored at 206 nm and the hypertrehalosaemic activity detected by bioassay. The amino acid compositions of hypertrehalosaemic hormone I and II were determined after acid hydrolysis with HCl or methanesulfonic acid. Both neurohormones are octapeptides. Hypertrehalosaemic hormone I contained the amino acids $Asp_{(2)}$, Ser, Glu, Pro, Val, Phe and Trp, whereas hypertrehalosaemic hormone II contained the amino acid residues Asp, $Thr_{(2)}$, Glu, Pro, Leu, Phe and Trp.

The presence of a factor in the corpora cardiaca of the American cockroach, *Periplaneta americana*, capable of elevating blood sugar levels in this species was demonstrated more than 20 years ago [1]. The factor was termed "hyperglycaemic hormone" despite the fact that it is usually the disaccharide trehalose that is affected by the corpus cardiacum extract and despite the lack of evidence either of release under physiological conditions or of a clear physiological role [2]. Various groups have attempted the isolation of the hypertrehalosaemic hormone but there is little precise information about its chemical nature; its structure is not known and no definite amino acid composition can be given [3].

All studies on the mode of action of this neuropeptide have been undertaken using crude extracts of corpora cardiaca or, at best, partially purified material [4–6]. The results achieved are not conclusive because of the possible contamination of the injected extracts with other material.

Recently, we have shown that extracts of cockroach corpora cardiaca separate on a reversed-phase HPLC C_{18} column and contain two absorbance peaks which have hypertrehalosaemic activity [7]. In the present study an attempt was made to purify these two peptides and determine their amino acid composition.

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Materials and Methods

Insects

Adult (male and female) cockroaches (*Periplaneta americana*) were supplied by Prof. Dr. Hansen (Universität Regensburg), Prof. Dr. Walther (Universität Ulm) and Fa. Thompson (Düsseldorf). The animals were kept in our laboratory at about 24 °C with a LD, 14:10 h cycle and fed with bran and dog flakes supplemented with fresh carrots.

Extraction of hormone and reversed-phase HPLC

50 pairs of corpora cardiaca were routinely dissected from the heads of *P. americana* for one HPLC analysis and prepared for HPLC as described previously [8]. The active material was dissolved in 50 µl of 2 M acetic acid and applied to a Nucleosil C_{18} column (dimensions: *i.d.* 4.6 mm, length 250 mm) with 7 µm particle size material. The HPLC chromatography system consisted of two Model 302 piston pumps with 5 S pump head, a manometric modul Model 802, a Model 811 mixing chamber (all Gilson, Inc.), a Rheodyne Model 7125 sample injector with a 50 µl sample loop, a LKB 2158 Uvicord SD equipped with a 206 nm filter as detector (8 µl HPLC flow cell; 2.5 mm pathlength) and a Gilson Microcol TDC 80 fraction collector. An Apple II plus microcomputer was used to control the pumps and to build up the desired gradient using the Gilson Gradient Program software. Aqueous solvent (solvent A) was 0.11% trifluoroacetic acid (Merck, Uvasol); solvent B was 0.10 trifluoro-

acetic acid in 60% acetonitrile (Roth; HPLC grade): this was applied as a linear gradient (25–80% B in 45 min) with a flow rate of 0.9 ml/min. For re-chromatography of bioactive fractions a linear gradient from 40 to 65% B in 22.5 min was chosen with a flow rate of 1.0 ml/min. The eluent was monitored at 206 nm at a sensitivity of 0.1 absorbance units full scale and recorded with an LKB Model 2110 one-channel recorder. The fractions were lyophilized by vacuum centrifugation (Savant speed-vac), resuspended in bidistilled water as required, and used for the carbohydrate bioassay.

Bioassay

Hypertrehalosaemic activity was tested by injection of the appropriate material after HPLC into male cockroaches as described earlier [9]. Carbohydrates were quantified as anthrone-positive material according to previously published methods [10, 11].

Amino acid analysis

Lyophilized fractions after HPLC containing 200 to 400 pmol were taken up into 50 µl of 5.7 M HCl and hydrolyzed in N_2-flushed evacuated and sealed glass tubes for 24 or 48 h at 110 °C. Amino acid analyses were performed with a Biotronik LC 500 (at Bonn) or a Beckman Model 121 M (at Köln) analyzer using ninhydrine detection. For tryptophan detection, the method was as previously outlined [8].

