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Primary structure of two neuropeptide hormones with adipokinetic and hypotrehalosemic activity isolated from the corpora cardiaca of horse flies (Diptera)

(invertebrate/hyperlipemic/hypotrehalosemic/amino acid sequence)

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ABSTRACT **The primary structures of two neuropeptides, *Tabanus atratus* adipokinetic hormone (Taa-AKH) and *Tabanus atratus* hypotrehalosemic hormone (Taa-HoTH), from the corpora cardiaca of horse flies (Diptera: Tabanidae) have been determined. Amino acid sequences of Taa-AKH (<Glu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-NH_2) and Taa-HoTH (<Glu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-Tyr-NH_2) (where <Glu = pyroglutamic acid) were determined by automated gas-phase Edman degradation of the peptides deblocked by pyroglutamate aminopeptidase and by fast atom bombardment mass spectrometry. The hormones were synthesized, and the natural and synthetic peptides exhibited identical chromatographic, spectroscopic, and biological properties. When assayed in adult face fly males, Taa-AKH and Taa-HoTH demonstrated hyperlipemic activity; in addition, Taa-HoTH also demonstrated a significant hypotrehalosemic activity.**

The corpora cardiaca of insects have been recognized as neuroendocrine structures that are the sites of production and release of several neurohormones, including the adipokinetic/red pigment-concentrating family of hormones (AKH/RPCH family) (1). We recently reported (2) the isolation of two peptides, *Tabanus atratus* adipokinetic hormone (Taa-AKH) and *Tabanus atratus* hypotrehalosemic hormone (Taa-HoTH), from the corpora cardiaca of horse flies (Diptera: Tabanidae).‖ Both peptides were purified by a sequence of three reversed-phase HPLC steps. Amino acid composition, spectral analysis, and preliminary biological data clearly placed these peptides in the AKH/RPCH family (2). This report describes the elucidation of the primary structure, chemical syntheses, and biological activity of Taa-AKH and Taa-HoTH.

MATERIALS AND METHODS

Insects. Adult horse flies were field collected in Colt, Arkansas, and shipped live to Beltsville, Maryland. Even though the collection represented 19 species in four genera, a majority of the flies were *T. atratus*, and chromatographic evidence indicated that the two peptides were identical across the species. Isolation of the peptides has been described earlier (2). Since Tabanids cannot presently be reared in the laboratory, and the physiological status of field-collected flies was unknown, laboratory-reared face fly (*Musca autumnalis*) adults (a species from the same order of insects) were used for the bioassays. These were maintained in incubators with a 16:8 light:dark cycle and 25 ± 1°C. Adults were sexed immediately after emergence and the females were discarded.

Enzyme Deblocking. Enzyme deblocking was performed with pyroglutamate aminopeptidase (EC 3.4.19.3) essentially according to the method of Hayes *et al.* (4). Enzyme (25 units, purchased from Sigma) was dissolved in 240 μl of Milli-Q water (Millipore). Purified samples of Taa-AKH (450 pmol) and Taa-HoTH (290 pmol) were each treated with 60 μl of the enzyme solution under N_2 at 37°C for 8 min. The reaction was quenched by dilution of the samples with 195 μl of Milli-Q water, and the resulting preparation was immediately injected on the HPLC equipped with a Zorbax C-8 150SP column (DuPont). HPLC conditions were the same as described previously for step C (2). Fractions of 1-min duration were collected and dried in a model SVC 200H Speed Vac concentrator (Savant).

Sequence Analysis. Peptide fragments resulting from enzyme deblocking were sequenced on a model 470A peptide sequencer (Applied Biosystems) using the 02RPTH program supplied by the vendor. The phenylthiohydantoin residues were analyzed by a model 120A on-line analyzer (Applied Biosystems). Fast atom bombardment mass spectrometry (FAB-MS) on purified native peptides was performed as described (5).

