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Purification and characterization of red pigment concentrating hormone from the Alaska pink shrimp *Pandalus eous*

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Abstract

Crustacean body color is controlled by a red pigment concentrating hormone (RPCH), which concentrates pigment granules in the chromatophore. In this study, RPCH in the sinus gland of the Alaska pink shrimp *Pandalus eous* was purified by reversed-phase HPLC and structurally determined. The amino acid sequence of the characterized *P. eous* RPCH (Pae-RPCH) was pELNFSPGW-NH_2, identical to those of decapod RPCHs known to date. In an *in vivo* bioassay, injection of Pae-RPCH dramatically changed the body color of shrimps from red to white. This bioassay using *P. eous* was easier and more sensitive than similar experiments conducted previously in other decapod crustaceans.

Key words: red pigment concentrating hormone; chromatophore; body color; sinus gland; eyestalk; *Pandalus eous*

Introduction

The body color of crustaceans is regulated by the movement of pigment granules in the epidermal chromatophore. The concentration and dispersion of pigment in the chromatophore are controlled by chromatophorotropic hormones produced and stored in the X-organ/sinus gland complex of the eyestalk (Katayama et al. 2013). In 1972, a red pigment concentrating hormone (RPCH) was purified from the northern shrimp *Pandalus borealis* and its primary structure was determined (Fernlund and Josefsson 1972). The northern shrimp RPCH was an octapeptide with an N-terminal pyroglutamylation and C-terminal amidation. This molecule was the first peptide hormone to be structurally determined in invertebrates. In 1976, an adipokinetic hormone (AKH) was characterized from the corpus cardiacum of the desert locust *Schistocerca gregaria* (Stone et al. 1976). The desert locust AKH was also composed of eight amino acid residues, and the N- and C-termini were modified by the same way as the shrimp RPCH. Moreover, AKH was observed to show high amino acid sequence similarity with RPCH, thus establishing an AKH/RPCH family (Gäde 2009). Until now, over 40 different types of AKH/RPCH family peptides were identified from many insect species. Since insect AKH/RPCH family peptides showed various biological activities, these molecules are

referred to as AKH, hypertrehalosemic hormone (HrTH) and cardioaccelerating hormone (CAH) (Gäde 2009). RPCHs isolated and structurally determined in over 10 decapod crustacean species had the same amino acid sequence as that of *P. borealis* RPCH (Gäde 2009). On the other hand, genome and comprehensive sequence analyses revealed RPCHs of *Daphnia pulex* and *D. magna* in the order Anomopoda to have different amino acid sequences (Colbourne et al. 2005; Christie et al. 2008). Therefore, more diverse RPCHs may exist in other crustaceans other than decapods and anomopods.

The Alaska pink shrimp *Pandalus eous* is a commercially important species in Japan. This shrimp species is widely distributed in the northern parts of the Pacific Ocean, including the Sea of Japan, the Okhotsk and Bering Seas, and along the coasts of Alaska and Canada. The Alaska pink shrimp *P. eous* in the Pacific Ocean and the northern shrimp *P. borealis* in the Atlantic Ocean were thought to be the same species for a long time. In 1992, however, it was suggested that *P. eous* and *P. borealis* were different species because of morphological differences between their adults and differences in the size of their larvae (Squires 1992). Until now, there have been no studies on eyestalk hormones using *P. eous*, although studies using *P. borealis* have been conducted for many years (Christie et al. 2010).

The Alaska pink shrimp *P. eous* is suitable for the study of RPCH because of the erythrophores distributed on its entire body surface. However, a RPCH molecule has never been characterized from *P. eous*. In this article, we isolated and structurally determined a RPCH molecule from the sinus glands of *P. eous.* We also examined the biological activity of this *P. eous* RPCH by *in vivo* injection of the synthetic peptide into live shrimps.

Materials and Methods

Animals

Live adult Alaska pink shrimps *P. eous* weighing an average of 19.36 g were purchased from Sado Kaiyo Shinsosui Co., Ltd. (Niigata, Japan). Sinus glands were dissected from shrimps under a stereo microscope using the same technique described previously (Yang et al. 1995) and stored at -20°C in a solution of 30 % acetonitrile/0.9 % NaCl until extraction.