Results

When a crude methanolic extract of 50 pairs of cockroach corpora cardiaca is subjected to reversed-phase HPLC, the two major peaks of absorbance at 206 nm correspond with those fractions showing hypertrehalosaemic activity in the bioassay (Fig. 1 B). Both peaks have different retention times to the known arthropod neuropeptides locust adipokinetic hormone (AKH) and the crustacean red pigment-concentrating hormone (RPCH) which were used as standards (Fig. 1 A). The peak that elutes with a retention time of 27.4 min in our system has been called hypertrehalosaemic hormone I, whereas the peak with a retention time of 32.8 min is designated as hypertrehalosaemic hormone II. The optical density ratio of hypertrehalosaemic hormone I:II at 206 nm is typically about 3:1. When the peak I and peak II material from the first HPLC run were individually re-chromatographed on HPLC, we used a system with a shallower gradient. In this system our standard peptides, AKH and RPCH, had retention times of 18.3 and 20.0 min (Fig. 2C). The peak I hypertrehalosaemic hormone material showed two small and one larger absorbance peaks at 206 nm but only the latter peak with a retention time of 14.0 min, possessed hypertrehalosaemic activity (Fig. 2A). There were also two minor and one major absorbance peaks when the peak II hypertrehalosaemic hormone material was re-chromatographed (Fig. 2B). Again, only the major peak with a retention time of 20.9 min had hypertrehalosaemic activity.

Table I. Amino acid composition of hypertrehalosaemic hormones I and II from the corpus cardiacum of the American cockroach after reversed-phase high-performance liquid chromatography. Values are given as molar ratios to Glu = 1. Amino acid residues not listed had a molar ratio < 0.30; n.d. = not detectable in this system.

Detected amino acids	Hypertrehalosaemic hormone I			Hypertrehalosaemic hormone II		
	Hydrolysis in		Composition	Hydrolysis in		Composition
	HCl	MSA [a]		HCl	MSA [a]	
Asp	1.68	1.71	2	0.84	0.83	1
Thr	–	–	–	1.55	1.79	2
Ser	0.83	0.78	1	–	–	–
Glu	1.00	1.00	1	1.00	1.00	1
Pro	0.80	0.83	1	0.98	0.99	1
Val	0.89	0.89	1	–	–	–
Leu	–	–	–	0.73	0.77	1
Phe	0.93	0.88	1	0.79	0.82	1
Trp	n.d.	0.63	1	n.d.	0.48	1

[a] MSA, methanesulfonic acid.

