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Note

Isolation and high-performance liquid chromatographic purification of a myotropic peptide from the hindgut of the crayfish, *Procambarus clarkii**

VICTOR L. SYLVIA*
Vet. Public Health Dept., Texas A&M University, College Station, TX 77840 (U.S.A.)
and
G. MARK HOLMAN
Veterinary Toxicology and Entomology Research Laboratory, Agricultural Research Service, P.O. Drawer GE, College Station, TX 77841 (U.S.A.)
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Rapid improvements in the separation and purification of bioactive peptides by reversed-phase high-performance liquid chromatography (HPLC) have been developed in recent years. For reviews, see refs. 1–3. The utility of HPLC as an analytical tool in the study of comparative peptide biochemistry in invertebrate organisms has only recently become appreciated. Mapping of polypeptides and proteins using reversed-phase HPLC coupled with radioimmunoassay (RIA)[4] or mass spectrometric detection[5] can essentially be used to determine the conservation of specific amino acid sequence across phylogenetic boundaries. Just as the structural similarities and differences between vertebrate proteins have been elucidated by electrophoretic means, HPLC serves as a technique for comparing related peptides found among invertebrate species.

Many structural relationships have already been reported for peptides of animal origin spanning considerable evolutionary distance. For instance, the molluscan cardioexcitatory peptide, Phe-Met-Arg-Phe-NH_2[6], is structurally similar to the active carboxyterminal tetrapeptide of the mammalian gut peptide cholecystokinin and the head activator neuropeptide found in coelenterates has been reported found in the human hypothalamus[7]. Immunochemical determination of structural relationships has most often been used in these type studies and has been criticized for its lack of specificity[8]. Antisera raised against one specific group of peptides may cross-react with different molecular derivatives. Therefore, whenever possible it is desirable to isolate and purify the individual peptides by chromatographic means.

This was the approach in our study. The crayfish hindgut contains an endogenous peptidic substance whose activity is analogous to that of the insect neuropeptide proctolin, Arg-Tyr-Leu-Pro-Thr. Originally isolated from the cockroach, proctolin elicits rhythmic contractures in cockroach hindgut musculature[9]. Since its

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discovery, proctolin has been shown to be active on muscle tissue in a variety of invertebrate and vertebrate organisms[10–14]. This preliminary report describes the chromatographic similarities between proctolin and the crayfish hindgut peptide(s).

EXPERIMENTAL

Instrumentation

A Tracor Model 985/951 microprocessor high-performance liquid chromatograph system equipped with the Model 970A variable-wavelength detector was used. Waters Assoc. μ-phenyl (300 × 3.9 mm I.D., 10-μm particle size) and Supelco LC-C_{18} (250 × 4.6 mm I.D., 5-μm particle size) columns were used as the stationary phases upon which gradient and isocratic elutions respectively were performed. Eluted fractions were collected in Nalgene tubes and dried with pre-purified nitrogen before bioassay. Chromatograms were recorded using a Hewlett-Packard Model 3390A recording integrator. Bioassays were quantitated using an isotonic muscle transducer and Moseley 680 stripchart recorder according to specifications previously described[10].

Chemicals

Synthetic proctolin was obtained from Peninsula Laboratories, San Carlos, CA, U.S.A. DEAE- and CM-Sephadex ion-exchange resins were obtained from Pharmacia (Piscataway, NJ, U.S.A.). All solvents were HPLC grade Omnisolve (E. Merck, Darmstadt, G.F.R.) except for the water which was 18 MΩ cm organic-free water produced by Millipore water purification system (Continental Water Systems, El Paso, TX, U.S.A.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

Extraction

Hindgut musculature removed from the crayfish *Procambarus clarkii* was homogenized in methanol–acetic acid–water (90:9:1, v/v) for 3 min using a Polytron homogenizer and then decanted into centrifuge tubes. After centrifugation (4°C, 30 min, 3000 *g*), the supernatant was decanted into a boiling flask and the solvent removed with a rotary evaporator at 30°C. Equal portions of ethyl acetate and water were added to the flask. The residue was dissolved and transferred to a separatory funnel. The aqueous phase was shell-frozen with a dry ice–acetone bath and lyophilized.

Preparative chromatography

DEAE-Sephadex (A-25) was converted to the acetate form by stirring in 50% acetic acid (100 ml/g) for 30 min. The gel was then allowed to settle and rinsed repeatedly with distilled water until stabilized at pH 4. CM-Sephadex (C-25) was converted to the hydrogen form in a similar manner.

