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Short Communication

# Mass spectrometric identification of pEGFYSQRYamide: A crustacean peptide hormone possessing a vertebrate neuropeptide Y (NPY)-like carboxy-terminus

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**Abstract**

In invertebrates, peptides possessing the carboxy (C)-terminal motif –R*X*RFamide have been proposed as the homologs of vertebrate neuropeptide Y (NPY). Using matrix assisted laser desorption/ionization mass spectrometry, in combination with sustained off-resonance irradiation collision-induced dissociation and chemical and enzymatic reactions, we have identified the peptide pEGFYSQRYamide from the neuroendocrine pericardial organ (PO) of the crab *Pugettia producta*. This peptide is likely the same as that previously reported, but misidentified, as PAFYSQRYamide in several earlier reports (e.g. [Li, L., Kelley, W.P., Billimoria, C.P., Christie, A.E., Pulver, S.R., Sweedler, J.V., Marder, E. 2003. Mass spectrometric investigation of the neuropeptide complement and release in the pericardial organs of the crab, *Cancer borealis*. J. Neurochem. 87, 642–656; Fu, Q., Kutz, K.K., Schmidt, J.J., Hsu, Y.W., Messinger, D.I., Cain, S.D., de la Iglesia, H.O., Christie, A.E., Li, L. 2005. Hormone complement of the *Cancer productus* sinus gland and pericardial organ: an anatomical and mass spectrometric investigation. J. Comp. Neurol. 493, 607–626.]). The –QRYamide motif contained in pEGFYSQRYamide is identical to that present in many vertebrate members of the NPY superfamily. Mass spectrometric analysis conducted on the POs of several other decapods showed that pEGFYSQRYamide is present in three other brachyurans (*Cancer borealis*, *Cancer irroratus* and *Cancer productus*) as well as in one species from another decapod infraorder (*Lithodes maja*, an anomuran). Thus, our findings show that at least some invertebrates possess NPY-like peptides in addition to those exhibiting an –R*X*RFamide C-terminus, and raise the question as to whether the invertebrate –QRYamides are functionally and/or evolutionarily related to the NPY superfamily.


*Keywords:* *Pugettia producta*; *Cancer borealis*; *Cancer irroratus*; *Cancer productus*; *Lithodes maja*; PAFYSQRYamide; Matrix assisted laser desorption/ionizaton fourier transform mass spectrometry (MALDI-FTMS); Sustained off-resonance irradiation collision-induced dissociation (SORI-CID); Pericardial organ (PO); Stomatogastric nervous system (STNS)

## 1. Introduction

The neuropeptide Y superfamily is one of the most well-studied peptide families in the animal kingdom (for reviews, see: Larhammar, 1996a,b; Larhammar et al., 1998; Hoyle, 1998; Hoyle, 1999; Cerda-Reverter and Larhammar, 2000; Holmgren and Jensen, 2001; Conlon, 2002; Larhammar and Salaneck, 2004; Conlon and Larhammar, 2005). In vertebrates, the neuropeptide Y (NPY) superfamily consists of the NPY, pancreatic polypeptide, peptide YY and polypeptide Y subfamilies. Members of each of these peptide subfamilies exhibit similar and

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highly conserved structures, many possessing the carboxy (C)-terminal motif –RQRYamide. Members of the NPY superfamily are widely distributed within vertebrate nervous systems; some members are also found in other tissues (*e.g.* pancreatic polypeptide). Physiological investigations have shown that the NPY superfamily is extremely pleiotropic, participating in the regulation of diverse physiological functions, including satiety and sexual behavior.

It has long been noted that antibodies generated against vertebrate NPY family members produce labeling in the nervous system as well as in other tissues, such as the gut, of invertebrates (*e.g.* Schoofs et al., 1988; Skuce et al., 1990). Using a vertebrate antibody to pancreatic polypeptide, Maule et al. (1991) isolated from the tapeworm *Moniezia expansa* a peptide possessing some sequence homology to members of the NPY family. The discovery of this peptide, PDKDFIVNPSDLVLDNKAA LRDYLRQINEYFAIIGRPRFamide, commonly termed neuropeptide F (NPF), was followed by the isolation of similar peptides, most possessing the C-terminus –R*X*RFamide, from other invertebrate species (*e.g.* Smart et al., 1992; Leung et al., 1992; Spittaels et al., 1996; Brown et al., 1999). The sequence similarity between the NPFs and members of the vertebrate NPY superfamily, as well recent molecular comparisons of protein and gene structure (both of preprohormones and peptide receptors), has led to the hypothesis that the NPFs are invertebrate homologs of the vertebrate NPYs (for reviews, see: Hoyle, 1999; de Jong-Brink et al., 2001; Hewes and Taghert, 2001; McVeigh et al., 2005). This hypothesis is further supported by physiological studies, which have shown that many functions served by NPY family members in vertebrates are likewise regulated by NPFs in invertebrates (*e.g.* Wu et al., 2003; Lee et al., 2004).