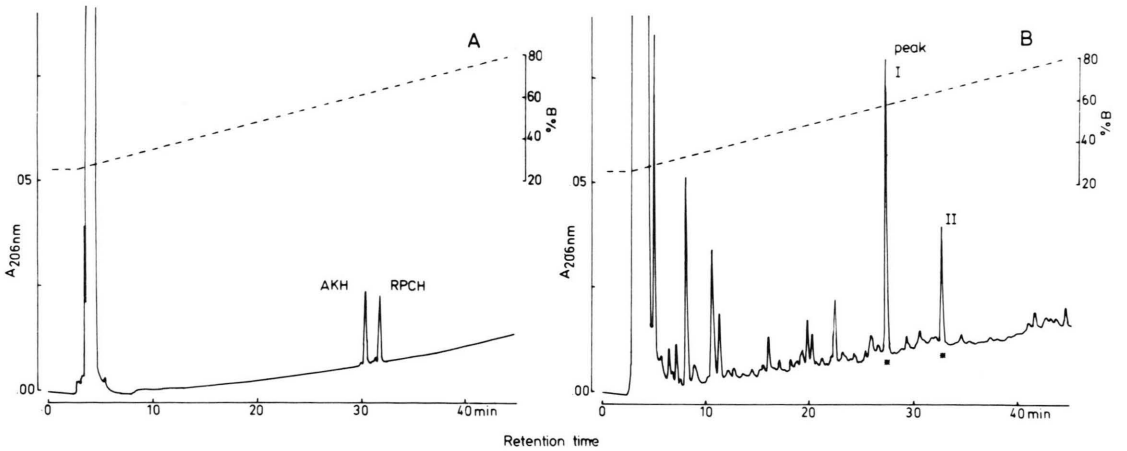

Fig. 1. The separation of hypertrehalosaemic hormones I and II from corpus cardiacum extract of *Periplaneta americana* using reversed-phase high-performance liquid chromatography. A. Separation of a mixture of 1 µg synthetic locust adipokinetic hormone (AKH) and crustacean red pigment-concentrating hormone (RPCH) as standards. B. Separation of a crude methanolic extract from 50 pairs of cockroach corpora cardiaca. The analysis was performed on a Nucleosil C_{18} column which was eluted with a linear gradient of 0.11% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in 60% acetonitrile (solvent B). The gradient ran from 25 to 80% B during 45 min at a flow rate of 0.9 ml/min. The elution was monitored at 206 nm. The fractions marked with an asterisk showed hypertrehalosaemic activity in the cockroach bioassay.

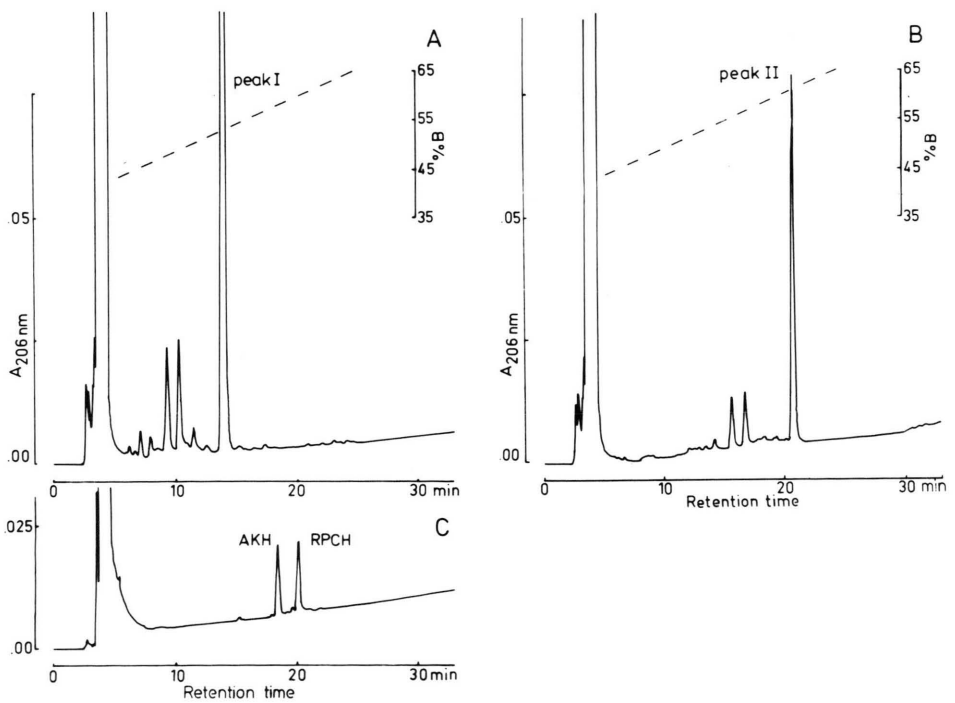

Fig. 2. Re-chromatography of hypertrehalosaemic hormone I (A) and II (B) peak material of 150 pairs of cockroach corpora cardiaca chromatographed prior on the HPLC system shown in Fig. 1. Additionally, separation of a mixture of 1 µg synthetic AKH and RPCH as standards is shown (C). The analysis was performed on a Nucleosil C_{18} column which was eluted with a linear gradient of 0.11% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in 60% acetonitrile (solvent B). The gradient ran from 40 to 65% B during 22.5 min at a flow rate of 1 ml/min. The elution was monitored at 206 nm.

Table II. Total haemolymph carbohydrate concentration in adult male American cockroaches before and 120 min after injection of distilled water and known amounts of purified hypertrehalosaemic hormone I and II, respectively. The values shown are the mean ± S.D. of three animals.

