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Crystallization and preliminary X-ray analysis of crustacean hyperglycaemic hormone from the kuruma prawn *Marsupenaeus japonicus* in its weakly active precursor form

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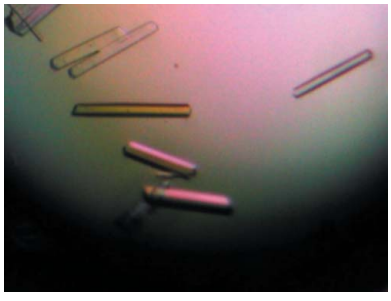
Crustacean hyperglycaemic hormone (CHH) plays a pivotal role in the regulation of glucose metabolism in crustaceans. Pej-SGP-I, one of the six known CHHs in the kuruma prawn *Marsupenaeus japonicus*, was heterologously expressed in *Escherichia coli* as an N-terminally His-tagged and Nus-tagged protein in its weakly active precursor form, Pej-SGP-I-Gly, which has an extra glycine residue at the C-terminus. The recombinant peptide was subjected to affinity purification, tag removal, further purification and crystallization by the sitting-drop vapour-diffusion method using NaCl as the main precipitant. The crystals diffracted to 1.95 Å resolution and the space group was assigned as primitive orthorhombic $P2_12_12_1$, with unit-cell parameters a = 40.19, b = 53.65, c = 53.63 Å. The Matthews coefficient (V_M = 1.73 Å^3 Da^{-1}) indicated that the crystal contained two Pej-SGP-I-Gly molecules per asymmetric unit, with a solvent content of 29.0%.

1. Introduction

The X-organ/sinus gland complex is a centre of the endocrine control system in decapod crustaceans; a variety of peptide hormones are synthesized in the X-organ and are then stored in and secreted from the sinus gland. The major group of peptide hormones produced in the X-organ/sinus gland complex are known as the crustacean hyperglycaemic hormone (CHH) family and includes CHH, moult-inhibiting hormone (MIH), mandibular organ-inhibiting hormone (MOIH) and vitellogenesis-inhibiting hormone (VIH). The CHH-family members regulate various physiological processes. CHH is involved in carbohydrate metabolism by increasing haemolymph glucose levels (Keller & Wunderer, 1978), MIH regulates moulting by suppressing the synthesis of ecdysteroid (moulting hormone) in the Y-organ (Webster, 1991), MOIH inhibits the synthesis of methyl farnesoate (a putative crustacean juvenile hormone) in the mandibular organ (Wainwright *et al.*, 1996) and VIH regulates reproduction by inhibiting vitellogenin synthesis (Soyez *et al.*, 1987). Peptides belonging to the CHH family have also been characterized in noncrustacean species, including ion-transport peptide in insects (Dircksen, 2009), CHH-like peptide in the nematode *Caenorhabditis elegans* (Chen *et al.*, 2005) and low-molecular-weight protein in black widow spider (Gasparini *et al.*, 1994). Our research goal is to reveal how the CHH-family peptides regulate various physiological processes in crustaceans.

In most cases, CHH-family peptides comprise 72–78 amino-acid residues and contain six Cys residues which form three intramolecular disulfide bonds. The CHH-family members can be classified into two types, types I and II, based on their primary structures, specifically the absence (type I) or presence (type II) of a Gly residue at position 12 in the mature peptide, and the presence (type I) or absence (type II) of the CHH precursor-related peptide in their precursors (Yang *et al.*, 1996, 1997; Lacombe *et al.*, 1999; Chen *et al.*, 2005). Type I peptides often possess an amidated C-terminus, which is post-translationally modified by α-amidating enzyme from an extra Gly residue at the C-terminus and is significant for maximal bioactivity. Generally, CHH belongs to type I, whereas MIH, MOIH and VIH belong to type II, although there are some exceptions (Chang *et al.*, 1990; Aguilar *et al.*, 1996; Liu *et al.*, 1997; Marco *et al.*, 2000).