The lyophilized residue was resuspended in 0.01% trifluoroacetic acid (TFA) and pumped through C_{18} Sep-Pak cartridges which were subsequently dried with pre-purified nitrogen and eluted with 20% acetonitrile in 0.01% TFA. The acetonitrile was removed with a rotary evaporator and the remaining aqueous phase was batched with the DEAE-Sephadex gel. The gel was then filtered through sintered

glass directly into a flask containing the CM-Sephadex gel. The CM-Sephadex was rinsed initially with distilled water and finally with 0.05 *M* HCl.

HPLC purification

The 0.05 *M* HCl fraction was lyophilized and resuspended in 0.01% TFA. This fraction could now be chromatographed through the μ-phenyl column under the following conditions: linear gradient 0.01% TFA to 50% acetonitrile in 0.01% TFA over 1 h, flow-rate 1.5 ml/min, UV detection wavelength 275 nm and ambient column temperature. Throughout the purification samples were bioassayed using an isolated crayfish hindgut preparation according to procedures described earlier[10]. Active fractions eluting from the μ-phenyl column were purified further with the 25-cm Supelco C_{18} analytical column under the following conditions: 20% acetonitrile in 0.01% TFA isocratic, flow-rate 1.0 ml/min, UV detection wavelength 275 nm and ambient column temperature. In a few experiments, synthetic proctolin was carried through the outlined purification scheme to serve as an internal standard.

RESULTS AND DISCUSSION

The pentapeptide proctolin is excitatory on the hindgut of the crayfish *Procambarus clarkii* with dose-dependent changes in both amplitude and frequency of contractures. A typical proctolinergic response is shown in Fig. 1. The effective dose range for the pentapeptide is 0.33–8.00 ng per 10 ml bath saline ($4.95 \cdot 10^{-11}$–$1.20 \cdot 10^{-9}$ *M*).

Hindgut tissue from 480 crayfish (wet weight 6.87 g) was purified for excitatory substances using the proctolin purification scheme previously described. Throughout

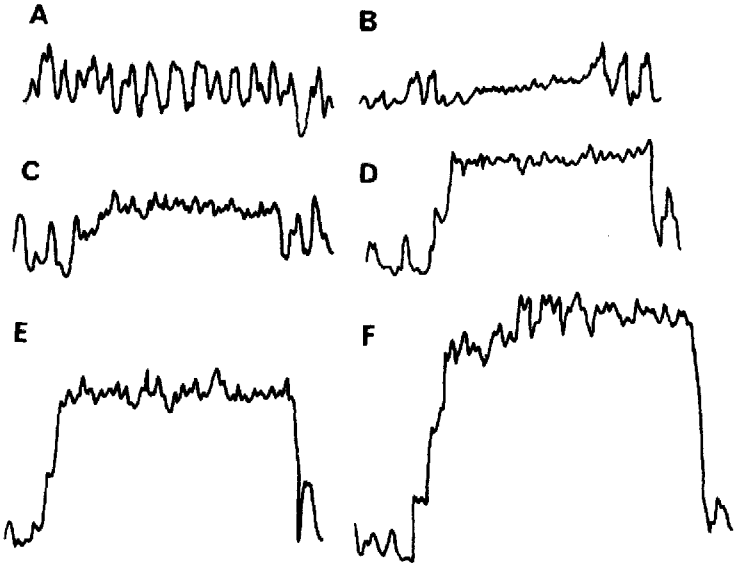

Fig. 1. Isolated crayfish hindgut responses to various dose levels of synthetic proctolin. A, Normal spontaneous hindgut activity; B, 0.0033 ng ($3.77 \cdot 10^{-11}$ *M*) threshold dose; C, 0.005 ng/ml ($5.69 \cdot 10^{-11}$ *M*); D, 0.01 ng/ml ($1.14 \cdot 10^{-10}$ *M*); E, 0.02 ng/ml ($2.28 \cdot 10^{-10}$ *M*); F, 0.04 ng/ml ($4.56 \cdot 10^{-10}$ *M*). Chart speed: 1 in./min. Sensitivity 50 mV per division. Chart progression right to left.

the cleanup procedure, aliquots equalling 1/50 of each sample were bioassayed. Crude hindgut extract prior to Sep-Pak treatments elicits a response comparable to 0.035 ng/ml proctolin (5.39×10^{-11} *M*). After Sep-Pak treatment the response is comparable to 0.065 ng/ml (1.00×10^{-10} *M*). Purification through μ-phenyl reversed-phase HPLC (Fig. 2a) resolved the active material as a broad impure peak with considerable tailing (t_R 13–15 min). When this fraction is chromatographed through Supelco LC-C_{18} (Fig. 3a), six peaks are observed, the last two being active. The second to last peak bioassayed nine-fold more active as the larger last peak.