Peptide Synthesis. Solid-phase syntheses of Taa-AKH and Taa-HoTH were performed on a model 990 synthesizer (Beckman) using reagents and cycles supplied by the manufacturer. The crude deprotected peptides (1 μg) were analyzed by FAB-MS in 1.5 μg of thioglycerol/glycerol (3:1) on a model 70/70 HS-MS (VG) equipped with Cu probe. The sample was bombarded with 6-kV Cs^+ ions at 1.65 kV. MH^+ for Taa-AKH: Calculated 931.47. Found 931.42; MH^+ for Taa-HoTH: Calculated 1151.55. Found 1151.25. The crude deprotected peptides were purified by a sequence of two reversed-phase HPLC steps. After chromatography by the conditions of HPLC step A (2), the resulting Taa-AKH and Taa-HoTH peaks were rechromatographed by the conditions of HPLC step C (2) to achieve final purification.

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Abbreviations: Taa-AKH, *Tabanus atratus* adipokinetic hormone; Taa-HoTH, *Tabanus atratus* hypotrehalosemic hormone; FAB, fast atom bombardment; AKH-RPCH family, adipokinetic/red pigment-concentrating family of hormones.

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‖These peptides, previously (2) referred to as DCCI and DCCII (Dipteran corpora cardiaca factors I and II), respectively, have been renamed, in this paper, Taa-AKH and Taa-HoTH according to a newly proposed insect-peptide nomenclature scheme (3).

Bioassays. Because of the limited amount of natural peptides available to us, dose–responses were determined with synthetic hormones. Synthetic Taa-HoTH and Taa-AKH were dissolved in insect saline (6) to obtain 0.1, 1.0, and 10.0 pmol/μl concentrations. Assays were carried out between the third and fifth hours of the photophase for the lipid mobilization assay. Two-day-old male face flies, fed sugar and water, were used. Flies were anesthetized with CO_2, and hormone was injected into the abdomen (from the ventral side) with a 10-μl syringe. Control flies were injected with 1 μl of saline. After 1 hr, the dorsal aorta in the abdominal region of a test fly was pierced with a sharp needle and hemolymph was collected into a 1-μl microcapillary tube. Total hemolymph lipids were measured by the phosphovanallin method (7). For trehalose (the major carbohydrate in insect hemolymph) assay, 1-day-old males that were provided only with water were used. Injection and hemolymph collection were done exactly as described for the lipids assay. Since the main sugar in insect hemolymph is trehalose, sugar levels were measured by the anthrone method and reported as trehalose (8). Percent changes in lipids and trehalose levels in the hemolymph relative to saline-injected controls were calculated. The change in response was computed relative to the saline control. Least squares polynomial regression (linear and quadratic) was used to determine if lipid or trehalose changed with increasing dose and to determine the shape of the response curve. Subsequently, natural and synthetic hormones were tested at 5 pmol doses, and the change in hemolymph lipid and trehalose levels was recorded.

RESULTS

As previously reported (2), Taa-AKH and Taa-HoTH were purified by a sequence of three reversed-phase HPLC steps and found to have the following amino acid compositions for Taa-AKH, $Glx_1Gly_1Leu_1Phe_1Pro_1Thr_2$, and for Taa-HoTH, $Glx_1Gly_2Leu_1Phe_1Pro_1Thr_2Tyr_1$. In addition, spectral evidence was presented for the presence of Trp and Phe in Taa-AKH and Trp, Tyr, and Phe in Taa-HoTH. Both peptides displayed red pigment-concentrating activity in the crab *Uca pugilator*. Taken as a whole, the structural and biological data clearly place these hormones in the AKH/RPCH family of invertebrate neuropeptides.

