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Short Communication

Rapid isolation of a neurohormone from mosquito heads by high-performance liquid chromatography

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ABSTRACT

Methods were developed for the isolation of the egg development neurosecretory hormone, EDNH, from heads of the mosquito *Aedes aegypti*. This hormone stimulates ecdysone production by ovaries. Methods used for the successful isolation of insulin-like peptides from vertebrate tissues were modified to develop a four-step procedure involving extraction in acidified ethanol, precipitation by neutralization, followed by sequential separation on size-exclusion, ion-exchange and reversed-phase high-performance liquid chromatography columns.

INTRODUCTION

The egg development neurosecretory hormone (EDNH), first identified in the mosquito by Lea [1], is necessary for egg development to occur normally after a blood meal. This hormone is produced by neurosecretory cells in the medial portion of the brain and released from neurohemal sites lying along the aorta [2]. We have shown that EDNH stimulates the ovary to produce ecdysone [3]. Several attempts have been made to isolate EDNH [4–8]. A functionally related molecule, Bombyxin (which was earlier known as 4K-PTTH), was isolated from *Bombyx mori* on the basis of the fact that it stimulated ecdysone production by the prothoracic glands of *Samia cynthia ricini* [9–10]. However, it has no effect on *B. mori* glands, and it has been suggested that its role in *B. mori* is to stimulate ecdysone production by the ovary [11]. Bombyxin is closely related to insulin [9]. This line of reasoning led us to consider whether methods used for the isolation of hormones related to insulin from vertebrate tissues might work in the isolation of EDNH. We report here that techniques developed for the initial extraction of insulin-like peptides were useful for extracting EDNH. In

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addition we report on improved techniques for using high-performance liquid chromatography (HPLC) to isolate EDNH.

EXPERIMENTAL

Animals

Larval mosquitoes (*Aedes aegypti* derived from the Rock strain) were mass reared at 26.5 ± 0.5°C in 20 shallow pans (38 × 144 cm) containing 1½ cm of water and approximately 2000 larvae. They were fed on a solution containing 45 g mouse and hamster chow (Agway 32000 meal), 35 yeast hydrolysate and 45 g lactalbumin hydrolysate suspended in 1 liter of distilled water, the whole autoclaved before use. The feeding schedule was:

Day	1	2	3	4	5	6	7
ml	20	10	0	30	30	10–50	0–50

Pupae were collected on day 8 and adults emerged on days 9–11. Adults were maintained on a 3% sucrose solution in 12 l containers and harvested on day 16.

Preparation of heads

Adults were collected by gentle vacuuming and frozen at −70°C. Heads were isolated by shaking frozen adults in a chilled erlenmeyer flask and sieving the body parts through U.S.A. Standard testing sieves. Sieves 20 and 25 contained thoraces and abdomens while sieve 40 contained female and male heads and legs. Heads were separated from legs by letting them roll to the side of the sieve and aspirating them into a collection flask. Contamination with other body parts was negligible. Heads were lyophilized and stored at −70°C.

Solutions

Extraction buffer: 1 ml conc. HCl was added to 40 ml absolute ethanol, water was then added to a total volume of 50 ml. Size-exclusion chromatography (SEC) buffer: 20 m*M* phosphate buffer (HPLC-grade phosphoric acid adjusted to pH 6.5 with 1 *M* NaOH) containing 200 m*M* NaCl, 1 m*M* thiodiglycol and 1 m*M* EDTA (thiodiglycol is added just prior to use)). Ion-exchange chromatography (IEX) buffers: 50 m*M* Tris stock (pH 7.2 obtained by mixture of Tris base and Tris acid); IEX buffer A = 200 ml Tris stock, 100 ml isopropanol diluted to 1 l and filtered; IEX buffer B = IEX buffer A plus 500 m*M* NaCl. Analytical reversed-phase (ARP) buffers: ARP buffer A = m*M* phosphate (HPLC-grade phosphoric acid adjusted to pH 6.5 with 1 *M* NaOH) containing 150 m*M* NaCl; ARP buffer B = equal volumes of ARP buffer A and isopropanol. Microbore reversed-phase (MRP) buffers: MRP buffer A = 0.01% trifluoroacetic acid; MRP buffer B = 60% acetonitrile in 0.01% trifluoacetic acid, (TFA). Solvents were HPLC grade. Buffers and solvents were filtered through a 0.2-μm filter and degassed with helium prior to use.

