Peptides xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Hyperglycemic activity of the recombinant crustacean hyperglycemic hormone B1 isoform (CHH-B1) of the Pacific white shrimp *Litopenaeus vannamei*

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ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form 22 May 2015
Accepted 26 May 2015
Available online xxx

Keywords:
Crustacean hyperglycemic hormone
Crustacean neurohormone
Recombinant expression
Dose–response hyperglycemic analysis
Litopenaeus vannamei

ABSTRACT

Crustacean hyperglycemic hormone (CHH) is the most abundant neuropeptide produced by the X-organ/sinus gland (XO/SG) complex in the crustacean eyestalk. CHH plays a principal role in the control of glucose metabolism. The CHH-B1 isoform is produced in the eyestalk of *Litopenaeus vannamei* by alternative splicing of the *chhB* gene and its cDNA sequence has revealed that this isoform has a non-amidated C-terminal residue (CHH-like peptide). In this work, a recombinant CHH-B1 (rCHH-B1) with a sequence identical to the native hormone was expressed in the methylotrophic yeast *Pichia pastoris* X-33 and purified from the culture medium by RP-HPLC. The identity of the purified rCHH-B1 was confirmed by N-terminal sequencing and by using an anti-CHH-B1 polyclonal antibody. An in vivo assay showed that the hyperglycemic effect was dependant of the dosage of rCHH-B1, and the maximal hyperglycemic response was obtained with 250 pmol treatment. These results suggest that the amino acid sequence of the C-terminus and its correct structure are both important for the hyperglycemic activity of naturally occurring non-amidated CHH peptides, such as CHH-B1. CHH-B1 appears to be the first reported CHH-like peptide with significant hyperglycemic activity produced in the sinus gland of a penaeid shrimp.

Introduction

Q3 In crustaceans, many physiological processes are regulated by neuropeptides that belong to the crustacean hyperglycemic hormone (CHH) family, mainly produced by the X-organ/sinus gland (XO/SG) complex in the eyestalks. The most abundant neuropeptide produced by this complex is CHH, which is involved in the control of glucose levels in hemolymph [11]. However, multiple functions have been reported for CHH, including regulation of lipid metabolism [31], molting [6,7], osmoregulation [33,36], reproduction [9,42] and stress response [1].

The multiple activities have been explained as being due to the existence of CHH isoforms that may originate by various mechanisms, including the transcription of multiple copies of genes [13], alternative splicing of genes [10], or by post-translational processing such as L to D isomerization [34,35], and N-terminal blocking [5]. The CHH genes are classified into two types according their exon–intron organization: the type I genes that have 4 exons and 3 introns, and the type II genes that have 3 exons and 2 introns. Most of the type I genes described so far encode two transcripts that, in most cases, share the signal peptide sequence, the CHH precursor-related peptide (CPRP) and the N-terminal region (exons I and II), but differ in the C-terminal region, which can be encoded by exon III or exon IV [4,10]. These alternative splicing isoforms

Abbreviations: PAM, point accepted mutation; PBS, phosphate saline buffer; PCR, polymerase chain reaction; RP-HPLC, reverse-phase high performance liquid chromatography; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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http://dx.doi.org/10.1016/j.peptides.2015.05.014
0196-9781/

have been found in various crustaceans, including *Carcinus maenas* [10], *Macrobrachium rosenbergii* [3], *Pachygrapsus marmoratus*, *Potamon ibericum* [38], *Gecarcinus lateralis* [22], *Callinectes sapidus* [8,53], *Scylla olivacea* [41], *Pandalopsis japonica* [16], *Procambarus clarkii* [19,47,48,50], *Portunus trituberculatus* [49], and *Litopenaeus vannamei* [21].

Isoforms originating by alternative splicing have different expression patterns, where at least one transcript has been detected in extra-eyestalk tissues. Interestingly, in all cases only one of the isoforms has an amide moiety at the C-terminus, which is characteristic of CHH neuropeptides, while the other isoform has a free C-terminus (CHH-like or CHH-L) [23,46]. In the Pacific white shrimp *L. vannamei*, three different CHH isoforms have been cloned and sequenced: CHH-A (GenBank accession no. AY434016), CHH-B1 (GenBank accession no. AY167045), and CHH-B2 (GenBank accession no. AY167046). The CHH-B1 and CHH-B2 isoforms are both produced in the eyestalk of *L. vannamei* by alternative splicing of the *chhB* gene (formerly referred as molt-inhibiting hormone, *mih*) [21]. The sequence analysis of mRNA transcripts indicated that the CHH-B1 isoform is a CHH-L neuropeptide, whereas CHH-B2 has a processing signal (GK) for α-amidation of the C-terminal valine amino acid residue (CHH peptide). CHH-B1 expression has been shown to be higher than that for CHH-B2 in eyestalks and the gene expression of these neurohormones seems to be highly dependent on the environmental salinity and temperature [21]. CHH-B1 has also been detected in tissues other than the eyestalk [37]. Those findings suggest that CHH-B1 plays an important physiological role in *L. vannamei*, and may also have some of the additional functions attributed to CHHs, which have not been studied previously in this species.