Tertiary-structure analysis of the CHH-family peptides will provide clues to their modes of action and their functional specificities. However, only one structure has been reported for the CHH-family peptides: the NMR solution structure of a type II peptide, Pej-MIH, from the kuruma prawn *Marsupenaeus japonicus* (PDB entry 1j0t; Katayama *et al.*, 2003). The locations of the six Cys residues and the arrangements of the three disulfide bonds are conserved among the CHH family, suggesting a common fold in type I and II CHH-family peptides (Katayama *et al.*, 2003). Therefore, subsequent structure predictions of other CHH-family peptides (Chen *et al.*, 2005; Nagaraju *et al.*, 2009, 2011) have been performed based on the Pej-MIH structure. However, the amino-acid sequence is not well conserved between types I and II, which will produce some structural differences between them, and such differences could be clarified by structural analysis of the type I peptide itself instead of homology modelling based on the Pej-MIH structure.

The sinus gland of *M. japonicus* contains at least eight CHH-family peptides. Six of them, Pej-SGP-I, Pej-SGP-II, Pej-SGP-III, Pej-SGP-V, Pej-SGP-VI and Pej-SGP-VII, are classified as type I and have been characterized as CHHs (Yang *et al.*, 1997; Nagasawa *et al.*, 1999) and also as VIHs (Pej-SGP-III, Tsutsui *et al.*, 2005; Pej-SGP-I, Pej-SGP-II, Pej-SGP-V, Pej-SGP-VI and Pej-SGP-VII, unpublished data), whereas two type II peptides, Pej-MIH (Pej-SGP-IV) and Pej-MIH-B, have been characterized as MIHs or MIH-like (Yang *et al.*, 1996; Ohira *et al.*, 2005). To reveal the structural basis for the functional differences between type I and type II peptides, tertiary structural information on a type I peptide is a prerequisite. We therefore initiated X-ray crystallographic analysis of Pej-SGP-I, a representative type I CHH-family peptide. Mature type I CHH-family peptides possess a C-terminal amide and show tenfold to 100-fold higher hyperglycaemic activity than their precursor peptides that have a Gly residue instead of the C-terminal amide (Katayama *et al.*, 2002; Ohira *et al.*, 2003; Nagai *et al.*, 2009). However, the yield of C-terminal amidation *in vitro* was as low as 30%. Thus, we selected Pej-SGP-I-Gly, the precursor form of Pej-SGP-I, as the initial target for crystallization. Here, we report the crystallization and preliminary X-ray analysis of Pej-SGP-I-Gly.

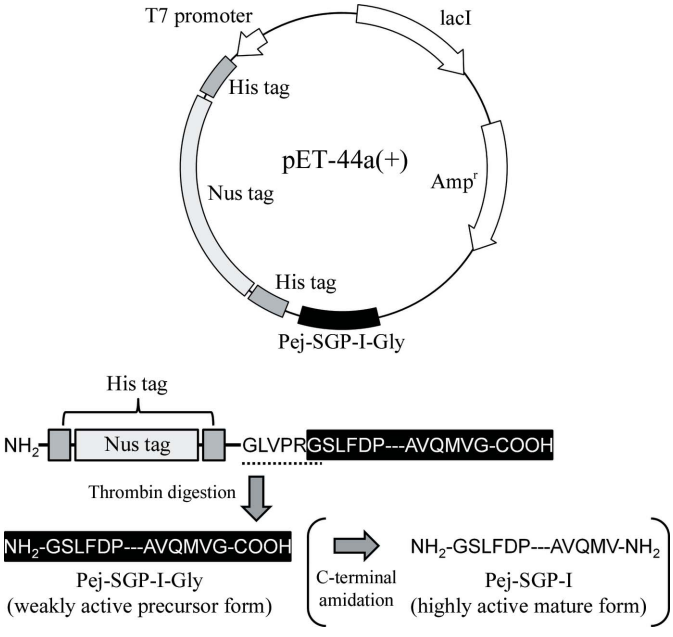


Figure 1
Schematic diagram of the expression vector and preparation steps for recombinant Pej-SGP-I-Gly. The Nus and His tags are shown as light grey and dark grey boxes, respectively. The black box represents Pej-SGP-I-Gly. The dotted underline indicates the thrombin recognition region. The C-terminal amidation shown in parentheses was not performed in this study. Amp^r, β-lactamase gene conferring ampicillin and carbenicillin resistance; lacI, *lacI* gene encoding *lac* repressor protein.