Extraction of heads

In a standard preparation, 0.5 g of lyophilized heads (about 14 000 heads) were homogenized in 20 ml of ice cold extraction buffer using a motor driven 50-ml PTFE

homogenizer for about 25 strokes. The extract was centrifuged at 4°C at 15 000 *g* for 10 min. The pellet was reextracted in 10 ml of extraction buffer. The two supernatants were combined and centrifuged again. While keeping the extract on ice, 600 μl of 500 m*M* Tris pH 7.7 was added and the pH was adjusted to 7.5 to 8.0 by slowly adding 1 *M* NaOH. The pH was checked for stability after 10 min. Absolute ethanol was added to a final concentration of *ca.* 95% and the solution was held on ice overnight. The precipitate containing EDNH activity was removed by centrifugation at 15 000 *g* and dried under vacuum. The precipitate was then homogenized in 5 ml of 6 *M* urea prepared just before use and deionized by stirring with 1/5 volume of Dowex MR-3 ion exchanger (Sigma). The homogenate was mixed for 30–45 min by gentle shaking at room temperature. The non-solubilized material was pelleted and the supernatant was recentrifuged.

High-performance liquid chromatography

Instrumentation. LKB Ultra Chrom GT; BioSeparation System (Pharmacia LKB, Piscataway, NJ, U.S.A.).

Programmed gradients. Program No. 1 (ion exchange): 0–100% IEX buffer B in 50 min starting 5 min after injection, flow-rate 0.5 ml/min. Progam No. 2 (analytical reversed phase): 0–10% ARP buffer B in 13 min, 10–36% ARP buffer B in 2 min, 36–56% ARP buffer B in 30 min, 56–100% ARP buffer B in 22 min, flow-rate 1 ml/min. Program No. 3 (microbore reversed-phase column): 0–8% MRP buffer B in 5 min, 8–100% MRP buffer B in 55 min, flow-rate 1 ml/min.

Size-exclusion chromatography. Column: Altex Spherogel TSK-G 2000 SWg, 30 cm × 21.5 mm (Beckman, San Ramon, CA, U.S.A.). Conditions: The column was equilibrated for 18 h with 500 ml SEC buffer. The head extract (3 ml) was prepared by centrifuging in a microfuge 2 × for 10 min and injected at a flow-rate of 1 ml/min for 5 min and then 1.5 ml/min for the remainder of the run. Fractions of 3 ml were collected. Active fractions were concentrated in a dialysis bag (3500 cut off) against polyethylene glycol compound (Sigma) at 4°C until the volume was reduced to 0.5 ml. They were then dialyzed against IEX buffer A for 2 h.

Ion-exchange chromatography. Column: Altex Spherogel (DEAE-55W, 7.5 cm × 7.5 mm, 10 μm (Beckman). Conditions: The column was equilibrated with IEX buffer B at 0.5 ml/min for 30 min followed by buffer A at 0.5 ml/min for 2 h. The sample was prepared for injection by removing the sample, including the precipitate, from the dialysis bag. The sample was diluted to 3 ml with buffer A, and mixed gently for 15 min at room temperature. After centrifugation in a microfuge for 10 min the sample was injected at 0.5 ml/min and eluted using program No. 1. Five minutes after starting the gradient, the loop was disconnected from the buffer flow. Fractions of 2 ml were collected. Neurotensin (20 μg) was added to the active fractions as a carrier.

Analytical reversed-phase chromatography. Column: Analytical Vydac C_8 pH stable 25 cm × 2.1 mm (Alltech, Deerfield, IL, U.S.A.). The column was equilibrated at 1 ml/min with methanol for 30 min, water for 10 min and then ARP buffer A for 30–60 min. Prior to the run, 20 μg of neurotensin (40 μg/ml) was injected for 2 min at 1 ml/min and eluted using program No. 2. After elution of neurotensin (which was saved for later use), the active fraction from the IEX column was injected and eluted as described above. Fractions of 3 ml were collected. Purified neurotensin (20 μg) was added to the active fractions which were then frozen.

Microbore reversed-phase chromatography. Column: Brownlee Aquapore RP-300 C_8, 30 × 2.1 mm column in an HPLC cartridge (Raining, Woburn, MA, U.S.A.). The column was washed with 5 ml of buffer B and then equilibrated with buffer A. Neurotensin, purified on a reversed-phase column, was injected in 0.01% TFA and eluted using program No. 3. Fractions containing activity from the analytical reversed-phase column were reduced in volume by lyophilization and made up to 0.01% TFA by adding 0.1% TFA, injected onto the column and eluted using program No. 3. Fractions of 3 ml were collected and 15 μg of BSA in water were added to fractions which were then frozen and lyophilized prior to bioassay.