The crayfish hindgut-stimulating peptide fraction, termed procambarin, purified in this manner is chromatographically distinct from synthetic proctolin (Figs. 2b, 3b). Synthetic proctolin possesses a longer retention time than procambarin in both reversed-phase HPLC systems employed. Quantitation of procambarin concentration in the hindgut tissue measured on the basis of proctolin-equivalent bioassay activity indicates the presence of approximately 98 pg of the peptide per hindgut. Our preliminary studies have only produced 46.80 ng of pure procambarin, an amount far exceeding the quantity necessary for bioassay detection but less than that required for structural analysis. At present our laboratory is processing sufficient quantities of crayfish hindgut tissue to employ amino acid analyses, peptide sequencing and other methods of structural elucidation.

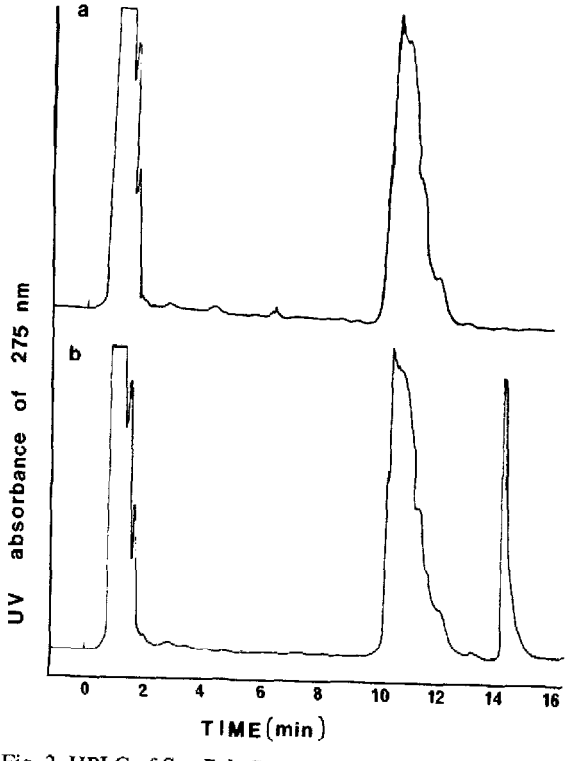

Fig. 2. HPLC of Sep-Pak C_{18}-purified procambarin (48 crayfish equivalents). Column, μ-phenyl (300 × 3.9 mm I.D., particle size 10 μm); 0.01% TFA to 50% acetonitrile + 0.01% TFA using a linear gradient over 20 min; flow-rate 1.50 ml/min; 275 nm; 0.005 a.u.f.s.; ambient column temperature. a, Sample alone; b, sample spiked with 10 ng proctolin.

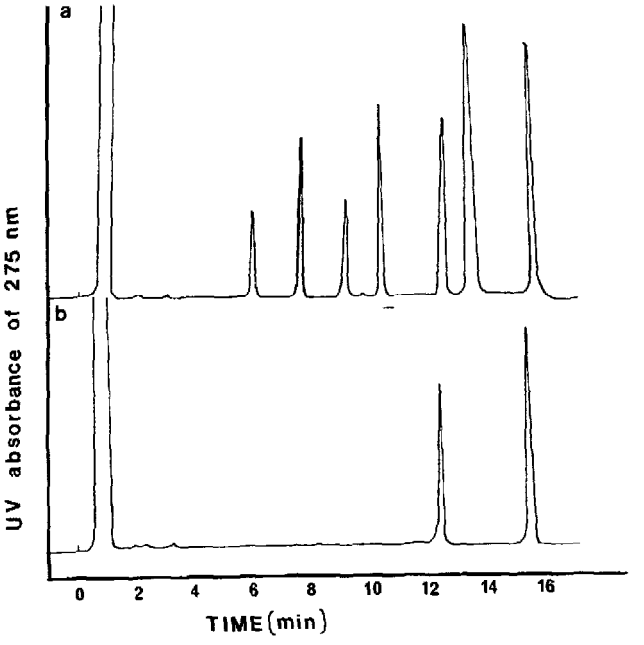

Fig. 3. HPLC of μ-phenyl-purified procambarin (160 crayfish equivalents). Column, Supelco LC-C_{18} (250 × 4.6 mm I.D., particle size 5 μm); 20% acetonitrile + 0.01% TFA isocratic; flow-rate 1.00 ml/min; 275 nm; 0.005 a.u.f.s.; ambient column temperature. a, Sample spiked with 10 ng proctolin; b, active component spiked with 10 ng proctolin.

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