Because of the presumed presence of an NH_2-terminal <Glu characteristic of the AKH/RPCH family, Taa-AKH and Taa-HoTH were treated with pyroglutamate aminopeptidase prior to sequencing. The elution profiles resulting from enzyme treatment of Taa-AKH and Taa-HoTH (Fig. 1) each displayed three peptide fragments: A, B, and C and A, B, and D, respectively (all other peaks resulted from the enzyme reagent and/or solvents). The A fragment peaks, having the same retention time, were each shown to be pure by superimposition of their normalized up-slope, apex, and down-slope UV spectra (data not shown) and identical by superimposition of their normalized apex UV spectra (Fig. 1 *Inset*). Automated Edman degradation of one of the A fragments (from Taa-HoTH) resulted in the assignment of the following sequence: Leu-Thr-Phe-Xaa-Pro. Although the fourth residue in this fragment could not be unambiguously determined (<5 pmol), sequence analysis of one of the B fragments (from Taa-HoTH), each of which had the same retention time, showed it to be a subset of fragment A beginning with Phe with the following sequence: Phe-Thr-Pro, thus allowing the assignment of the undetermined fourth residue in fragment A to Thr. Fragments C and D from Taa-AKH and Taa-HoTH were shown to be pure by superimposition of their normalized up-slope, apex, and down-slope UV spectra and to have the following respective sequences: Gly-Trp and Gly-Trp-Gly-Tyr. The presence of Trp in fragments C and D was confirmed spectrophotometrically by comparison of their

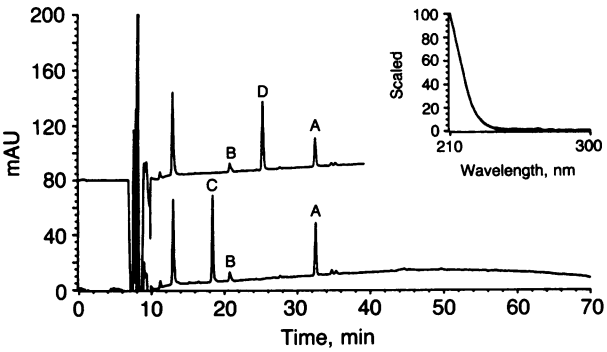

FIG. 1. Elution profiles of pyroglutamate aminopeptidase digests of 450 pmol of Taa-AKH (lower chromatogram) and 290 pmol of Taa-HoTH (upper chromatogram) by HPLC conditions of step C of ref. 2 (0.200 A_{210} full-scale, Zorbax C-8, 150SP column) and overlay (*Inset*) of normalized Apex UV spectra of the resulting A fragment peaks between 210 and 300 nm. mAU, milliabsorbance units.

zero-order UV spectra with Trp (Fig. 2 for fragment C) and by the presence of minima at 280 and 290 nm in their respective second-derivative UV spectra.

FAB-MS (Figs. 3 and 4) indicated molecular weights of 930 and 1151 for Taa-AKH and Taa-HoTH, respectively. The amino acid, spectroscopic, sequence, and FAB-MS data are consistent with the NH_2-terminal <Glu, COOH-terminal carboxamide octa- and decapeptides shown below.

Taa-AKH: <Glu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-NH_2

Taa-HoTH: <Glu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-Tyr-NH_2

As seen in the FAB-MS data of Taa-AKH (Fig. 3), sequence ions consistent with the loss of <Glu and Leu (Y_8 and Y_7) from the amino terminus and the loss of NH_2 (as $CONH_2$) and Trp (A_7 and A_8) from the COOH terminus of the proposed structure were evident. Similarly, for Taa-HoTH (Fig. 4), sequence ions consistent with the loss of <Glu, Leu, Thr, and Phe (Y_{10-7}) from the NH_2 terminus and the loss of NH_2 (as $CONH_2$), Tyr, Gly, and Trp (A_{10-7}) from the COOH terminus of the proposed structure were observed.