Bioassay of fractions

Ovaries were dissected from 3–6 day old mosquitoes, rinsed five times with sterile saline and divided into groups of 20 ovaries which were incubated for 6 h at 25°C in 50 μl of saline containing a dilution of the fraction being tested. Fractions were assayed in duplicate. The incubation medium was then assayed for the presence of ecdysone using a radioimmunoassay [3]. As high doses of EDNH are inhibitory [3], several doses were assayed to ensure that the linear portion of the response was obtained. Protein content of the fractions was determined using the method of Bradford [12] using BSA as a standard, or, for the microbore column, by area of the peak using neurotensin as a standard. To show that fractions active in the ovary bioassay also stimulated egg development *in vivo*, extracts were injected into blood-fed, decapitated, females as described by Wheelock and Hagedorn [13].

RESULTS

Previous studies had shown that EDNH retains activity after boiling, is fairly hydrophobic and contains disulfide bonds [4]. Although boiling achieved some degree of purification we wanted to avoid boiling because it caused erratic chromatographic behavior. Extraction with saline exposes the peptide to enzymatic acitivity and is not selective. We therefore extracted in acid ethanol which has been used in purification of insulin-like growth factors [14]. Acid–ethanol extracts molecules in the size range of EDNH; it is therefore somewhat selective. The pellet containing EDNH activity was extracted with 6 *M* urea to avoid non-specific interaction of EDNH with other proteins, as suggested by work with relaxin [15]. Using this technique, over 90% of the activity was extracted and 3- to 6-fold purification was achieved, as compared to saline extracts. The specific activity of the extract was 2 to 4 head equivalents/μg of protein. Acetone extracts were less successful. Although high recoveries were achieved with acetone, subsequent elution patterns on columns were not reproducible.

Having developed an efficient method for the initial extraction we then turned to methods for isolating EDNH from the acid–ethanol–urea extract. After trying a number of columns and elution techniques, we developed a purification scheme that involved the sequential use of size exclusion, ion exchange, and C_8 reversed-phase HPLC columns.

SEC of the initial extract showed that the acid–ethanol method removed the bulk of the proteins leaving only molecules in the size range of EDNH (Fig. 1A). Despite the coelution of EDNH activity with the major peak of protein, this column

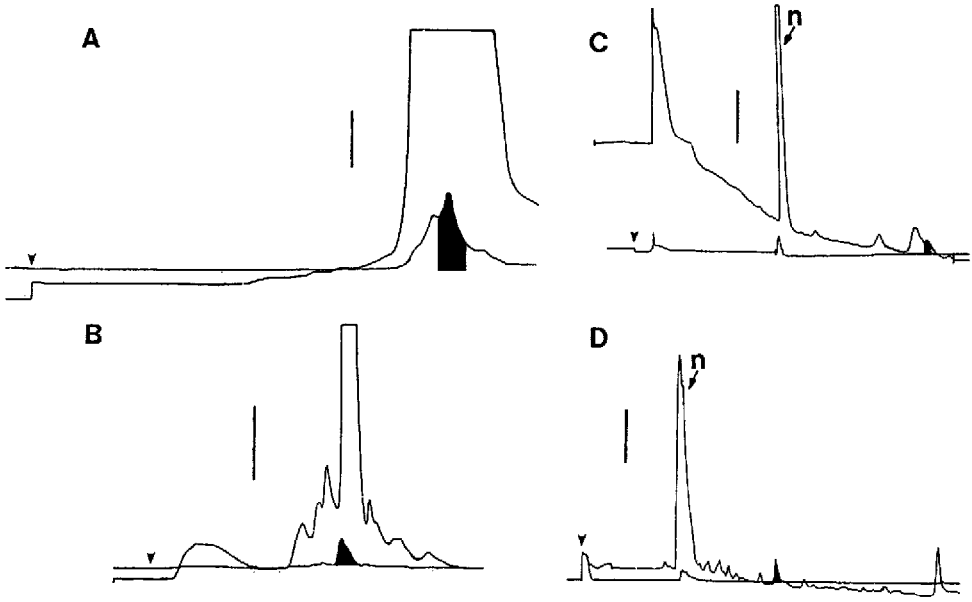

Fig. 1. Separation of EDNH activity from head extracts of *Aedes aegypti* using a size-exclusion column (A), ion-exchange column (B), analytical reversed-phase C_8 column (C), and a microbore reversed-phase C_8 column (D). The arrow head indicates the time of injection; n = neurotensin, an added carrier. The traces represent absorbance at 280 (A and B) and 206 (C and D) nm at two different sensitivities. The bar on the left of each figure represents 0.2 absorbtion units. Shaded areas indicate fractions with EDNH activity.

