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ISOLATION AND PRIMARY STRUCTURE OF A NEUROPEPTIDE HORMONE FROM *HELIOTHIS ZEA* WITH HYPERTREHALOSEMIC AND ADIPOKINETIC ACTIVITIES[a]

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A neuropeptide was isolated from the corpora cardiaca of the corn earworm moth *Heliothis zea*, and purified by sequential gradient elution in three reversed phase-high performance liquid chromatographic steps. The primary structure, pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-Asn-NH_2 was determined by automated gas-phase Edman degradation of the peptide deblocked with pyroglutamate aminopeptidase, and confirmed by fast atom bombardment mass spectrometry. The hormone was synthesized and the natural and synthetic peptides had identical chromatographic and spectroscopic properties. Both natural and synthetic hormones caused the elevation of trehalose and lipid levels in the hemolymph of adult *H. zea* males.

Trehalose and lipids (mostly in the form of diglycerides) provide the source of energy for insect flight (1). Adipokinetic and hypertrehalosemic activities have been reported in the extracts of a neurohemal organ, the CC of several insect species (2). These activities are attributed to a group of neurohormones belonging to the AKH/RPCH family, characterized by C- and N-terminus blocked peptides which contain 8-10 amino acid residues. We have recently reported (3) on the isolation and structure of a neuropeptide, Hez-AKH, (pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-NH_2) from the CC of the corn earworm *Heliothis zea*, a major pest of a number of agricultural crops. Hez-AKH was shown to have adipokinetic activity and was identical in structure to

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Abbreviations Used: AKH, adipokinetic hormone; CC, corpora cardiaca; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high performance liquid chromatography; HrTH, hypertrehalosemic hormone; Hez-AKH, *Heliothis zea*-AKH; PTH, phenylthiohydantoin; RPCH, red pigment concentrating hormone; UV, ultraviolet.

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the adipokinetic hormone reported from *Manduca sexta* (4). We now report the isolation and primary structure of a second peptide hormone from the CC of *H. zea*. The hormone, Hez-HrTH, while conforming to the general structural characteristics of other AKH/RPCH-family peptides, is the first fully characterized hormone from a moth showing both hypertrehalosemic and adipokinetic activities.

MATERIALS AND METHODS

Experimental animals. *H. zea* adults were used for this study, and were reared as described previously (3). The insects were maintained under a 16:8 light:dark photoperiod, 26° C and 50% ± 10% RH.

Purification of Hez-HrTH. CC complexes were dissected from adult moths into methanol:water:acetic acid (90:9:1) and processed as described previously (3). Hez-HrTH was purified by a three-step reversed phase, gradient HPLC procedure as described previously (3), with several minor changes. Samples were concentrated in a model SVC 200H Speed Vac concentrator (Savant[b]) and in later work a Vydac 218 TP54 C-18 column (Separations Group) was substituted for the Zorbax C-8 150 SP column (Dupont) in the final HPLC step.

Structure determination. Purified Hez-HrTH was subjected to amino acid analysis and FAB-MS as described previously (3). The purified Hez-HrTH was also deblocked with pyroglutamate aminopeptidase (3), and the resulting products were sequenced by gas-phase Edman degradation (3).

Peptide synthesis. Hez-HrTH was synthesized using t-Boc chemistry on a Model 430A peptide synthesizer (Applied Biosystems) using reagents and cycles supplied by the manufacturer. The resulting peptide was purified by reverse phase HPLC.

Quantitative analysis. Synthetic Hez-HrTH (2 x ca. 300 pmoles) was analyzed by the conditions of HPLC step C (Vydac 218 TP54 C-18 column). Aliquots of the Hez-HrTH peak were dried *in vacuo* in the Speed Vac and subjected to amino acid analysis. Peak integration was used to calculate the average pmoles/area-count for the synthetic hormone, which was then used to quantify any other natural and synthetic hormone samples.