Final structural proof came from synthesis and demonstration of identical chemical and biological properties for the native and synthetic hormones. Elution profiles of samples containing equal amount of native and synthetic Taa-AKH and Taa-HoTH displayed one peak in each case. Each peak displayed superimposition of its up-slope, apex, and down-slope UV spectra, indicating peak homogeneity. In addition,

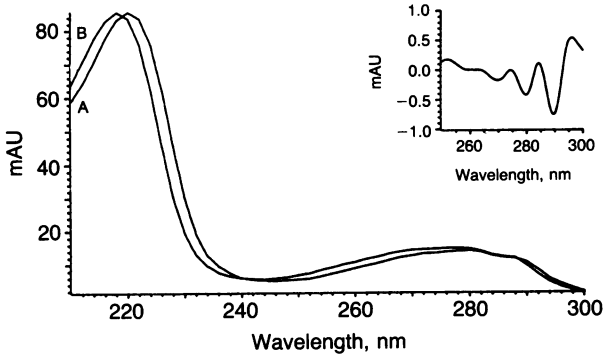

FIG. 2. Zero-order UV spectra between 210 and 300 nm of fragment C, Gly-Trp (spectrum A) from the digest of Taa-AKH with pyroglutamate aminopeptidase and tryptophan (spectrum B) and the second-derivative spectrum (*Inset*) of fragment C. mAU, milliabsorbance units.

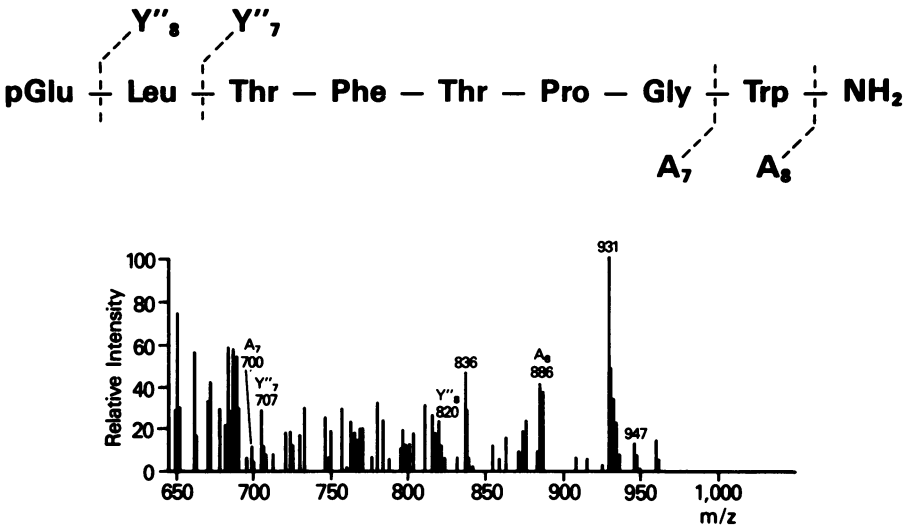

FIG. 3. Partial FAB-MS of Taa-AKH. The nomenclature of fragment ions is that of ref. 9. pGlu = <Glu.

each peak demonstrated coincidence of its elution profile and retention time at 200, 210, 215, 220, 230, 254, and 280 nm, again indicting peak homogeneity. Synthetic Taa-AKH and Taa-HoTH displayed essentially identical FAB-MS as the native peptide. In addition, digestion of the synthetic peptide with pyroglutamate aminopeptidase resulted in the production of the same HPLC fragments observed from the native peptide. Sequence analyses of these fragments confirmed the structure of the synthetic peptides.

Taa-AKH caused a significant increase in hemolymph lipids at all three test doses, with maximal elevation at 1 pmol that was not statistically different from the 10 pmol dose (Fig. 5*A*) Even though the elevation in lipids caused by Taa-HoTH was significant compared to that of saline, response was not dose dependent. Taa-AKH did not affect the hemolymph trehalose level at any of the test doses (Fig. 5*B*). However, there was a significant reduction in the level of hemolymph trehalose caused by Taa-HoTH, at the 1 and 10 pmol doses. When natural and synthetic Taa-AKH and Taa-HoTH were tested for lipid-mobilizing activity, we found that the two natural peptides and the synthetic Taa-AKH caused a significantly higher elevation of total lipids compared to synthetic Taa-HoTH at the 5 pmol dose (Table 1). The average increase in total lipids in response to Taa-AKH and Taa-HoTH was 43% and 33%, respectively. In the test for trehalose, the natural and synthetic Taa-AKH did not cause any significant change. However, natural and synthetic Taa-HoTH lowered the hemolymph trehalose by an average of 25% compared to the saline-injected control flies (Table 1). There was no significant difference between the responses obtained from natural and synthetic peptides.

