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Effect of a Glycine Residue Insertion into Crustacean Hyperglycemic Hormone on Hormonal Activity

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ABSTRACT—Crustacean hyperglycemic hormone (CHH) and molt-inhibiting hormone (MIH) have similar amino acid sequences and therefore comprise a peptide family referred to as the CHH family. All MIHs unexceptionally have an additional glycine residue at position 12, which is lacking in all CHHs. In order to understand the relevance of the absence of the glycine residue for hyperglycemic activity, a mutant CHH having a glycine residue insertion was prepared, and its hyperglycemic activity was assessed. This mutant CHH had the same disulfide bond arrangement as the recombinant CHH produced in *Escherichia coli* cells, and exhibited a similar circular dichroism spectrum to the recombinant CHH, indicating that the two CHHs possessed similar conformations. The mutant CHH showed a hyperglycemic effect weaker than the recombinant CHH by about one order of magnitude. These results suggest that the insertion of a glycine residue is one of the indices for structural and functional divergence of the CHH family peptides.

Key words: crustacean hyperglycemic hormone, kuruma prawn, mutational analysis

INTRODUCTION

In crustaceans, the hemolymph glucose level is regulated by crustacean hyperglycemic hormone (CHH). CHHs have been isolated from many crustacean species, and have amino acid sequences similar to that of molt-inhibiting hormones (MIHs). Therefore, CHHs and MIHs comprise a peptide family referred to as the CHH family (Keller, 1992). CHHs consist of 72 or 73 amino acid residues and have an amidated C-terminus. On the other hand, the C-terminus of most MIHs is not amidated, and a glycine residue is inserted at position 12.

The tertiary structure of MIH of the kuruma prawn *Marsupenaeus japonicus* has recently been determined (Katayama *et al*., 2003). The MIH carries five α-helices, and Gly^{12} is located in the short N-terminal helix. The tertiary structure of one of the *M. japonicus* CHHs was modeled based on the structure of the MIH. By comparison of tertiary and surface structures of MIH and CHH, the N-terminal helix of the MIH was presumed to be one of the significant regions for conferring biological activity (Katayama *et al*., 2003).

In the present study, in order to examine the significance of the absence of a glycine residue at position 12 in natural CHHs, a mutant *M. japonicus* CHH (CHH-Gly^{12}) with an additional glycine residue inserted between positions 11 and 12 was prepared, and its hyperglycemic activity was assessed.

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MATERIALS AND METHODS

Construction of an expression plasmid

Cloning and characterization of a cDNA encoding Pej-SGP-I, one of the CHHs from *M. japonicus*, were reported previously (Ohira *et al*., 1997). Two primers, 12-F and 12-R, were designed based on the nucleotide sequence of Pej-SGP-I cDNA, with the inclusion of three nucleotides (GGT in 12-F, and ACC in 12-R, underlined in Table 1) encoding a glycine residue to be inserted at position 12. Two primers, F1 and R1, which completely matched the untranslated region of the cDNA were designed as forward and reverse primers, respectively. Another set of primers, F2 and R2, were designed to contain 20 nucleotide residues encoding the N- and C-terminal regions, respectively, and restriction enzyme sites (an *Nco* I site in F2, and an *Eco*R I site in R2, indicated by italics in Table 1). The nucleotide sequences of these primers are shown in Table 1. Three rounds of PCR were conducted. First, two rounds of PCR were separately conducted with two sets of primers, F1 and 12-R, and 12-F and R1, using a plasmid containing the Pej-SGP-I cDNA as template. Each PCR product was separated on a 2.0% agarose gel, and extracted from the gel using a kit of Quantum Prep Freeze'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, USA) according to instructions of the manufacturer. Two PCR products thus obtained were mixed with PCR buffer, dNTP mixture and Taq polymerase. The mixture was allowed to react under the following program: 5 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30