Extraction and purification of *P. eous* red pigment concentrating hormone (Pae-RPCH)

Sinus glands were homogenized, and subsequently peptides were extracted (Yang et al. 1995). The extract was subjected to solid-phase extraction (Ohira et al. 2006). The eluate was concentrated by a centrifugal evaporator and applied to reversed-phase HPLC (RP-HPLC) separation on a Senshu Pak PEGASIL C4 SP100 column (4.6 × 250 mm, Senshu Scientific, Tokyo, Japan) with a 40-min linear gradient of 10-60 % acetonitrile in 0.05 % trifluoroacetic acid (TFA), a 1-min linear gradient of 60-80 % acetonitrile in 0.05 % TFA, and 5-min holding at 80 % acetonitrile in 0.05 % TFA at a flow rate of 1 mL/min. Column effluent was monitored at 225 nm, and each peak eluate was collected into 1.5 mL tube. For increasing the purity of Pae-RPCH, a peak fraction collected in the first RP-HPLC were subjected to a second RP-HPLC under the same conditions except that a Capcellpak C18 MGII column (4.6 × 150 mm, Osaka-Soda, Osaka, Japan) was used instead of the Senshu Pak PEGASIL C4 SP100 column.

Mass spectrometry analysis

Mass spectra of the purified native Pae-RPCH and an enzymatic digest of Pae-RPCH by pyroglutamate aminopeptidase digestion (see

below) were measured on a matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (AXIMA®-CFR, Shimadzu, Kyoto, Japan) with α-cyano-4-hydroxycinnamic acid (Tokyo Chemical Industry, Tokyo, Japan) as a matrix in the positive ion mode.

Pyroglutamate aminopeptidase digestion

The native Pae-RPCH (800 pmol) was dissolved in 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 10 mM dithiothreitol (DTT), and then incubated with 0.2 mU of *Pfu* pyroglutamate aminopeptidase (Takara Bio, Otsu, Japan) at 50°C for 12 h. To this solution, an equal amount of the same enzyme was added, and the mixture was incubated again for another 12 h. To stop digestion, half volume of 1 M HCl was added. The reaction product was applied to RP-HPLC under the same conditions as used in the second round of purification of the native Pae-RPCH.

N-terminal amino acid sequencing

N-terminal amino acid sequences of the native Pae-RPCH and the enzymatic digest were analyzed on an Applied Biosystems model 491HT protein sequencer (Applied Biosystems, Foster City, CA, USA) in the pulsed-liquid mode.

***In vivo* bioassay for RPCH**

Synthetic Pae-RPCH (pELNFSPGW-NH_2) was prepared by Fmoc-based solid-phase peptide synthesis (SPPS) and purified by RP-HPLC. The synthetic Pae-RPCH was lyophilized and dissolved with saline solution. Two doses of synthetic Pae-RPCH (0.1 and 1.0 ng/individual, respectively) and saline solution, which was used as the negative control, were injected into four shrimps in each group. All injected shrimps were kept in aerated seawater at 2-3°C. Pictures of shrimps in each treatment group were taken before and 30 min after injection.

