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A convenient method for preparation of biologically active recombinant CHH of the kuruma prawn, *Marsupenaeus japonicus*, using the bacterial expression system

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ABSTRACT

Crustacean hyperglycemic hormone (CHH) not only plays an important role in the modulation of hemolymph glucose level but also functions in other biological events including molting, reproduction and stress response. Of the six CHHs characterized in *Marsupenaeus japonicus*, an expression system for recombinant Pej-SGP-VII (rPej-SGP-VII-amide) has not yet been established. Here, we established a procedure using a Nus-tag for solubilization, thereby soluble and biologically active rPej-SGP-VII-amide could successfully be obtained by a simpler procedure than previous ones used for producing other recombinant Pej-SGPs (Pej-SGP-I, III and IV). It was found that rPej-SGP-VII-amide thus obtained had the correct arrangement of intramolecular disulfide bonds and helix-rich secondary structure. The established expression system for rPej-SGP-VII-amide may be applicable for the preparation of other recombinant CHHs.

1. Introduction

In crustaceans, the major neuroendocrine system is located within the X-organ/sinus gland complex in the medulla terminalis of the eyestalk. The CHH-family [20] identified from the sinus gland is a group of neuropeptides that includes various neuropeptides such as crustacean hyperglycemic hormone (CHH) [19], molt-inhibiting hormone (MIH) [41], vitellogenesis/gonad-inhibiting hormone (VIH/GIH) [37], and mandibular organ-inhibiting hormone (MOIH) [40]. Since CHH increases hemolymph glucose levels in crustaceans, this peptide hormone is thought to modulate hemolymph glucose level. In addition, it has been reported that CHH is involved in several other biological events such as reproduction [7], molting [3,4], lipid metabolism [36], and stress response [24]. Although more than 100 peptides belonging to the CHH-family have been identified [2], CHH-family peptides have never been found in organisms other than Arthropods.

Six Cys residues forming three intramolecular disulfide bonds are completely conserved among the members of the

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Abbreviations: HEPES, 2-(4-[2-hydroxyethyl]-1-piperazinyl)ethanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline; RP-HPLC, reverse-phase high performance liquid chromatography; SDS, sodium dodecylsulfate; SGP, sinus gland peptide; TFA, trifluoroacetic acid.

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CHH-family of peptides. The CHH-family of peptides is classified into two subtypes, type I and type II based on their primary structures [43] and cDNA sequences [2,9,21]. Most type I peptides are often post-translationally modified by amidation at their C-terminus. Concerning the structure–function relationship, most CHHs are type I, while most MIHs, VIHs and MOIHs are type II, indicating that these subtypes coincide well with their biological activities, probably due to the structural differences.

In the kuruma prawn, *Marsupenaeus japonicus*, eight CHH-family peptides have been isolated and identified [28,31,35,42,43,44]. Seven of these peptides have been identified from the SG extract and designated Pej-SGP-I to -VII depending on their elution order from reverse-phase high performance liquid chromatography (RP-HPLC). Pej-SGP-VII is different from other peptides in that 2 M urea is required for extraction [35]. We previously reported that Pej-SGP-I, II, III, V, VI and VII are type I peptides and exhibit hyperglycemic activity [28,43], while Pej-SGP-IV is type II and exhibits molt-inhibiting activity [44]. Therefore, we have designated Pej-SGP-IV as a MIH and the others as CHHs in *M. japonicus*. However, each peptide does not always correspond to a single biological activity. For example, Pej-SGP-V and VI also exhibit weak molt-inhibiting activity as well as hyperglycemic activity [34]. Similarly, Pej-SGP-III has vitellogenesis-inhibiting activity [39]. Therefore, it is likely that these peptides might exert pleiotropic functions *in vivo*. The exact assignment of each peptide's biological function remains to be elucidated.