Bioassay. Two-day, post-eclosion males were ligated between head and thorax, 18 hours before testing, to exclude any endogenous hormone. Bioassays were conducted between 7 and 9 hrs into photophase. Both natural and synthetic hormones were dissolved in insect saline (5) and dilutions were made to obtain 2.5, 5.0 and 10 pmoles/10 µl. Various concentrations of the hormone were injected through the ventral side of the abdomen with a 25 µl syringe. Control insects were injected with 10 µl saline. The insects were held individually in 1 oz plastic cups. After one hour, hemolymph was drawn from each insect by piercing the dorsal intersegmental membrane of the abdomen with a sharp needle. Two hemolymph samples from each insect were collected in 2 µl-Drummond micropipettes. One sample was tested for lipids by the phospho-vanillin method (6), and the second sample from the same insect was tested for trehalose by the anthrone method (7). The test was replicated six times. Hemolymph samples from male moths, injected with saline or 5 pmoles Hez-HrTH, were also subjected to HPLC analysis for glucose and trehalose, using a 250 x 4 mm Bio-Sil Amino 5S column (Bio-Rad) with guard column on a chromatographic system consisting of a Model M6000 pump and R401 differential refractometer (Waters). Hemolymph (19-30 µl) from 3 moles was pooled and added to centrifuge tube containing 60 µl of 70% acetonitrile in water. After centrifugation at 14,000 x g in a tabletop centrifuge (Eppendorf), the entire supernatant was injected onto the column. The column was eluted isocratically with 70% acetonitrile in water at ambient temperature.

RESULTS AND DISCUSSION

HPLC analysis of an extract of 273 CC complexes of *H. zea* indicated the presence of a second peak (peak A, figure 1) in addition to the previously identified Hez-AKH (peak B). As in the purification of Hez-AKH (3), the

[b]Mention of a commercial product in this paper does not constitute an endorsement by the USDA.

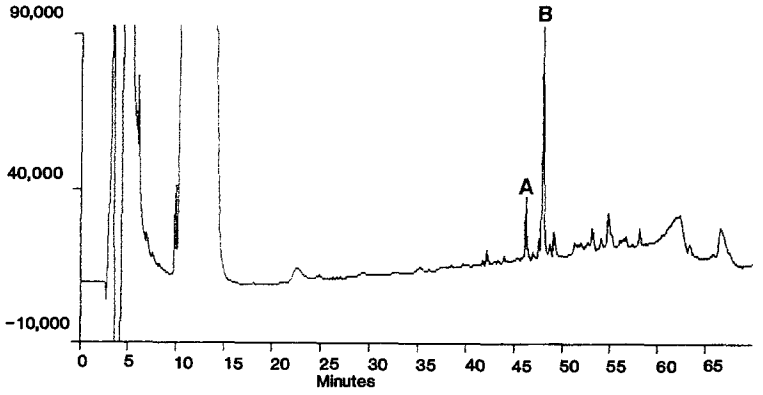

Figure 1. Elution profile of an extract of 273 CC complexes from *H. zea* by HPLC step A ($0.100A_{214}$ full scale). Peak A, Hez-HrTH; peak B, Hez-AKH.

material in peak A (Hez-HrTH) was purified by using a three step reversed phase gradient HPLC procedure (figure 2). The purified Hez-HrTH peak

demonstrated superimposition of its normalized upslope, apex and downslope UV spectra indicating peak homogeneity (figure 2, insert).

Amino acid analysis of purified Hez-HrTH indicated the following composition: Asx (1), Glx (1), Gly (2), Leu (1), Phe (1), Ser (2) and Thr (1). Although Trp was not determined by this method, its presence in the peptide was shown spectrophotometrically (8,9), particularly in the second derivative spectrum between 250-310 nm, which displayed strong minima at 290 and 280 nm. In addition, there was spectral evidence for Phe by the presence of a weak minimum at 255 nm. Finally FAB-MS of Hez-HrTH indicated a molecular weight of 1077, consistent with an amino-terminal pGlu, C-terminal carboxamide, Trp-containing decapeptide (figure 3).