Table 1. Nucleotide sequences of primers used in this study

Primer	Sequence
F1	CGTCCCTACGCTTTTACACT
F2	*CCATGG*CAAGCTTCATAGACAACACCTG
R1	GACAGCAGATTAGTGGGAAT
R2	*GAATTC*TCACTGTCCGGCGTTCAGGA
12-F	CGTCTTCGGTGACCGACAGCTCCTGCGAAG
12-R	TGTCGGTCACCGAAGACGCCAGTGCACGAA

Italics in F2 and R2 indicate the restriction enzyme recognition sequences. Nucleotide sequences encoding an additional glycine residue in 12-F and 12-R are underlined.

sec at 72°C. To this tube, a set of primers, F2 and R2, were added, and PCR was performed with the following program: 20 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. The amplified PCR product was cloned with a pCR2.1 plasmid (Invitrogen, USA), and the nucleotide sequence of the PCR product was analyzed. Subsequently, the insert was released from the pCR2.1 plasmid by digestion with *Nco* I/*Eco*R I, and then ligated into the *Nco* I/*Eco*R I site of an expression plasmid pProEX HTa (Invitrogen, USA).

Preparation of CHH-Gly^{12}

Expression, refolding reaction, cleavage of His-tag, amidating reaction and purification of CHH-Gly^{12} were performed according to the method used for preparation of recombinant CHH (Katayama *et al*., 2002). The amidating enzyme was a kind gift from Drs. Ohsuye and Furukawa of Daiichi Suntory Pharma Co. Ltd., Japan.

Bioassay for hyperglycemic activity

Kuruma prawns, *M. japonicus*, were obtained at a fish market in Tokyo, Japan, and maintained in an aquarium at 20°C during the experiment. Hyperglycemic activity was assessed essentially according to the method described previously (Yang *et al*., 1995; Katayama *et al*., 2002).

Trypsin digestion

CHH-Gly^{12} (15 μg) was dissolved in 300 μl of 25 mM HEPES buffer (pH 6.8). To this solution, 2 μl of TPCK-treated trypsin (Promega, USA) solution (1 mg/ml) was added, and the mixture was incubated at 37°C for 20 h. The digestion was stopped by the addition of 2 μl of trifluoroacetic acid (TFA). The tryptic digests were separated by reverse-phase HPLC (RP-HPLC) on a Capcell-Pak C_{18} column (2.0×250 mm, Shiseido, Japan) with a 30-min linear gradient of 0–40% acetonitrile in 0.05% TFA, and a 10-min linear gradient of 40–80% acetonitrile in 0.05% TFA at a flow rate of 0.2 ml/min.

Amino acid sequence analysis

The N-terminal amino acid sequences of peptides were analyzed on an Applied Biosystems model 476A protein sequencer in the pulsed-liquid mode. The phenylthiohydantoin derivative of cystine (PTH-cystine) was detected in amino acid sequencing using an Applied Biosystems model 492HT protein sequencer with a modified HPLC program (Katayama *et al*., 2002).

Mass spectral analysis

Mass spectra were measured on a matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE™ STR, Applied Biosystems, USA) with 3,5-dimethoxy-4-hydroxycinnamic acid or α-cyano-4-hydroxycinnamic acid as a matrix in the positive ion mode.

Circular dichroism spectral analysis

The recombinant peptide (about 15 μg) was dissolved in 250 μl of PBS. The circular dichroism (CD) spectrum was recorded from 200 to 260 nm on a spectropolarimeter (Jasco J-720, Japan) at room temperature with a 1-mm path length cell.

RESULTS AND DISCUSSION

Preparation of CHH-Gly^{12}

An expression plasmid containing the CHH-Gly^{12} insert was constructed. Using this construct, CHH-Gly^{12} was expressed as a fusion protein with an N-terminal histidine tag (His-tag). After breakage of the *E. coli* cells that expressed CHH-Gly^{12}, the lysate was centrifuged to obtain soluble and insoluble fractions. These fractions were subjected to SDS-polyacrylamide gel electrophoresis, and a heavily stained band (≈11.5 kDa) likely representing CHH-Gly^{12} was detected only in the insoluble fraction (Fig. 1), indicating that this peptide was expressed as inclusion bodies in *E. coli* cells. Since a refolding reaction was considered necessary for obtaining the mutant peptide in its natural conformation, it was performed according to the method used

