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Molecular Cloning of Two Pigment-Dispersing Hormone (PDH) Precursors in the Blue Crab *Callinectes sapidus* Reveals a Novel Member of the PDH Neuropeptide Family+

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SUMMARY. A cDNA library was established from the eyestalk ganglia of the blue crab *Callinectes sapidus*. Screening resulted in the isolation of a clone (497 bp excluding poly(A) tail) which encodes a β-PDH previously found in several crustacean species. It displays high sequence similarity with a clone isolated from an eyestalk cDNA library of the shore crab *Carcinus maenas*, indicating the close phylogenetic relationship of both species. A second clone (414 bp exclusive of the poly(A) tail) encodes a novel β-PDH analog which displays 400-fold less potency in crab bioassays. Both cDNAs encode open reading frames of 234 bp for the prepropeptides, consisting of signal peptides, PDH-precursor-related peptides, and PDH sequences.

Pigment-dispersing hormones (PDHs) in crustaceans and pigment-dispersing factors (PDFs) from insects are a family of structurally related neuropeptides (1). In crustaceans these peptides induce integumental color change by triggering pigment dispersion within epithelial chromatophores and aid in the photomechanical adaptation of the compound eye by eliciting light-adaptational movement of screening pigments in certain extraretinular eye pigment cells. The functions of PDFs in insects have not been clearly demonstrated, although there is suggestive evidence for their involvement in the regulatory functions related to the visual system, possibly associated with a circadian pacemaker system (2). The elucidation of primary structures of PDHs and PDFs from representative arthropod species, the apparent diverse roles of these peptides among arthropods, and the discovery of PDH-like immunoreactive material in the pituitary of dogfish (3) have generated considerable interest in understanding the molecular evolution of the PDH peptide family, including the structural and genetic basis of sequence heterogeneity.

+The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EBI and NCBI databases with accession numbers L36716 and L36717.

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Abbreviations: PDF: pigment-dispersing factor; PDH: pigment-dispersing hormone; PPRP: PDH-precursor-related peptide.

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The identified PDHs are separable into two groups: α-PDH and its analogs; and the more acidic β-PDH and its analogs. α-PDH, initially called light-adapting distal retinal pigment hormone, was isolated from eyestalks of the shrimp *Pandalus borealis* and identified as NSGMINSILGIPRVMTEAamide (4). An octadecapeptide differing from α-PDH at six positions and designated as β-PDH (NSELINSILGLPKVMNDAamide) was subsequently identified as the major form in the eyestalks of the fiddler crab *Uca pugilator* (5). Whereas α-PDH has been found only in species of *Pandalus* (4, 6), β-PDH and peptides closely similar to β-PDH have been shown to occur widely in crustaceans and insects. Thus, the sequence of β-PDH is conserved among brachyuran crabs -- *Uca pugilator* (5), *Cancer magister* (7), *Callinectes sapidus* (8), and *Carcinus maenas* (9, 10) -- and in a crayfish, *Pacifastacus leniusculus* (1). Peptides with close sequence similarity and net charge identity to β-PDH have been identified in several species of crayfish, shrimp and insects (1).

Although three forms of PDH (α-PDH, an analog of α-PDH, and an analog of β-PDH) were purified from eyestalks of *Pandalus jordani* (6) and sequenced by Edman degradation, only one form of PDH (β-PDH or an analog of β-PDH) could be successfully purified and fully characterized in each of the other crustacean species and insects studied (1). The present report shows the occurence of two PDH peptides in the blue crab by means of molecular cloning.

MATERIALS AND METHODS

Poly(A^+)RNA from eyestalks of the blue crab *Callinectes sapidus* was isolated by the use of guanidine thiocyanate and oligo(dT)cellulose (Stratagene). About 5 μg of poly(A^+)RNA were used for creating the cDNA-library with the Uni-ZAP XR vector (Stratagene). 50,000 clones were screened with a radioactively labelled cDNA probe, generated from the PCR amplification product of five fractions of the cDNA-library with primer BS2 (5'-AGCGG ATAACAATTTCACACAGGA-3') corresponding to nucleotides 824-847 of the pBluescript II SK- vector and primer P1-CSA (5'-GGGAATTC(A/C/G/T)CC(A/C/G/T)GC(A/G)TC(A/G)TT CAT-3') corresponding to amino acids 15-18 of ß-PDH (5). The PCR was performed for 5 cycles with an annealing step at 68 °C, 5 cycles at 64 °C and 40 cycles at 60 °C. The denaturation step in each cycle was 40 s at 94 °C, the amplification step 3 min at 72 °C. The amplification product was digested with Pst I and the 300 bp fragment was isolated from an agarose gel by the use of low melting agarose. Labelling of the DNA fragment with ^{32}P was performed according to standard procedures (11, 12). Prehybridization and hybridization of the nylon membrane replicas (Stratagene) were performed in 2x PIPES Buffer (0.5 M NaCl, 0.02 M PIPES, pH 6.5), 50 % formamide, 1% SDS, and denatured, sonicated salmon sperm DNA (100 μg/ml). Washes were performed 15 min at room temperature in 2x SSC, 0.1 % SDS, and subsequently 15 min at 68 °C in 2x SSC, 0.1% SDS, 1x SSC, 0.1% SDS, and 0.25x SSC, 0.1% SDS. Plaques which gave positive hybridization signals were cored and used for a secondary screening. The conversion of recombinant Uni-ZAP XR clones to a pBluescript phagemid was performed according to the manufacturer's instructions. The sequences were determined by the dideoxy sequencing method (13) using PDH-cDNA-specific oligonucleotides as primers.