2. Materials and methods

2.1. Protein expression and purification

The recombinant Pej-SGP-I-Gly peptide was prepared according to a recently reported method (Nagai *et al.*, 2009) with slight modifications. The forward primer (5′-*CCCGG**GGC**TCGCTCTTCGACC CTT-3′) was designed to include a *Sma*I restriction site (shown in italics) and an additional three bases which encode a Gly residue (bold), and an *Eco*RI restriction site (shown in italics) was attached to the reverse primer (5′-*GAATTC*CTACCCGACCATCTGTAC-3′). The polymerase chain reaction (PCR) was performed using these two primers and a previously prepared template cDNA for Pej-SGP-I (Katayama *et al.*, 2002). The resultant PCR product was subcloned into pGEM-T Easy vector (Promega, Madison, Wisconsin, USA). After verification of the nucleotide sequence, the plasmid was digested with *Eco*RI and *Sma*I and the object DNA fragment was inserted into a *Sma*I/*Eco*RI-digested pET-44a(+) vector (Novagen, Madison, Wisconsin, USA) to express N-terminally Nus-tagged Pej-SGP-I-Gly (Fig. 1). *Escherichia coli* strain BL21 (DE3) STAR (Invitrogen, Carlsbad, California, USA) was then transformed with the recombinant plasmid. The bacterial cells were grown in 1 l Luria–Bertani medium containing 50 mg carbenicillin (Nacalai Tesque, Kyoto, Japan) at 310 K. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 m*M* when the OD_{600} reached approximately 0.6 and the culture was then continued for a further 4 h at 300 K. The cells were collected by centrifugation, resuspended in thrombin buffer (50 m*M* Tris–HCl, 150 m*M* NaCl, 2.5 m*M* $CaCl_2$ pH 7.1) containing 0.2 *M* NaCl and disrupted by sonication. After centrifugation at 7000*g* for 10 min at 277 K, the supernatant was filtered using a 0.45 µm filter, mixed with 7 ml (bed volume) Ni Sepharose 6 Fast Flow (GE Healthcare, Uppsala, Sweden) pre-equilibrated with the same buffer in a 50 ml tube and incubated at 277 K for 16 h with gentle agitation. The resin was transferred to an empty column (23 mm internal diameter × 100 mm), washed with thrombin buffer containing 0.4 *M* NaCl and 1 m*M* imidazole and subsequently equilibrated with thrombin buffer. To liberate Pej-SGP-I-Gly from the His-Nus-His-tag moiety (Fig. 1), on-column digestion was performed using 160 U thrombin (GE Healthcare) at 292 K for 48 h; the column was placed in a polypropylene container filled with nitrogen gas to prevent methionine oxidization during the digestion. Pej-SGP-I-Gly was then eluted with thrombin buffer containing 0.4 *M* NaCl, 50 m*M* imidazole and 10% acetonitrile. The eluate was applied onto a Shodex Asahipak ODP-50 column (6 mm internal diameter × 150 mm; Showa Denko, Tokyo, Japan) for RP-HPLC separation with a 4 min linear gradient of 6–21% acetonitrile in 0.05% trifluoroacetic acid (TFA) followed by a 16 min linear gradient of 21–33% acetonitrile in 0.05% TFA at a flow rate of 0.8 ml min^{-1}. The elution was monitored by absorbance at 280 nm. The Pej-SGP-I-Gly peak was identified by mass-spectrometric analysis; in addition, SDS–PAGE was carried out to confirm its purity (data not shown).