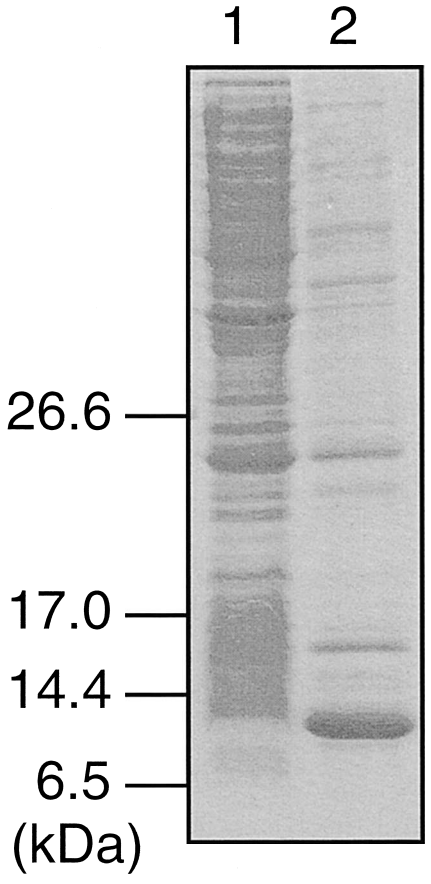

Fig. 1. SDS-polyacrylamide gel electrophoresis (15% acrylamide) of soluble and insoluble fractions after cell breakage from a 0.1-ml culture. Lanes 1 and 2 are the soluble and insoluble fractions of cells carrying an expression vector containing the CHH-Gly^{12} insert, respectively. The positions of molecular weight markers are indicated.

Table 2. Amino acid sequence and TOF-MS analyses of tryptic fragments of CHH-Gly^{12}.

Peak No.	Retention time (min)	Sequence	Molecular ion peak, *m/z* ((M+H)$^+$)	
			observed	theoretical
1	29.48	VXDDXFNVFR	4139.3	4139.2
		EPNVATEXR		
		QXMAYVVPAHLHNEHR		
2	33.15	GAMGSLFDPSXTGVFGDR	3029.5	3030.6
		SNXYNNPVFR		

X: Unidentified residue, but expected to be Cys.

for preparation of recombinant CHH (Katayama *et al*., 2002). Subsequently, a His-tag attached at the N-terminus of CHH-Gly^{12} was removed by digestion with TEV protease. CHH-Gly^{12} was amidated with peptidylglycine α-amidating enzyme, and the resulting peptide was purified by RP-HPLC. The peptide showed an expected single molecular ion peak at *m/z* 8751.3 and an expected N-terminal sequence, Gly-Ala-Met-Gly-Ser-Leu-Phe-Asp-Pro-Ser-, which was identical to that of natural CHH except for the four N-terminal residues derived from the nucleotide sequence of the expression plasmid. Since the recombinant CHH having four additional residues at the N-terminus showed hyperglycemic activity as high as the natural peptide (Katayama *et al*., 2002), it was unlikely that these four residues in CHH-Gly^{12} affected hormonal activity.

In order to examine whether the recombinant peptide possessed natural conformation, we analyzed the arrangement of disulfide bonds in CHH-Gly^{12} by identification of its tryptic digests, as was done for the recombinant CHH (Katayama *et al*., 2002). Two major peaks were observed in the elution profile of tryptic digests of CHH-Gly^{12} on RP-HPLC. Table 2 shows the results of mass spectral and amino acid sequence analyses. Fragment 2 contained one disulfide bond between Cys^7 and Cys^{44}. In the amino acid sequence analysis of fragment 1, PTH-cystine was detected at both cycles 5 and 8, unambiguously establishing the two disulfide bonds between Cys^{24} and Cys^{40}, and between Cys^{27} and Cys^{53}. This disulfide bond arrangement was identical to that of the recombinant CHH (Katayama *et al*., 2002). The circular dichroism (CD) spectrum of CHH-Gly^{12} exhibited a pattern typical for α-helix-rich proteins (data not shown), and it was calculated that the α-helix content in CHH-Gly^{12} was 44.7%. These results were similar to those of the natural and recombinant CHHs (Katayama *et al*., 2002). All these results indicate that CHH-Gly^{12} possesses a conformation similar to the conformations of natural and recombinant CHHs.