CHH-B1 has been cloned previously and expressed in the methylotrophic yeast *Pichia pastoris*, but with 28 additional amino acids at the C-terminus (*c-myc*/polyhistidine tags) that allowed the easy identification and purification of the neurohormone with commercial antibodies and metal affinity chromatography, respectively. Even though the recombinant peptide showed hyperglycemic activity in vivo, it did not show molt-inhibiting activity [30]. In the present work, we report the cloning in *P. pastoris*, expression and purification of the *L. vannamei* recombinant CHH-B1 isoform with a free C-terminus (rCHH-B1) and identical to the native neurohormone, as well as an examination of its hyperglycemic activity using a dose–response bioassay with bilaterally ablated shrimp.

Materials and methods

Animals

The *L. vannamei* shrimp used in these experiments were acquired as post-larvae from the commercial farm Acuacultura Mahr in La Paz, México. The post-larvae were grown until they reached juvenile stage in tanks filled with aerated seawater at 35‰ and 28 ± 1 °C. For the hyperglycemia assay, 200 sub-adult shrimp were transferred to individual containers (3.5 L) submerged in tanks with aerated seawater at 35‰ and 26 ± 1 °C. The animals were fed twice a day with a commercial diet supplemented with 4% squid meal to enrich protein content in the food. Daily, debris and feces were removed, and the seawater was completely exchanged. The shrimp were acclimated to these conditions for 10 days before starting the bioassay.

Construction of the expression vector for rCHH-B1

Eyestalks from juvenile shrimp were dissected for total RNA extraction. After removal of the cuticle and non-neural tissues, eyestalks were homogenized with Tri Reagent (Sigma–Aldrich, Saint Louis, MO, USA) and RNA was isolated according to the manufacturer's instructions. Total RNA (1 μg) was treated with DNAse I, Amplification Grade (Invitrogen, Life Technologies, Carlsbad, CA, USA) and first strand cDNA was synthesized using SuperScript III Reverse Transcriptase and an Oligo $(dT)_{20}$ primer (Invitrogen, Life Technologies), according to the manufacturer's protocol. The *chh-B1* transcript was amplified by PCR using the forward primer 5′-TTGAGAAGCTGCTGTCGTCCT-3′ and the reverse primer 5′-CTTGTTTCCTCCACATTAGCG-3′ [21]. The PCR reaction (50 μL) consisted of 1X Green GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, 1.25 U of GoTaq® DNA Polymerase, and 2.0 μL of the reverse transcription reaction mix. The PCR was carried out at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 45 s and 72 °C for 45 s, and a final extension step at 72 °C for 5 min. The amplified PCR product of about 500 bp was purified using a PureLink™ PCR Purification kit (Invitrogen, Life Technologies), and used as template in a second PCR reaction. For cloning the mature *chh-B1* transcript into the pPICZαA vector (Invitrogen, Life Technologies), specific primers were designed. The forward primer 5′-CCGCTCGAGAAAAGA**GACACCTTCGACCACTCCTGCAAGG**-3′ includes a *Xho*I site (underlined), and deletes the *Ste*13 signal cleavage site (two E–A repeats) between the α-factor secretion signal sequence and the mature *chh-B1* transcript (bold). The reverse primer 5′-GCTCTAGA**TTA**GGGATAGCGCAGAAA-3′ includes a *Xba*I site (underlined) and stop codon (bold). The mature *chh-B1* transcript was amplified by PCR (100 μL) using the cDNA PCR fragment (1.0 μL) previously purified. PCR mix and settings were identical to those described above. The purified *chh-B1* fragment and pPICZαA vector were digested with *Xho*I and *Xba*I restriction enzymes (Promega) and ligated with T4 DNA ligase (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's protocol. The ligation product was used to transform *Escherichia coli* DH5α cells by electroporation in a Bio-Rad Micropulser (2.5 kV, 1 pulse, ~5 ms). Transformant colonies were screened for zeocin (Invitrogen, Life Technologies) resistance and evaluated by PCR using 5′AOX1 and 3′AOX1 primers (5′AOX1, 5′-GACTGGTTCCAATTGACAAGC-3′; 3′AOX1, 5′-GCAAATGGCATTCTGACATCC-3′). The plasmid vector construction was verified by sequencing (SeqxCel, San Diego, CA, USA). The pPICZαA-CHH-B1a vector was linearized with *Bst*XI (New England BioLabs) for integration into the *P. pastoris* X-33cell genome by electroporation (2.0 kV, 1 pulse, ~5 ms), as described in the manual (version G) of the EasySelect Pichia Expression Kit (Invitrogen, Life Technologies). The transformants were screened for zeocin resistance on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar, 100 μg/mL zeocin) and integration of the construct into the yeast genome was evaluated by PCR and sequence analysis.

Sequence analysis

The amino acid sequence of mature CHH-B1 was aligned against CHH and CHH-like (CHH-L) isoforms originated by alternative splicing. The sequences were obtained from the NCBI GenBank database (http://ncbi.nlm.nih.gov). The multiple sequence alignment was performed with the software BioEdit 7.25 [15], using standard parameters such as the PAM250 residue weight table, and gap penalty of 10.

Expression of rCHH-B1

The expression of rCHH-B1 was induced according to the method described by Sánchez-Castrejón et al. [30] with some modifications. A colony of *P. pastoris* X-33 containing pPicZαA-CHH-B1a inserted into its genome was grown in 3 mL of YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 100 μg/mL zeocin) for 18 h

Please cite this article in press as: Camacho-Jiménez L, et al. Hyperglycemic activity of the recombinant crustacean hyperglycemic hormone B1 isoform (CHH-B1) of the Pacific white shrimp *Litopenaeus vannamei*. Peptides (2015), http://dx.doi.org/10.1016/j.peptides.2015.05.014