In our study, we used matrix assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS), in combination with sustained off-resonance irradiation collision-induced dissociation (SORI-CID) and chemical and enzymatic reactions, to identify a peptide from the neuroendocrine pericardial organ (PO) of the crab *Pugettia producta*. This peptide, pEGFYSQRYamide, possesses sequence homology to NPY, notably including the C-terminal motif –QRYamide. Using MALDI-FTMS, we show that this peptide is present in several other brachyuran species, and is present in at least one anomuran as well. Collectively, our results indicate that in some invertebrates, NPY-like peptides possessing C-termini identical to that of vertebrate NPY, but distinct from those of the NPF peptides, are present. These results thus raise the question as to whether these -QRYamides are also functionally and/or evolutionarily related to the NPY superfamily. Moreover, our identification of a crustacean peptide with a true NPY C-terminus raises the question as to whether previous mapping studies using antibodies to NPY family members in invertebrate tissues are detecting only the NPFs, or if they may be detecting a combination of NPFs and members of the -QRYamide family. Some of these data have appeared previously in abstract form (Messinger et al., 2006).

## 2. Materials and methods

### 2.1. Animals

Northern kelp crabs *P. producta* (Decapoda, Brachyura) were collected by hand off dock pilings and in kelp beds at Friday Harbor Laboratories (Friday Harbor, WA). Jonah crabs *Cancer borealis* (Decapoda, Brachyura), Atlantic rock crabs *Cancer irroratus* (Decapoda, Brachyura) and Northern stone crabs *Lithodes maja* (Decapoda, Anomura) were purchased from local seafood dealers in Brunswick, ME. Red rock crabs *Cancer productus* (Decapoda, Brachyura) were collected by hand or trap at multiple sites throughout the greater Puget Sound area of Washington State. Regardless of species, animals were kept in aerated natural seawater aquaria at 8–10 °C until dissection.

### 2.2. Tissue dissection and MALDI sample preparation

Before dissection, animals were anesthetized on ice for approximately 30 min. Tissue samples were dissected from the animal in chilled (10 °C) physiological saline (442 mM NaCl, 11 mM KCl, 13 mM $CaCl_2$, 26 mM $MgCl_2$, 12 mM Trizma base, 1.2 mM maleic acid, pH 7.4). POs were obtained by isolating the lateral walls of the pericardial chamber, pinning them in a wax-lined Pyrex dish, and manually dissecting the nerve roots comprising each PO from the surrounding connective and muscle tissues. Isolated POs were removed from the saline with fine forceps, rinsed sequentially in two 25 µl droplets of 0.75 M (135 mg/ml) fructose and placed on a face of the 10-facetted probe tip, minimizing co-transfers of solution. The tissue was sliced 10–20 times with a 0.1 mm needle, gathered together, covered with a 0.5 µl droplet of 2,5-dihydroxybenzoic acid matrix solution (DHB, Sigma–Aldrich, MO) (1.0 M DHB, 1:1 acetonitrile:2% phosphoric acid in water), and allowed to co-crystallize with the matrix at room temperature (approximately 20 °C).

### 2.3. Tissue extraction

Individual POs were extracted, delipidated and concentrated using the following procedure. A single PO was placed in 30 µl of extraction solvent (65% methanol, 5% acetic acid, and 30% water) and homogenized manually using Vannas-type spring scissors. The mixture was centrifuged for five minutes to form a pellet of any remaining tissue, the supernatant was transferred to a new microcentrifuge tube, and the remaining pellet was washed with 5 µl of nanopure water. Nanopure water (20 µl) was added to the supernatant followed by 25 µl of chloroform to form an organic and aqueous layer. The aqueous layer was removed and dried using a SpeedVac. Dried extracts were either dissolved in 50:50 acetonitrile:water for MALDI-FTMS analysis or were subjected to chemical derivatization or enzymatic digestion prior to analysis by MALDI-FTMS. For MALDI-FTMS, samples were prepared by mixing reconstituted extract 1:1 with 1.0 M DHB matrix (prepared as described earlier) on a face of a 10-facetted MALDI probe tip.

### 2.4. Chemical and enzymatic reactions

Acetylation was achieved by adding acetic anhydride (5 µl; Alltech, Deerfield, IL) and nanopure water (2.5 µl water) to a dried extract from a single PO for one hour. Enzymatic digestion used pyroglutamate aminopeptidase (10 mU, Sigma–Aldrich, St. Louis, MO) reconstituted in 50 µl of buffer solution (50 mM sodium phosphate, pH 7.0, 10 mM DDT, 1 mM EDTA). Enzyme solution (5 µl) was added to a dried extract from a single PO and reacted overnight at 37 °C. Samples were prepared for MALDI-FTMS as described earlier.