Because the amount of CHH-family peptides obtained from a SG extract is extremely small, a significant amount of recombinant peptides, identical to natural peptides in terms of chemical structure and biological activity, are required for functional analyses. Although several expression systems for recombinant CHH-family peptides from crustacean species have already been established, these systems have sizeable drawbacks. For example, in the *Escherichia coli* expression systems [11,12,17,26,32], since the expressed proteins aggregate in an insoluble form, an inefficient refolding step is needed to obtain biologically active recombinant peptides; consequently, the final product is obtained in a poor yield. Although the expressed proteins are soluble and obtained in a biologically active form in the yeast [22,38] or baculovirus/insect cell [23] expression systems, their yields are much less than those obtained in *E. coli*. In addition, it has not been determined whether the structural characteristics, including secondary structure and disulfide bond arrangement, and biological activities of these recombinant peptides are comparable to the natural ones. Most recombinant CHHs have lower hyperglycemic activity than the natural ones, probably due to the lack of amidation at the C-terminus.

In our previous studies, expression systems for recombinant Pej-SGP-I, III and IV peptides were established [17,30,32]. However, these systems are, as described above, problematic and not applicable to the production of the other Pej-SGPs. Therefore, we aimed at establishing a simpler and more efficient system than the previous ones to prepare any recombinant CHH in *M. japonicus*. Since Pej-SGP-VII is the most abundant of the Pej-SGPs in the SG extract and has both relatively strong hyperglycemic activity and vitellogenesis-inhibiting activity (unpublished data), we selected Pej-SGP-VII as a target molecule.

In this report, a method for preparation of soluble and biologically active recombinant Pej-SGP-VII (rPej-SGP-VII) is described. Taking advantage of higher expression, we selected an *E. coli* expression system. Also, to overcome the difficulties of product insolubility, we adopted a relatively new pET expression system commercially available that was designed to increase solubility of insoluble recombinant proteins in *E. coli* and to be expressed as a cleavable fusion protein with NusA [6].

2. Materials and methods

2.1. Animals

Kuruma prawns *M. japonicus* (body weight, approximately 20 g) were purchased at a fish market in Tokyo, Japan. The prawns were kept in a tank with natural seawater at 20 °C during the experiment as reported previously [31].

2.2. Construction of the expression plasmid for rPej-SGP-VII

Total RNA was extracted from the eyestalk using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription (RT) with total RNA (1 μg) was carried out using SuperScript III Reverse Transcriptase (Invitrogen). The first strand cDNA was stored at −20 °C until needed.

Fig. 1 shows the construction method for the rPej-SGP-VII-Gly expression plasmid. Polymerase chain reaction (PCR) was performed with SGP-VII-F and SGP-VII-R primers using the eyestalk cDNA. The following program was used for PCR amplification; 20 s (80 s only for the first cycle) at 94 °C, 30 s at 52 °C and 30 s at 72 °C, for 30 cycles. Two oligonucleotide primers were designed based on the nucleotide sequence of the Pej-SGP-VII cDNA. The forward primer (SGP-VII-F: 5′-*CCCGGG*CAGCCTTCGACCCGTCCT-3′) includes the Sma I site (italicized). The reverse primer (SGP-VII-R: 5′-*GAATTC*C**TA**GCCCACCGTCTGCACGAGGGC-3′) includes the EcoR I site (italicized), stop codon (bold), and three additional bases (underlined) after the stop codon encoding the amidating donor residue, Gly. Since Pej-SGP-V cDNA was also amplified involuntary, the amplified PCR product was treated with Mva I to specifically remove Pej-SGP-V cDNA from the products before subcloning into a pGEM-T Easy vector (Promega, Madison, WI, USA). Then the inserted nucleotide sequence was confirmed. Subsequently, the Sma I/EcoR I site containing the Pej-SGP-VII-Gly cDNA sequence was digested with these restriction enzymes, and the digested cDNA was inserted into the Sma I/EcoR I-digested pET-44a (+) (Novagen, Madison, WI, USA).

2.3. Expression of His-Nus-His-tagged rPej-SGP-VII-Gly

Competent cells of *E. coli* strains, BL21 (DE3), BL21 (DE3) pLysS (Novagen), BL21 Star (DE3) (Invitrogen), BL21-CodonPlus (DE3) (Stratagene, La Jolla, CA, USA) and Rosetta-gami (DE3)