2.2. Mass-spectrometric analysis and amino-acid sequence analysis

Mass spectra were measured on a matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectrometer (Voyager-

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
No. of crystals used	1
Beamline	NW12A, Photon Factory-Advanced Ring
Wavelength (Å)	1.000
Detector	Quantum 210
Crystal-to-detector distance (mm)	166.2
Rotation range per image (°)	1.0
Total rotation range	360
Exposure time per image (s)	5
Data processing	
Resolution range (Å)	50.00–1.95 (1.98–1.95)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 40.19, b = 53.65, c = 53.63
Mosaicity (°)	0.563
Total No. of measured intensities	127994 (5224)
Unique reflections	9245 (439)
Multiplicity	13.8 (11.9)
Mean $I/\sigma(I)$	61.63 (4.2)
Completeness (%)	99.8 (99.5)
$R_{\rm merge}$† (%)	4.6 (39.1)
Overall B factor from Wilson plot (Å^2)	31.8

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

DE STR, Applied Biosystems, Foster City, California, USA) with 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix in the positive-ion mode.

The N-terminal amino-acid sequence of Pej-SGP-I-Gly was analyzed using a Model 491 cLC protein sequencer (Applied Biosystems) in the pulsed-liquid mode.

2.3. Crystallization

The sparse-matrix screening kits Index HT and Crystal Screen HT (Hampton Research, Aliso Viejo, California, USA) were used for initial crystallization trials in conjunction with the sitting-drop vapour-diffusion method. HPLC-purified Pej-SGP-I-Gly solution was concentrated using a VC-360 centrifugal concentrator (TAITEC, Saitama, Japan), lyophilized using an FDU-1000 freeze-dryer (EYELA, Tokyo, Japan) and subsequently dissolved in distilled water to a protein concentration of 7 mg ml^{-1}. Each drop, comprising 0.5 µl protein solution and 0.5 µl reservoir solution, was set up manually, equilibrated against 100 µl reservoir solution in Intelli-Plates (Art Robbins Enterprises, Sunnyvale, California, USA) and incubated at 293 K. Small needle-shaped crystals grew within a week in a drop corresponding to condition E8 of Crystal Screen HT (10% ethanol, 1.5 *M* NaCl without any buffer). Further optimization was carried out by varying the pH, the type of alcohol and the NaCl concentration.

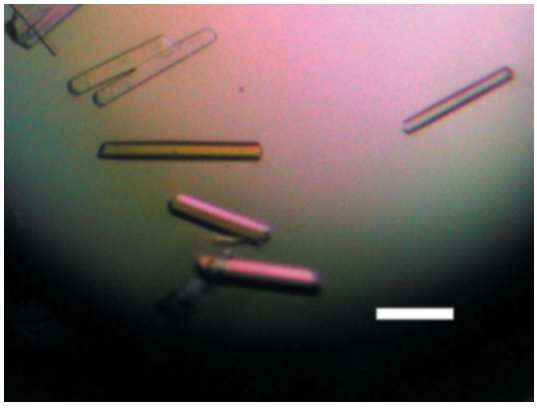


Figure 2
Crystals of Pej-SGP-I-Gly grown at 293 K in the optimized reservoir solution. The scale bar corresponds to 50 µm.

2.4. X-ray data collection

X-ray diffraction experiments were performed on beamline NW12A of Photon Factory-Advanced Ring, Tsukuba, Japan. The crystals were mounted in nylon loops (Hampton Research) and flash-cooled in a nitrogen stream at 95 K. The wavelength of the X-rays used was 1.000 Å and the crystal-to-detector distance was 166.2 mm. Diffraction data comprised of 360 images were collected with a rotation angle of 1.0° and an exposure time of 5 s per image using a Quantum 210 CCD X-ray detector (Area Detector Systems Corporation, Poway, California, USA). The diffraction data were indexed and scaled with the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

The purified peptide was identified as Pej-SGP-I-Gly by MALDI–TOF mass-spectrometric analysis; its molecular-ion peak was observed at m/z = 8487.3, which was consistent with the theoretical mass value of 8485.6. In addition, sequencing analysis revealed that the peptide had the desired amino-acid sequence in the N-terminal ten residues. The yield of Pej-SGP-I-Gly was approximately 1 mg from 1 l culture.