Treatment	Blood Carbohydrates [mg/ml]		
	0 min	120 min	Difference
Control			
10 µl distilled water	15.1 ± 3.7	16.4 ± 2.1	1.3 ± 2.6
Hypertrehalosaemic hormone I			
2 pmol	14.6 ± 2.2	32.9 ± 4.0	18.3 ± 4.6
10 pmol	17.2 ± 2.3	42.3 ± 6.1	25.1 ± 3.9
Hypertrehalosaemic hormone II			
2 pmol	14.6 ± 0.9	31.3 ± 4.3	16.7 ± 3.4
10 pmol	14.7 ± 4.4	49.7 ± 5.0	35.0 ± 5.1

The active materials from the second HPLC separations were individually lyophilized and aliquots used for amino acid analysis. At least two different extracts of corpora cardiaca were used as the starting material. Most preparations were not only analyzed after hydrolysis in 5.7 M HCl for 24 h and 48 h, but also after hydrolysis in 4 M methanesulfonic acid in order to detect tryptophan. The results of representative amino acid analyses are summarized in Table I. Cockroach hypertrehalosaemic hormone I contained the following amino acid residues: 2 Asp, Ser, Glu, Pro, Val, Phe and Trp, whereas hypertrehalosaemic hormone II had the following composition: Asp, 2 Thr, Glu, Pro, Leu, Phe and Trp.

The purified material from both hypertrehalosaemic hormones was used to quantify the bioassay on a molar basis. As is shown in Table II, both peptides produced a greater than 100% increase in blood carbohydrate concentration when as little as 2 pmol were injected.

Discussion

In contrast to various groups which have attempted the isolation of cockroach hypertrehalosaemic hormones by conventional separation methods [3], our reversed-phase HPLC system proved successful in the purification of two peptides with hypertrehalosaemic activity. Apparent homogeneity of both compounds was achieved by re-chromatography of the separated peptides after the first HPLC step and this was established by subsequent amino acid analyses. Both peptides are octapeptides and have a pronounced hypertrehalosemic effect in cockroaches when as little as 2 pmol is injected. Thus, we feel confident that we have purified the long known, but not characterized, hypertrehalosaemic hormones of the American cockroach.

However, peptides with identical amino acid compositions, but characterized as myoactive peptides, have recently been isolated from cockroach corpora cardiaca by O'Shea *et al.* [12]. These authors used fast atom bombardment mass spectroscopy, and report a molecular weight of 972 for their myoactive factor I (which is identical to our hypertrehalosaemic hormone I) and 987 for their myoactive factor II (identical to hypertrehalosaemic hormone II). By this spectroscopic method they also determined that both peptides are blocked: at the amino terminal of each peptide there is a pyroglutamate residue, and the carboxy terminals are amidated. In addition, each aspartate residue of the acid hydrolysis proved to be an asparagine residue.

From this additional information, it becomes clear that cockroach hypertrehalosaemic hormones show structural similarities to those peptides already known of arthropods: locust adipokinetic hormone, pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2 [13], and crustacean red pigment-concentrating hormone, pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2 [14].

O'Shea and his colleagues [12] showed that their myoactive peptides had adipokinetic activity when injected in locusts, but their material was not tested in cockroaches for hypertrehalosaemic activity. Our results show that the myoactive and hypertrehalo-

saemic peptides are identically in their amino acid composition. Furthermore, we may call our peptides hypertrehalosaemic hormones, since it was shown in the previous study [12] that isolated corpora cardiaca superfused with saline containing calcium released the myoactive factors I and II.

It is of further interest to note that the amino acid composition of compound I is identical to that of neurohormone D [15], a factor from cockroach corpus cardiacum that has a cardioexcitatory action. We therefore believe that the peptides isolated from the corpus cardiacum of the American cockroach *Periplaneta americana* may have multiple functions: modulation of heart rate, myotropic and hypertrehalosaemic activity. Future work must determine if all these effects are simultaneously displayed, or if the amount of hormone present in the haemolymph determines the action *via* differential thresholds for the various actions.

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Note added in proof

This manuscript was first submitted to Biochem. Biophys. Res. Commun. as a rapid communication, but rejected with the following comment: "This work is carefully executed, but is not of a type that requires rapid publication. It can safely wait for normal publication schedules."

During the time that this manuscript was under review several new data were published:

1. The amino acid sequences for the myoactive peptides M I and M II [16],
2. evidence that M I and M II were very likely identical to cockroach hypertrehalosaemic hormones I and II [17],
3. structure elucidation (identical to M I and M II) of two peptides with cardioaccelerating and hyperglycaemic activity [18],
4. the sequence analysis of neurohormone D proved to be identical to M I and hypertrehalosaemic hormone I [19].

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