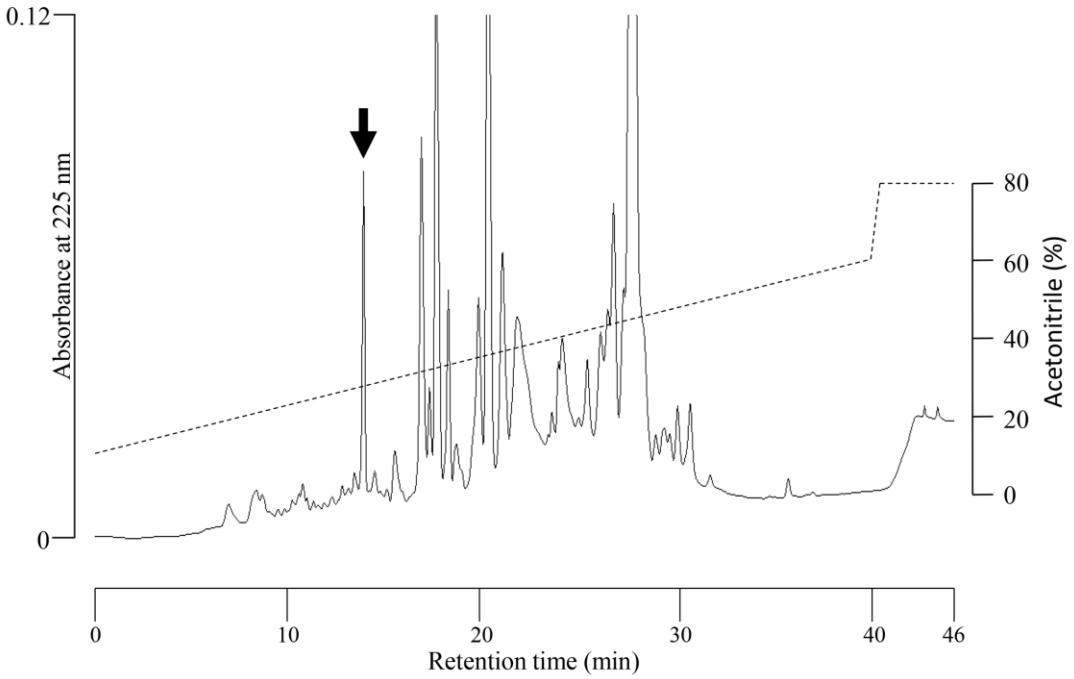

Fig. 1. RP-HPLC chromatogram of the sinus gland extracts from 143 eyestalks of the Alaska pink shrimp *Pandalus eous*. The dotted line and arrow indicate the concentration of acetonitrile and the peak fraction containing Pae-RPCH, respectively.

Results

Sinus gland extract from *P. eous* was fractioned by RP-HPLC (Fig. 1). Mass spectra of all recovered peak products analyzed by MALDI-TOF MS indicated that a single peak fraction contained RPCH, since its detected molecular ion peak (observed at *m/z* 952.48 $[M+Na]^+$) was close to the mass range of the AKH/RPCH family peptides (920-1,160) (Gäde 2009). The purity of this peak fraction was insufficient, because non-relevant mass spectra were also observed by MALDI-TOF MS analysis. Therefore, peak products were further separated by RP-HPLC using another column and a single peak fraction was collected (Fig. 2). A peptide in this peak fraction was subjected to N-terminal amino acid sequence analysis, but no sequence data was obtained. This result strongly suggested that the N-terminus of the peptide was blocked. Since N-terminal amino acids of all molecules belonging to the AKH/RPCH family are pyroglutamates (Gäde 2009), the peptide was digested by *Pfu* pyroglutamate aminopeptidase. As expected, a deblocked peptide was afforded (Fig. 3) and subsequently its amino acid sequence (residues 2–8) was identified as LNFSPGW. The C-terminal Trp residue was found to be amidated since a protonated monoisotopic ion peak was observed at *m/z* 819.32 in MALDI-TOF MS analysis (calculated value 819.39 $[M+H]^+$). Thus, the amino acid sequence of this peptide was determined as pELNFSPGW-NH_2. The molecular mass calculated from the determined structure (952.41 $[M+Na]^+$) is also consistent with the mass spectrometry result described above. The determined amino acid sequence was identical to the sequences of RPCH molecules previously characterized in decapod crustaceans, and