Callinectes-PDH I (β-PDH) was obtained from a previously described preparation (5). *Callinectes*-PDH II was synthesized on 4-(2', 4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (0.38 mmol/g resin; Novabiochem, La Jolla, CA) using Fmoc strategy and an automated

peptide synthesizer (431A, Applied Biosystems, Foster City, CA). Protocols for the synthesis, cleavage from resin, and purification of the peptide were essentially those recommended by the manufacturer. The peptide appeared to be homogeneous (single peak, u.v. detection at 214 nm) on analytical HPLC. Acid hydrolysis of the purified peptide yielded the correct ratios of amino acids upon analyses.

Bioassays were performed with the fiddler crab *Uca pugilator* collected from Port St. Joe, Florida. The crabs were maintained in a semiterrestrial system and fed oatmeal. The synthetic peptides were taken up in physiological saline (14) and 50 μl doses were injected into groups consisting of five eyestalkless specimens. The stages of melanophore pigment dispersion were microscopically assessed according to the described five-point scale of Hogben and Slome (15). The observed responses were quantified as a standard integrated response according to Fingerman et al. (16).

RESULTS AND DISCUSSION

A cDNA-library consisting of 3 million independent clones was succesfully generated from the eyestalk ganglia of the blue crab, *Callinectes sapidus*. Screening of 50,000 clones of this cDNA-library with a specific PDH-probe resulted in the isolation of two different clones. Both cDNAs present an open reading frame (ORF) of 234 bp which encode two different PDH-precursor molecules (called PDH I and PDH II). The nucleotide sequences are shown in Fig. 1 and the deduced amino acid sequences in Fig. 2.

The PDH I-cDNA has a length of 497 bp (excluding the poly (A) tail). The ORF is preceded by a 5'-flanking sequence of 30 bp and followed by a 3'-untranslated region of 230 bp with a variant polyadenylation site (ATTAAA) 15 bp upstream from the poly (A) tail (17). The ORF encodes the 78-amino acid PDH I-prepropeptide, consisting of a 22-amino acid signal peptide, a 33-amino acid peptide of unknown function (PPRP, PDH-precursor related peptide) and the PDH I sequence.

The PDH II-cDNA consists of a total of 414 bp (excluding the poly (A) tail). A 5'-flanking sequence of 49 bp is present, as well as a 3'-untranslated region of 128 bp following the stop codon with a putative polyadenylation site (AATAAA) 18 bp upstream from the poly (A) tail (17). The ORF encodes the 78-amino acid PDH II-prepropeptide, consisting of a 21-amino acid signal peptide, a 34-amino acid PPRP and the PDH II sequence.

The putative signal peptides display well known features of such sequences, in particular the central hydrophobic region, a basic amino acid (Arg) near the amino terminus and a typical signal peptide cleavage site (Gly^{-1}, Thr^{-3}; 18). Removal of the signal sequences by signal peptidases results in two different propeptides. The PDH I-propeptide consists of 56 amino acid residues with a calculated molecular weight of 6346 Da and is composed of the PPRP, a dibasic cleavage site (Lys-Arg), PDH I and another dibasic cleavage site (Arg-Arg). The PDH II-propeptide consists of 57 amino acid residues with a calculated molecular weight of 6156 Da. Its compositon is similar to that of the PDH I-propeptide (PPRP and PDH II which is flanked by two dibasic cleavage sites).