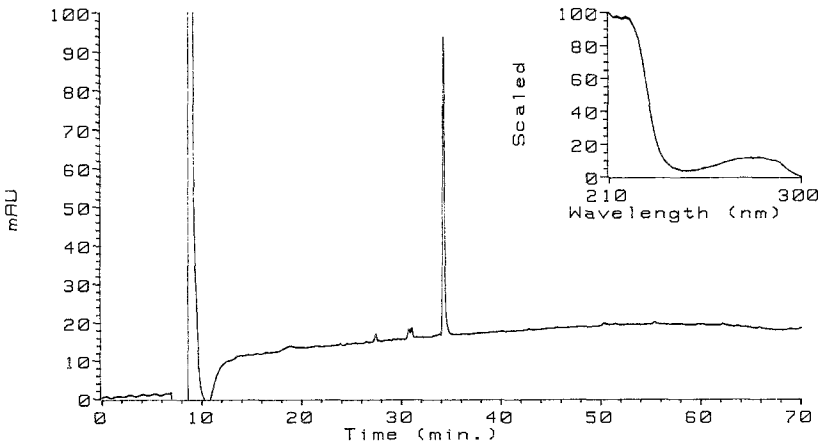

Figure 2. Elution profile (Vydac 218 TP54 C-18 column) of purified Hez-HrTH resulting from 656 cc complexes from *H. zea* by HPLC step C ($0.100A_{210}$ full scale) and overlay (insert) of normalized UV spectra of the upslope, apex and downslope of the purified peak eluting at 34.3 min.

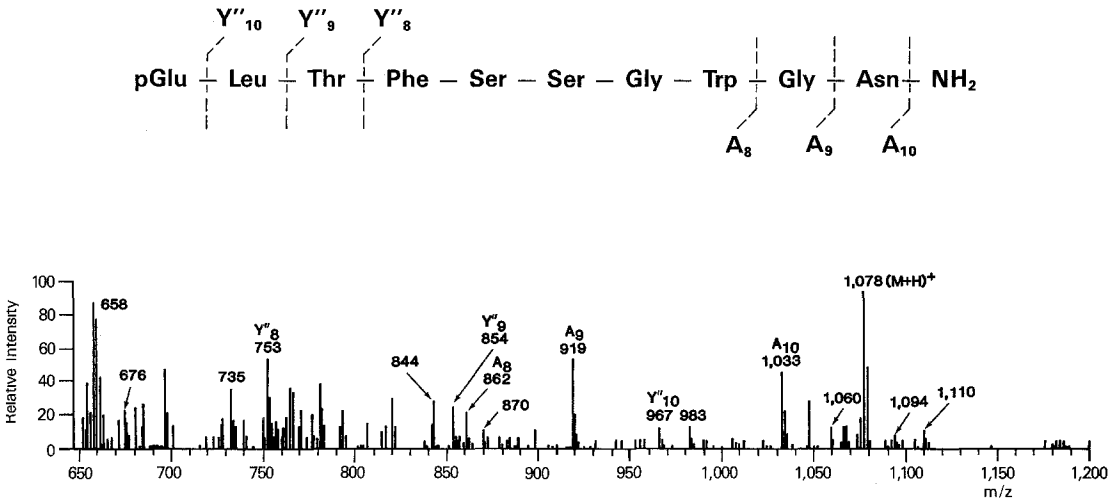

Figure 3. Partial FAB-mass spectrum of Hez-HrTH. Nomenclature for fragment ions is that of reference 15.