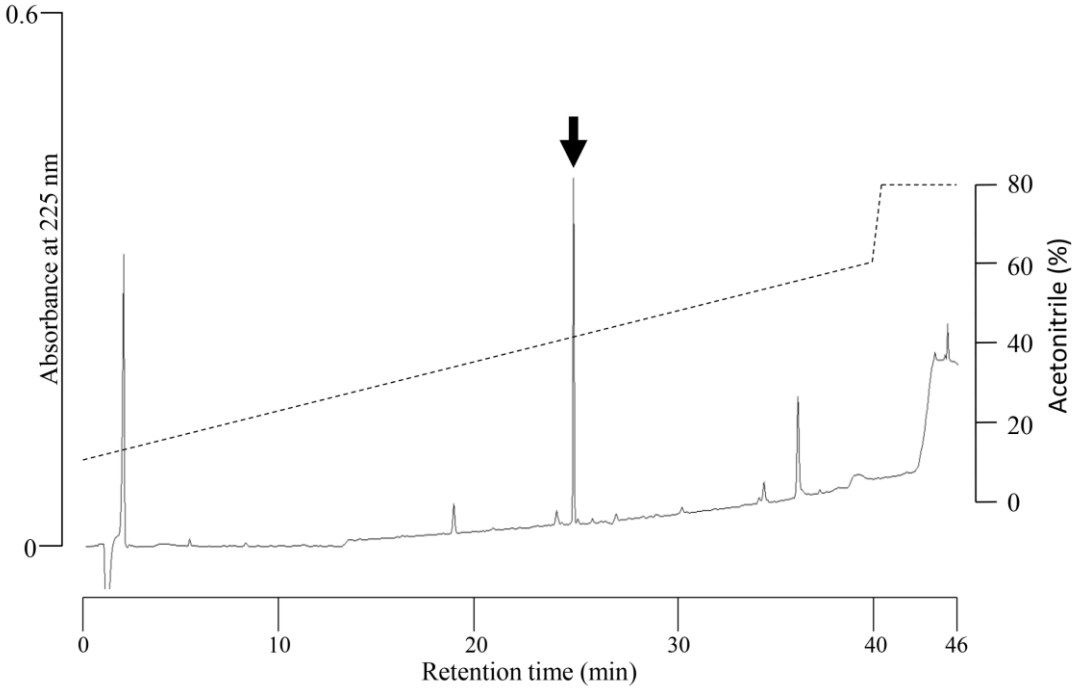

Fig. 2. RP-HPLC chromatogram of the peak fraction recovered from the first separation (Fig. 1). The dotted line and arrow indicate the concentration of acetonitrile and the peak fraction containing Pae-RPCH, respectively.

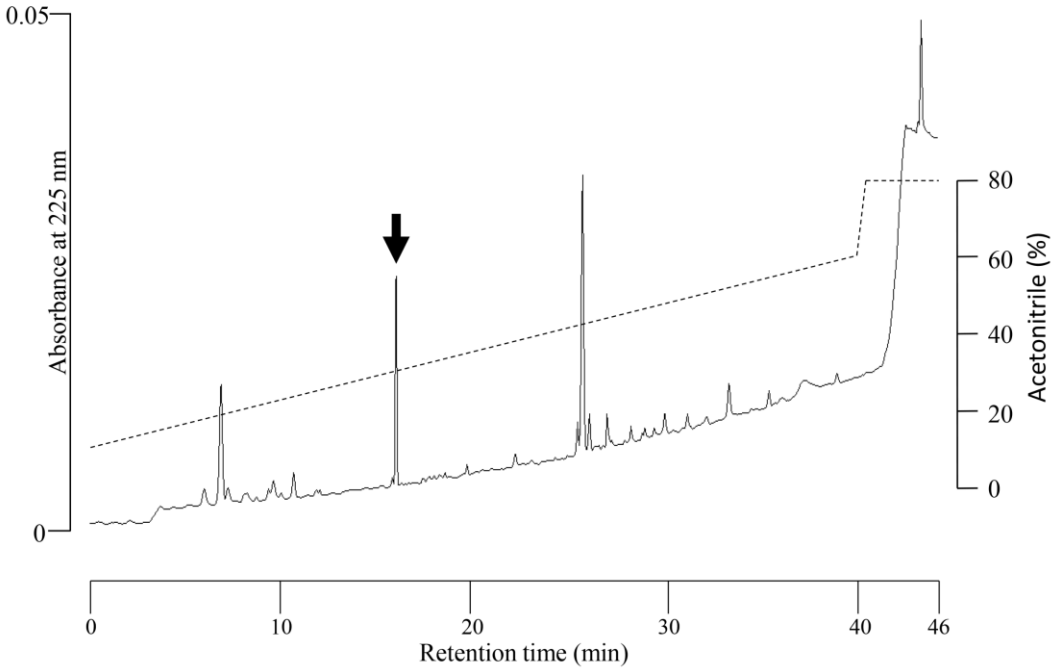

Fig. 3. RP-HPLC chromatogram of the deblocked Pae-RPCH by *Pfu* pyroglutamate aminopeptidase digestion. The dotted line and arrow indicate the concentration of acetonitrile and the peak fraction containing the deblocked Pae-RPCH, respectively.

therefore this peptide was designated as Pae-RPCH in this study.