Tiny crystals of Pej-SGP-I-Gly were obtained in a week using a reservoir solution consisting of 10% ethanol and 1.5 *M* NaCl. Subsequently, the pH and precipitant concentration of the reservoir solution were optimized. The optimized reservoir solution consisted of 0.1 *M* citrate buffer pH 2.5, 5%(*v*/*v*) ethanol, 25%(*v*/*v*) ethylene glycol and 1 *M* NaCl. After optimization, larger needle-like crystals with typical dimensions of 100 × 20 × 10 µm were obtained (Fig. 2).

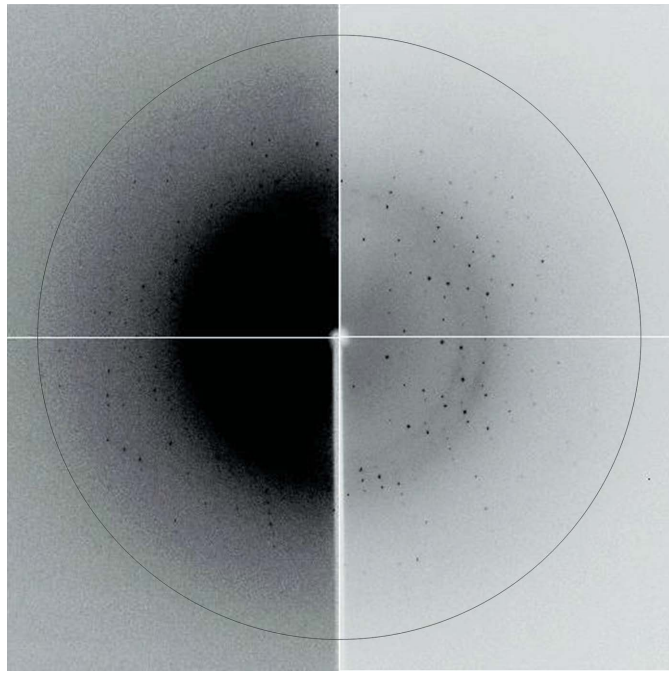


Figure 3
A diffraction image of the Pej-SGP-I-Gly crystal. The left and right halves are shown with different contrast. The edge of the detector and the ring correspond to resolutions of 1.8 and 1.95 Å, respectively.

Because the 25%(*v*/*v*) ethylene glycol in the optimized reservoir solution acted as a cryoprotectant, crystals were directly subjected to flash-cooling in a nitrogen stream after mounting in nylon loops. The best crystal diffracted X-rays to a resolution of 1.95 Å on beamline NW12A of the Photon Factory-Advanced Ring (Fig. 3). The X-ray diffraction data set from the crystal was processed and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 40.19, b = 53.65, c = 53.63 Å, based on indexing and systematic absences. The data-collection statistics for the best crystal are shown in Table 1. The asymmetric unit was estimated to contain two Pej-SGP-I-Gly molecules, with a Matthews coefficient of 1.74 $Å^3$ Da^{-1} (Matthews, 1968) and a solvent content of 29.2%.

Structure determination of Pej-SGP-I-Gly by molecular replacement using the coordinates of Pej-MIH (30% sequence identity; PDB entry 1j0t; Katayama *et al.*, 2003) as a search model is in progress. In this study, Pej-SGP-I-Gly, a weakly active precursor form of Pej-SGP-I, was used in the initial crystallization trials because the yield of the C-terminal amidation reaction was as low as 20–30%. Because the CD spectra of Pej-SGP-I-Gly and Pej-SGP-I were almost identical (Katayama *et al.*, 2002), their secondary structures and thus their overall folds and their crystallization conditions may be similar. The crystallization of Pej-SGP-I, a highly active C-terminally amidated mature form, is currently under way based on the results of this study.

The synchrotron-radiation experiments were performed on beamline NW12A of Photon Factory-Advanced Ring with the approval of the Photon Factory, KEK (Proposal No. 2008S2-001). This work was partly supported by the Targeted Proteins Research Program (TPRP) of the Ministry of Education, Culture, Sports, Science and Technology of Japan. NT and CN acknowledge the support of a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

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