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PDH I                                          CACAGCTCATCCTAACAACACCCAGACAAG   30
PDH II              CATATCTGCCAGCCAGCCTGCCTGTCAGCTCACCAACTTAGAGAGAAAA   49

          SP ——►
PDH I     ATG CGC AGT TCC GTG ATC GTA GCC GTG CTG GTG GTG GTG GCT CTC   75
PDH II    ATG CGT AGC GGT GTT TTC GTG GCC GTG CTT GTG GTG GTG GTC TTC   94

                                ◄—— SP | PPRP ——►
PDH I     GCA GCC CTA CTC ACC CAG GGG|CAG GAG CTC AAG TAC CAA GAA CGT  120
PDH II    *** GCT CTC CTC ACC CAG GGG|CAG GAG CTT CAT GTT CCC GAG CGT  136

PDH I     GAG ATG GTG GCC GAG CTG GCG CAG CAG ATA TAC CGC GTG GCA CAG  165
PDH II    GAG GCC GTG GCC AAC CTG GCG GCA CGC ATC CTG AAG ATT GTC CAC  181

                                                ◄—— PPRP      PDH ——►
PDH I     GCT CCG TGG GCG GCC GCC GTT GGC *** CCC CAC|AAG CGC|AAC TCT  207
PDH II    GCT CCC CAT GAC GCC GCC GCT GGT GTC CCT CAC|AAA CGC|AAC TCA  226

PDH I     GAG CTG ATC AAC TCC ATC CTG GGG CTG CCC AAG GTG ATG AAC GAC  252
PDH II    GAA CTC ATC AAT TCG CTG CTC GGC ATC TCT GCG CTG ATG AAC GAG  271

          ◄— PDH
PDH I     GCC GGC|AGG AGA|TAA GAAGTGTACTCTTTTCCACCACACCACCTTCCCAACATG  306
PDH II    GCC GGC|AGG CGG|TGA AGGGAGAACCCTTAGTGCCCTTCCCTAGTGCTTGCTGAA  325

PDH I     ACCTTCTACCTTCTGCCGGGAACAGGACGAGGCCAGCGGGCGCGTAGAGCATCACCATC  365
PDH II    GCGGACAGTTTCCGCCATATTTCAGGAGTGTTTCATTGTCTACATCTGTCTTAACCCAT  384

PDH I     TCCGGGCGACACCATCACTTCCTGTCAATTGTTTAGTCAGTCATATAATCAATCAATTA  424
PDH II    TTAATGAATAAACTACACGGCAGCAACAGT(A)n                           414

PDH I     GTCAGTCAACCAGTCAGCGAACGTCGGCCTCTCATGTACCTTGAGTGGACTCATTAAAA  483

PDH I     GCATATAGTATAGT(A)n                                           497
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Fig. 1. Comparison of the two different cDNAs encoding prepro-PDH I and PDH II from *Callinectes sapidus*. The nucleotide numbering is shown on the right. The putative polyadenylation sites are underlined. Asterisks indicate blanks in order to match identical bases. Proteolytic cleavage sites are boxed.

The PDHs are located at the C-termini of the prepropeptides. The mature PDH peptides consist of 18 amino acids as it is also the case with all other known pigment-dispersing peptides (1). This is achieved by the removal of the Arg-Arg residues at the C-termini and the subsequent use of the remaining Gly^{19} as an amide donor.

The deduced PDH I sequence of *Callinectes* is identical to the already known β-PDH-sequences of *Uca pugilator* (5), *Cancer magister* (7), *Callinectes sapidus* (8), *Carcinus maenas* (9, 10) and *Pacifastacus leniusculus* (1), previously determined by Edman degradation. There is a striking similarity between the *Carcinus*-PDH-precursor and the *Callinectes*-PDH I-precursor (96 %, Fig. 2). This demonstrates the close phylogenetic relationship between both species. PDH II represents a new member of the PDH neuropeptide family. The amino acid substitutions at positions 12 and 13 (Ser^{12}, Ala^{13}) have not been observed previously. PDH II shows a sequence similarity of 67 % to the *Carcinus*-PDH, 72 % to the *Orconectes limosus*-PDH, 78 %