Biological activity of Pae-RPCH was examined by an *in vivo* injection assay. Since the amount of purified native Pae-RPCH from the sinus glands was limited and insufficient for the injection assay, synthetic Pae-RPCH was prepared chemically. Injection of 1.0 ng of synthetic Pae-RPCH dramatically changed body color from red to white in all tested shrimp (Fig. 4, panels C and c). The effect of 0.1 ng Pae-RPCH was also significant, but their body color was slightly more pinkish than that of 1.0 ng injection (Fig. 4, panels B and b). In contrast, injection of saline solution as a negative control induced no change to shrimp body color (Fig. 4, panels A and a).

Discussion

In this study, a RPCH, named as Pae-RPCH, was isolated from the sinus glands of the Alaska pink shrimp *P. eous*. The amino acid sequence of Pae-RPCH, pELNFSPGW-NH_2, was completely identical to that of another decapod crustacean RPCH, previously determined (Gäde 2009). Although RPCH molecules have been characterized from more than 10 decapod crustacean species, their amino acid sequences are completely conserved. On the other hand, amino acid sequences and peptide lengths of insect AKH/RPCH family peptides are diverse. In dipteran insects, for example, there are three types of AKH with different amino acid sequences (Gäde 2009). In addition, lepidopteran insects have three types of AKH/RPCH family peptides (AKH and HrTH) consisting of 10 amino acids and have a different peptide length from dipteran AKHs (Gäde 2009). The insect AKH and HrTH act to mobilize lipids and carbohydrates from storage into the hemolymph for energy demands such as during long flights and fasting. Thus, the role of insect AKH and HrTH are very important, but their amino acid sequences vary. In general,