Biological activity of CHH-Gly^{12}

The dose-response relationship of CHH-Gly^{12} in hyperglycemic activity is shown in Fig. 2. Injection of 100 pmol of CHH-Gly^{12} increased the hemolymph glucose levels slightly, and its hyperglycemic activity reached a maximum at 300 pmol. It was demonstrated that the recombinant CHH showed low hyperglycemic activity at 10 pmol and high activity at 100 pmol (Katayama *et al*., 2002). These results indicated that the hyperglycemic activity of CHH-Gly^{12} was lower than that of the recombinant CHH by about one order of magnitude.

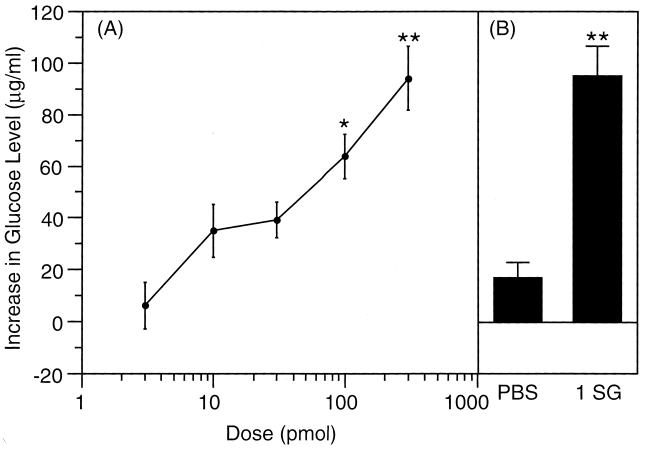

Fig. 2. (A) Dose-response relationship of CHH-Gly^{12}. (B) Negative control, injection of PBS; positive control, injection of sinus gland extract (one sinus gland equivalent) of *M. japonicus*. Results are expressed as the mean±S.E. (*N*=7–14). Asterisks indicate values significantly different from the negative control. Symbols, (*) and (**), indicate *P*<0.01 and *P*<0.001, respectively, which were calculated with the program of Fishers PLSD.

In the tertiary structure of *M. japonicus* MIH, the Gly^{12} residue is positioned in the short N-terminal helix, and it was presumed that this helical structure might be lost by the absence of the Gly^{12} residue (Katayama *et al*., 2003). Comparison of the tertiary and surface structures of the MIH with those of the modeled CHH suggests that this N-terminal helix is one of the significant regions for conferring MIH activity, and therefore the glycine residue at position 12 is likely to be one of the indices for diversification of the functions of the CHH family peptides. Thus, the present results are consistent with the hypothesis on the structure-activity relationship of the CHH family peptides.

The mutational analysis of *M. japonicus* MIH demonstrated that the residues at positions 13, 14, 71 and 72 were significant for conferring molt-inhibiting activity (Katayama *et al*., 2004). It was presumed that these residues were some of indices of dividing the functions of CHH-family peptides,

and were not conserved between CHHs and MIHs. Therefore, it is likely that CHH-Gly^{12} could not show the molt-inhibiting activity, and additional mutations at these positions would be required for exhibiting the molt-inhibiting activity.

Recently, it was proposed that molecular evolution of the CHH family peptides was similar to that of the growth hormone (GH) family proteins including GH and prolactin (PRL) (Chan *et al*., 2003); GH and PRL are structurally similar but functionally completely different. This is also true for the CHH family, but there are some exceptions; CHH of the lobster *Homarus americanus* showed both CHH and MIH activity (Chang *et al*., 1990). Therefore, it is likely that the CHH family peptides may be still on their way to attaining complete functional diversity. Studies on structural differences between CHH and MIH receptors and molecular mechanism of hormone-receptor interaction are required for revealing the significance of the glycine residue insertion or deletion in the functional and structural diversion.

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