The primary structure of Hez-HrTH was determined by sequence analysis of the peptide fragments obtained by enzyme deblocking of the natural hormone. The elution profile obtained after digestion of Hez-HrTH with pyroglutamate aminopeptidase (figure 4) displayed a major product, Hez-HrTH minus pGlu (peak B) eluting ca. 3 min earlier than unreacted Hez-HrTH (peak C) and a minor product, Hez-HrTH minus pGlu-Leu-Thr (peak A) eluting at 27 min. Sequential Edman degradation of the material in peak B indicated the presence of a single peptide fragment. The data were analyzed by the method of Smithies et al. (10). All amino acids expected from the amino acid analysis and UV spectroscopy were found in the PTH analysis with the exception of Glx. The minor product eluting at 27 min was sequenced and shown by the same analysis to be a subset of the natural peptide, starting at

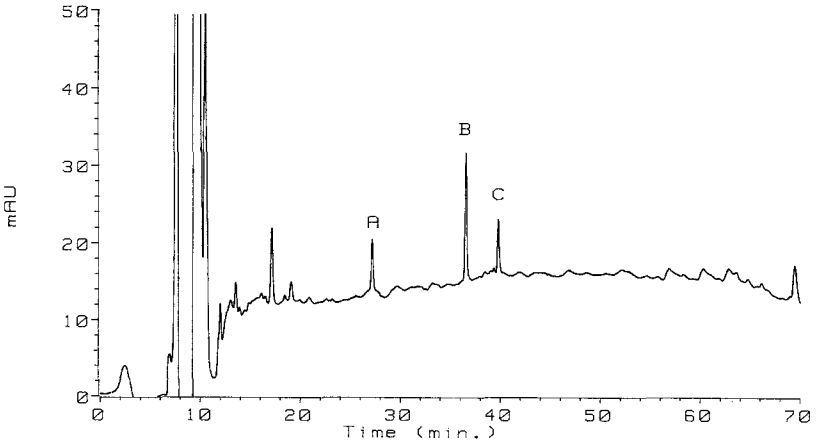

Figure 4. Elution profile (Zorbax C-8 150 SP column) of pyroglutamate aminopeptidase digest of Hez-HrTH by HPLC step C ($0.050A_{210}$ full scale). Peak A, Hez-HrTH minus pGlu-Leu-Thr; peak B, Hez-HrTH minus pGlu; peak C, Hez-HrTH.

Phe. Similar fragments were found in the digest of Hez-AKH (3). Examination of the FAB-MS (figure 3) revealed the presence of sequence ions consistent with the proposed primary structure as, pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-Asn-NH_2. The sequence was confirmed by synthesis. The synthetic peptide was identical to the natural hormone in retention time, UV spectrum and FAB-MS.

HPLC analysis of the *H. zea* hemolymph revealed that the major carbohydrate was trehalose (> 90%). This confirmed earlier reports that the major circulatory carbohydrate of most insects is trehalose (11,12). It was further observed that trehalose was the only saccharide that responded significantly to Hez-HrTH. Whereas, the glucose levels remained relatively constant, trehalose titers nearly doubled one hour after the injection of the hormone into the insects (figure 5). Both synthetic and natural Hez-HrTH caused significant elevation of hemolymph lipids at all three doses tested (figure 6A). Maximal elevation was caused by a dose of 5 pmol, with the response tapering off at higher and lower doses. The effect was more pronounced when tested for trehalose elevation. Synthetic and natural Hez-HrTH at 5 pmol dose caused 68 and 73% increase in hemolymph trehalose, respectively (figure 6B). Our