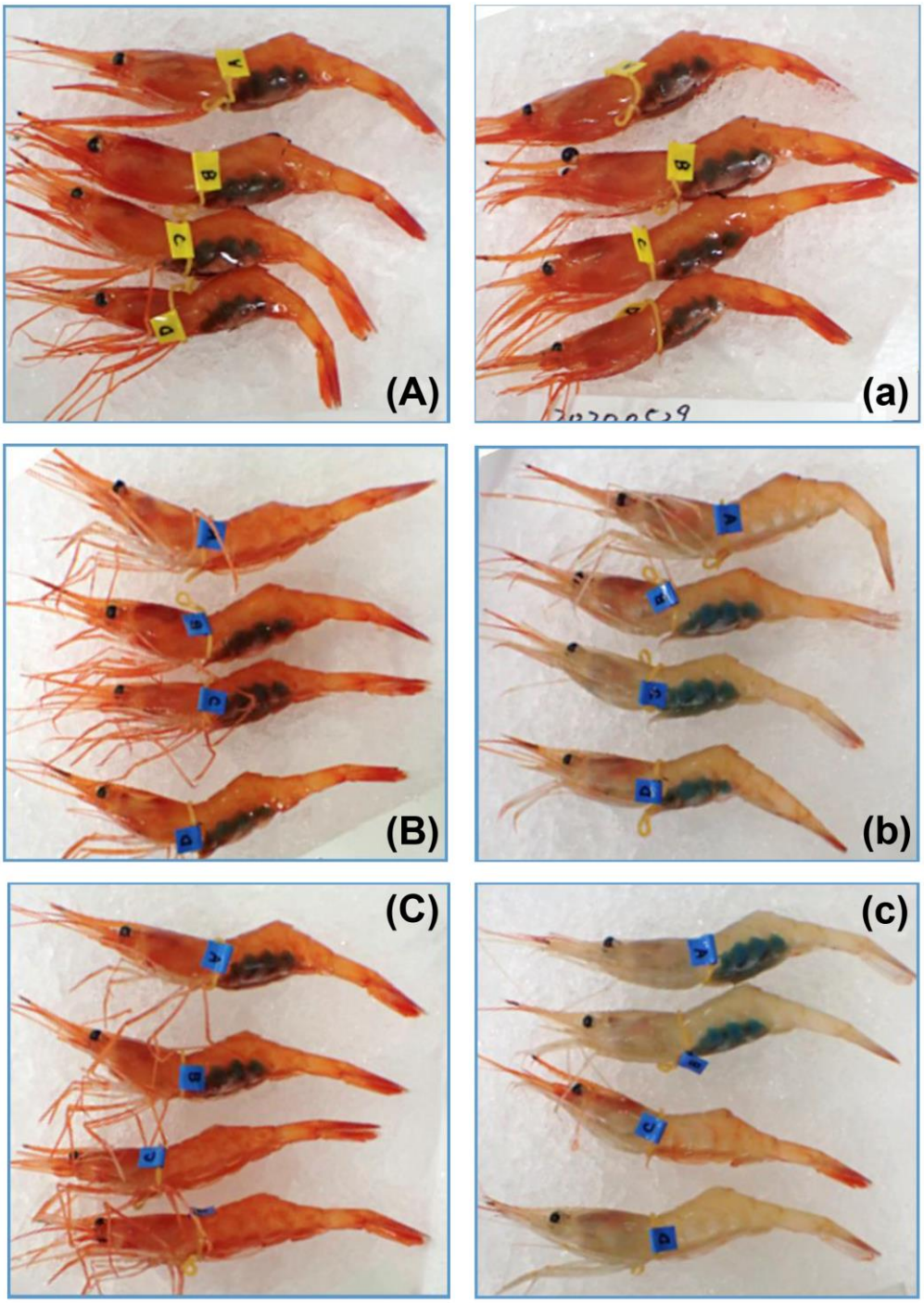

Fig.4. Pictures of the Alaska pink shrimps before injection (A, B, and C) and 30 min after injections of saline solution (a), 0.1 ng (b) and 1.0 ng (c) of the synthetic Pae-RPCH.

amino acid sequences are conserved in molecules that have essential roles. The currently known function of RPCH is to change body color by concentrating pigment granules in the chromatophore. However, the amino acid sequence of RPCH from the spiny-cheek crayfish *Orconectes limosus*, which displays little change in body color, is also conserved (Gaus et al. 1990). This suggests that RPCH may have important physiological functions other than the regulation

of body color. Recently, a RPCH receptor was identified and shown to be highly expressed in the ovary of the green shore crab *Carcinus maenas* (Alexander et al. 2018). It was also reported that RPCH promoted ovarian growth of the whiteleg shrimp *Litopenaeus vannamei* (Chen et al. 2018). These results suggest that RPCH may play an important role in controlling ovarian maturation in addition to regulation of body color.

The biological activity of RPCH was examined by an *in vivo* injection bioassay. In the bioassay for Japanese tiger shrimp *Marsupenaeus japonicus*, both eyestalks were ablated one day before RPCH injection to artificially disperse pigment granules in the chromatophore (Yang et al. 1999). In the examination of *C. maenas* RPCH activity, PDH was pre-injected 40 min before RPCH injection (Alexander et al. 2018). In *P. eous*, on the other hand, pigment granules in the erythrophore are naturally dispersed. Therefore, RPCH was injected into untreated shrimps in this study. As a result, the body color of injected shrimps changed considerably, from red to white (Fig. 4). From this result, it can be inferred that *P. eous* does not require any pretreatments for the RPCH bioassay. In addition, the injection of only 0.1 ng of RPCH induced a significant change in body color. In a study of *C. maenas*, body color of a crab injected with 0.1 pmol (0.093 ng) of RPCH was almost the same as that of an animal administered with saline, which was used as the negative control (Alexander et al. 2018). Considering these results, the bioassay system using *P. eous* for RPCH activity is more simple and more sensitive than that used for other decapod crustacean species examined previously.

Conclusion

In this study, Pae-RPCH was purified from the sinus glands of the Alaska pink shrimp *P. eous* and its amino acid sequence was determined. In addition, synthetic Pae-RPCH was injected into intact shrimps and its biological activity was assessed. The bioassay system established in this study was easier and more sensitive than assays performed previously in the other decapod crustacean species. Therefore, it is considered that this bioassay can be a useful tool for elucidating the structure-activity relationship of AKH/RPCH family peptides.

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