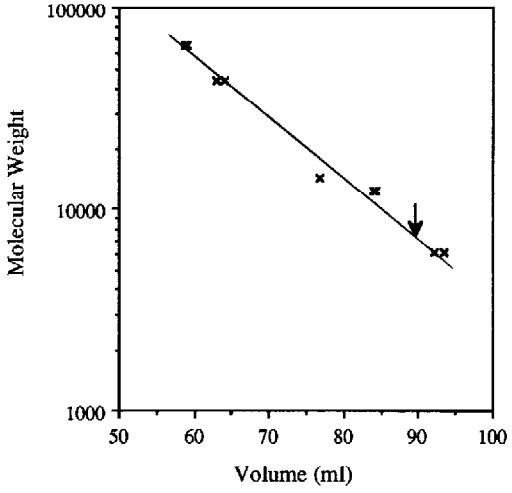

Fig. 2. Molecular weight determination by size-exclusion chromatography. Standards used included bovine serum albumin (65 000 dalton), ovalbumin (43 500), soybean trypsin inhibitor (14 300), cytochrome *c* (12 400), and insulin (6100). Points indicate results from two separate experiments. The arrow indicates the elution of EDNH activity.

achieved 3- to 7-fold purification with up to 90% recovery of acitivity. The specific activity of the active fractions from the SEC column in several experiments ranged between 8 and 25 head equivalents/μg of protein. The elution of the active fraction indicated an approximate molecular weight of 7500 (Fig. 2) in agreement with previous results [4].

The IEX column (Fig. 1B) achieved an 8-fold purification while allowing the recovery of up to 80 to 100% of the EDNH activity. The specific activity increased to 100 to 200 head equivalents/μg of protein.

Losses were greater on the analytical C_8 column (Fig. 1C) with recoveries of about 50%. But the specific activity of the active fraction of this column rose to over 600 head equivalents/μg of protein, and a 30-fold purification was achieved in this step. At this stage a greater than 10 000-fold purification had been achieved with recoveries of about 25%. Injection of 1.0 μl of the active fraction into females that were blood fed and decapitated caused egg maturation in 14 out of 17 injected females, thus showing that the purified material had the expected effects *in vivo* [13].

The active fraction from the C_8 column was still not pure as shown by the presence of multiple peaks in a subsequent separation of the active fraction on a microbore C_8 column (Fig. 1D). This column is a good candidate for the final step in isolation of EDNH.

DISCUSSION

The size and characteristics of EDNH, and the fact that the only source of this hormone is whole heads of mosquitoes, have made this a very difficult molecule to isolate and characterize. Nevertheless, EDNH is clearly an important insect hormone. In the mosquito is is thought to be released within hours after a blood meal where upon it stimulates the production of ecdysone by the ovary, thus beginning the process of vitellogenin synthesis [13]. The ovaries of many insects have been shown to produce ecdysteroids at some stage during the growth of the oocyte and the presence of factors with activities similar to EDNH have been detected.

Several groups have attempted to isolate EDNH with varying degrees of success [4–8, 13]. Our first effort, utilizing classical liquid chromatography, was hampered by lack of separation power of the techniques [4]. Separations were greatly improved by the development of HPLC [5], but recoveries were very low. As reported here, the development of HPLC columns specifically designed for peptide and protein separations our recoveries improved, but we also believe that the use of carrier protein avoids losses during the final steps of the isolation procedures. Neurotensin was added to the active fractions from the ion-exchange columns and the C_8 column. Neurotensin was chosen as a carrier because it elutes long before EDNH in both the ion-exchange and C_8 columns. We also found it important to add bovine serum albumin as a carrier to fractions that were to be bioassayed.

EDNH is functionally related to the prothoracicotropic hormone (PTTH) that stimulates ecdysone production by the prothoracic glands in immature insects. Definitive evidence that they are related awaits a sequence of EDNH. A hormone isolated from heads of adult *B. mori* was originally described as PTTH [10] but may, in fact, be more related to EDNH [11]. This molecule, recently renamed bombyxin, is closely related to insulin both in primary and secondary structure [9]. The work on Bombyx-

in suggested to us that methods used to isolate insulin-like peptides [14,15] might be successfully used for the isolation of EDNH. We have found that this was indeed true for both the acid–ethanol extraction of whole heads [14], and the use of urea to reduce hydrophobic interactions between EDNH and other molecules [15]. Another advantage of these techniques is that they avoid the use of a boiling step which we [4], and others [8], have used in the past and which may adversly affect the peptide.

The methods presented here for isolation of EDNH are relatively rapid compared to the more complex methods that have been used in the past [8,9]. It takes less than a week from extraction of the heads to the microbore C_8 column. Doing the bioassay after each column run will add several days per assay. We found, however, that the pattern of UV absorbance is a reproducible guide to active peaks, so that once the active peaks have been identified, the bioassay need not be done each time.

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