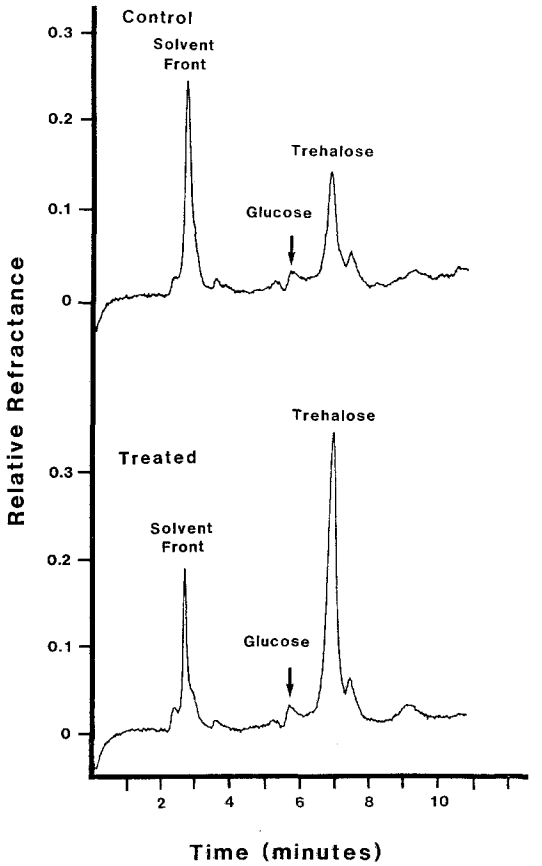

Figure 5. Glucose and trehalose titer in hemolymph pooled from 3 adult male *H. zea* one hour after injection of either physiological saline (top) or 5 pmol synthetic Hez-HrTH (bottom) determined by HPLC.

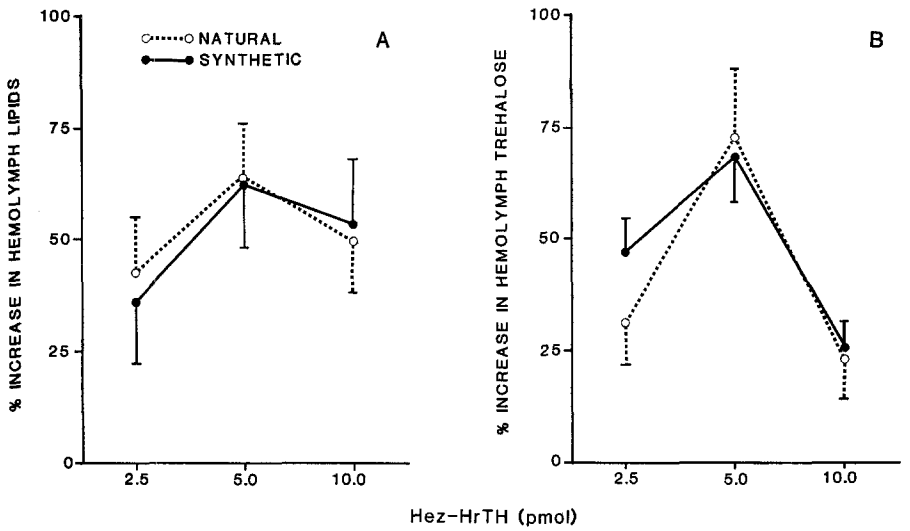

Figure 6. Hyperlipemic (A) and hypertrehalosemic (B) activities of natural and synthetic Hez-HrTH in male *H. zea.* Vertical bars represent standard error. N = 6.

consideration of this hormone as an HrTH is based on higher elicitation of its hypertrehalosemic effect over the hyperlipemic effect. In the cockroach *Blaberus discoidalis*, a dose of ca. 1.5 pmol of its hypertrehalosemic hormone was required to cause a 50% elevation in hemolymph carbohydrates (13). Two hormones (periplanetin CC-1 and CC-2) isolated from another species of cockroach *Periplaneta americana*, elicited both hyperlipemic and hyperglycemic activities (14). However, in all previously reported cases the dose responses have been linear. Hez-AKH and Hez-HrTH occur in a proportion of ca. 3:1 (based on peak height), that is similar to the ratio of periplenetin CC-1 to CC-2 (14). The exact roles of these hormones in relation to the development and activity of *H. zea* require further elucidation.

ACKNOWLEDGEMENT

The peptide nomenclature used in this paper is based on a system proposed by Ashok Raina and Gerd Güde (in preparation).

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