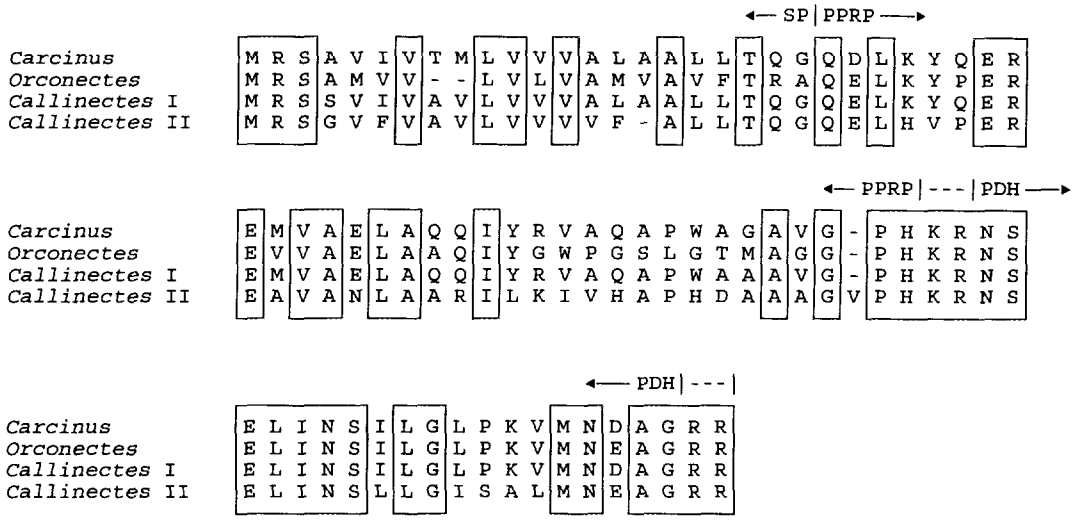

Fig. 2. Alignment of the prepro-PDHs from *Carcinus maenas* (9), *Orconectes limosus* (19), and *Callinectes sapidus*. All precursors consist of the signal peptide (SP), the PDH-precursor related peptide (PPRP) and the PDH. Identical positions are boxed. Blanks are inserted in order to get a maximum homology.

to the PDH of *Penaeus aztecus* and 67 % to the α-PDH in *Pandalus jordani* and *P. borealis*. In view of the Glu residue in position 3, PDH II can be considered to be a β-PDH analog.

A search in data bases for *Callinectes*-PPRP-like peptides showed only homology to the already known PPRPs of the *Carcinus*-PDH-precursor (9) and the PDH-precursor of *Orconectes limosus* (19). A comparison of the deduced amino acid sequences is presented in Fig. 2. The biological functions of PPRPs remain unknown.

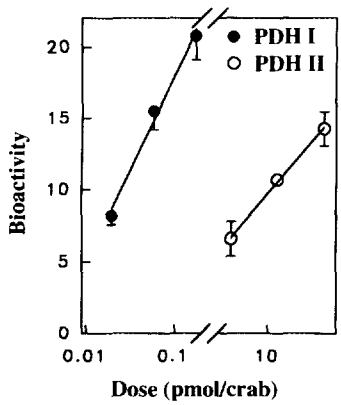

Fig. 3. Comparative dose-responses. Dose-related melanophore pigment-dispersing activity elicited by synthetic peptides, PDH I and PDH II. Each data point (mean ± SD) represents the mean of three groups of five crabs. The data points were used to plot a dose-response curve determined by first-order regression. Comparative potencies were determined by the quantities of peptide calculated to achieve a SIR of 10.

The presence of multiple PDHs, assessed in terms of separable active zones in ion-exchange chromatography, have been reported previously (20). So far, only in one crustacean species, the shrimp *Pandalus jordani*, were three isoforms purified from eyestalks (6). The present study is the first report of PDH-isoforms in a brachyuran crab. An explanation for the presence of multiple PDH-forms cannot be given yet.

A synthetic preparation of the newly identified *Callinectes*-PDH II displayed 400-fold less potency relative to *Callinectes*-PDH I (β-PDH) in assays for melanophorotropic activity in fiddler crabs (Fig. 3). The *Callinectes*-PDH II differs from β-PDH at six positions (8, 11, 12, 13, 14, and 17; Fig. 2). Of particular note are the radical substitutions occuring at positions 12 and 13 (Ser^{12}, Ala^{13}). All other characterized PDHs and PDFs possess Pro at position 12 and either Lys or Arg at position 13. Residue replacements at these positons led to reduction in potency: 25-fold loss when Ser^{12} was substituted for Pro^{12} (J.P. Riehm et al., unpublished); 3-fold loss when norvaline was substituted for Arg^{13} (21). A 7-fold reduction was noted when Glu^{17} was substituted for Asp^{17} (22). Previous tests with synthetic analogs indicated that the substitution of Leu^{8} for Ile^{8}, or Ile^{11} for Leu^{11}, or Leu^{14} for Val^{14} does not affect the potency of β-PDH (1). The reduced potency of *Callinectes*-PDH II appears to be attributable to the interactive effects of multiple residue substitutions.

On the basis of the sequence information of the two PDH-clones it will be possible to synthesize specific probes for PDH I and II. Currently experiments are in progress to investigate the expression of the PDH-genes in different tissues.

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