DISCUSSION

The structures of Taa-AKH and Taa-HoTH were determined by a combination of automated gas-phase Edman degradation of the peptides deblocked by pyroglutamate aminopeptidase and FAB-MS. A similar approach had been used earlier to determine the structures of two other AKH/RPCH family peptides from *Heliothis zea* (9, 10): Mas-AKH (<Glu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-NH_2) and Hez-HrTH (<Glu-Leu-Thr-Ser-Ser-Gly-Trp-Gly-Asn-NH_2). However, unlike the *Heliothis* peptides, Taa-AKH and Taa-HoTH contain a Pro-Gly bond that was rapidly cleaved by the above enzyme under the conditions used by Hayes *et al.* (4). Prolinase activity of this enzyme has been found by Hayes *et al.* (T. K. Hayes, personal communication) and is not surprising in light of the similarity in structure of <Glu and Pro.

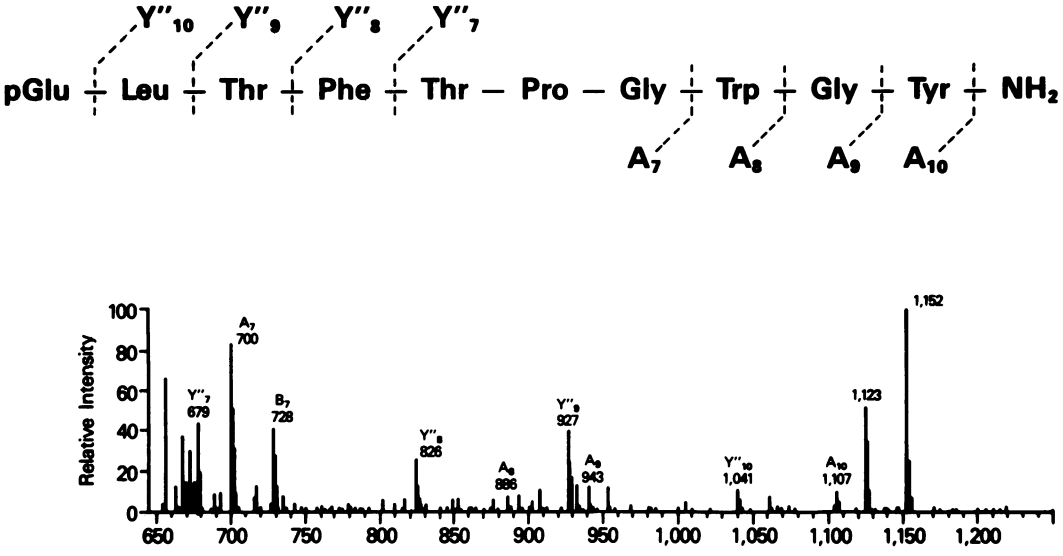

FIG. 4. Partial FAB-MS of Taa-HoTH. The nomenclature of fragment ions is that of ref. 9. pGlu = <Glu.

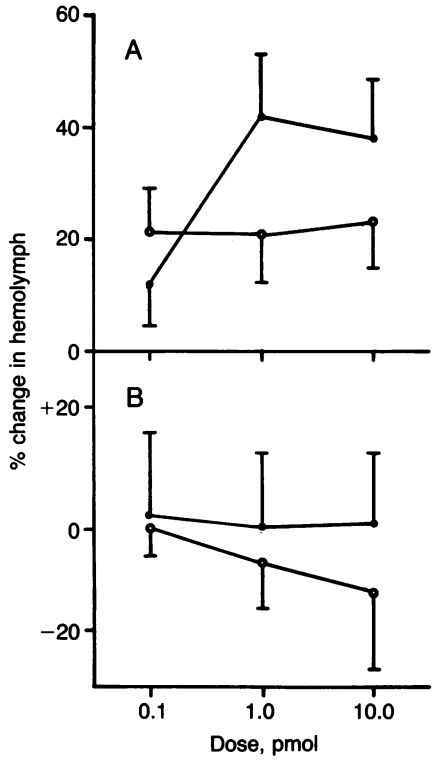

FIG. 5. Dose–response of synthetic Taa-AKH (●) and Taa-HoTH (○) in male face fly adults. Changes in hemolymph lipid (*A*) and trehalose (*B*) were measured 1 hr after the injection of the peptides. Vertical bars indicate SD; $n = 16$.

The additional fragments (C and D) thus generated facilitated structural determination of the peptides. Enzyme digestion of Taa-AKH and Taa-HoTH (data not shown) under less vigorous conditions than those reported by Hayes *et al.* (4) (e.g., lower temperature and enzyme concentrations) resulted in the isolation in each case of a non-Trp-containing (determined by UV spectroscopy) fourth fragment presumed to be the blocked intermediate, <Glu-Leu-Thr-Phe-Thr-Pro, which could not be sequenced. Fragments similar to the minor fragment B (Phe-Thr-Pro) resulting from the enzymatic cleavage of the Thr-Phe (and the Pro-Gly) bond have previously been reported in the *Heliothis* peptides (9, 10).

Table 1. Bioassay of natural and synthetic Taa-AKH and Taa-HoTH on male face fly adults

Injected hormone	% change in relation to saline-injected control flies	
	Lipid	Trehalose
Taa-AKH		
Natural	+41.2 ± 12.9	+4.4 ± 6.7
Synthetic	+44.7 ± 21.2	+3.2 ± 8.9
Taa-HoTH		
Natural	+40.5 ± 29.4	−27.0 ± 6.9
Synthetic	+24.6 ± 16.8	−23.0 ± 6.6

Flies were injected with 5 pmol of natural or synthetic hormone in 1 μl of saline. Control flies were injected with 1 μl of saline. Changes in lipid and trehalose levels were converted to % changes; $n = 10$. Data are presented as mean ± SEM.

The proposed sequences of Taa-AKH and Taa-HoTH were confirmed by FAB-MS. Although the amounts of native peptides (*ca.* 200 pmol) available were sufficient for determination of the molecular ion, the quantities were insufficient for *a priori* structural determination because of numerous matrix background peaks. However, it was possible to use the spectra to confirm the proposed sequence by observation of key sequence ions consistent with the proposed sequences.

The remarkable degree of sequence homology that exists in the AKH/RPCH family of peptides has previously been discussed (11). Taa-AKH and Taa-HoTH possess all key structural features of these peptides: <Glu-1, Leu-2, Phe-4, Pro-6, and Trp-8 together with amidated COOH terminus. In fact, Taa-AKH and Taa-HoTH are identical in their first six residues to Pea-CAH-II (periplanetin CC-II) from the cockroach *Periplaneta americana*. The most significant difference, however, is the presence of Tyr in Taa-HoTH, which, to our knowledge, has not been reported in any other AKH/RPCH peptides.

Two peptides ($M_r \approx 1500$) were reported in the corpora cardiaca of the tsetse fly *Glossina morsitans* (12, 13). One of these inhibited lipid synthesis and a second enhanced lipid mobilization. Taa-AKH and Taa-HoTH elicited a hyperlipemic response when assayed in the face fly but only the former elicited a dose–response. It is not uncommon among insects to have two structurally different peptides exhibiting similar biological activity. The peptides AKH-I and AKH-II from *Locusta migratoria* show adipokinetic activity (11, 14). However, because Taa-AKH caused a significantly greater increase in hemolymph lipids and expressed a dose–response, we designated it as the adipokinetic hormone of these flies. In the trehalose assay Taa-AKH showed almost no response, whereas Taa-HoTH significantly reduced the hemolymph trehalose and the response was dose dependent. This peptide is, therefore, designated as the hypotrehalosemic hormone in these flies.

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