Comparative Biochemistry and Physiology, Part B 194–195 (2016) 58–64

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Cloning and characterization of a riboflavin-binding hexamerin from the larval fat body of a lepidopteran stored grain pest, *Corcyra cephalonica*

V. Venkat Rao [a], Thuirei Jacob Ningshen [b], R.K. Chaitanya [c], B. Senthilkumaran [a], Aparna Dutta-Gupta [a,*]

[a] Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India
[b] Department of Biotechnology, Manipur University, Canchipur, Manipur 795003, India
[c] Centre for Animal Sciences, Central University of Punjab, Bhatinda, Punjab 151001, India

ARTICLE INFO

Article history:
Received 14 August 2015
Received in revised form 29 December 2015
Accepted 25 January 2016
Available online 27 January 2016

Keywords:
Fat body
Hexamerin
20-Hydroxyecdysone
Juvenile hormone
Riboflavin
Stored grain pest management

ABSTRACT

In the present study, a riboflavin-binding hexamerin (RbHex) was cloned and characterized from the larval fat body of *Corcyra cephalonica*. The complete cDNA (2121 bp) encodes a 706-amino acid protein with a molecular mass ~82 kDa. Expression of RbHex 82 was predominant in fat body among larval tissues. Further, it is prominently expressed during the last instar larval development. Homology modeling and docking studies predicted riboflavin binding site of the hexamerin. Spectrofluorimetric analysis further confirmed riboflavin release from the hexamerin fraction. Quantitative RT-PCR studies demonstrated hormonal regulation of RbHex 82. 20-Hydroxyecdysone (20HE) had a stimulatory effect on its transcription whereas JH alone did not show any effect. However, JH in the presence of 20HE maintains the RbHex 82 expression which indicates the JH's role as a *status quo* factor. This study is the first to report the characterization of riboflavin-binding hexamerin in a lepidopteran pest. Further, the possibility of RbHex 82 as a pest control target is discussed.

1. Introduction

Hexamerins belong to an arthropod protein superfamily, which includes hemocyanins, dipteran hexamerin receptors and phenoloxidases (Decker and Terwilliger, 2000; Burmester, 2002). These proteins with a native molecular mass of about 500 kDa exist as either homo- or hetero-hexamer subunits ranging 70–90 kDa (Telfer and Kunkel, 1991; Burmester and Scheller, 1999). Insect hexamerins are predominantly synthesized and expressed in the fat body and secreted into the hemolymph (Burmester, 1999; Telfer and Kunkel, 1991; Wang and Haunerland, 1991).

Hexamerins are primarily known to function as storage proteins that provide energy for non-feeding periods (Munn et al., 1967; Munn and Greville, 1969) but recent evidence demonstrate them as a versatile molecule. Hexamerins may transport hormones such as ecdysteroids (20HE) (Enderle et al., 1983) and juvenile hormone (JH) (Braun and Wyatt, 1996). In termites, hexamerins play a major role in caste determination in cooperation with JH (Zhou et al., 2006). Few studies implicate hexamerins' involvement in immune response (Beresford et al., 1997; Ma et al., 2005; Phipps et al., 1994; Poopathi et al., 2014). Hakim et al. (2007) have reported that aryphorin hexamerins have mitogenic effect and they stimulate stem cell proliferation *in vitro*. Recently, Martins et al. (2011) demonstrated the intranuclear localization of a hexamerin, Hex70a, in the ovaries and testes of the honeybee, and suggested that the hexamerin might have tissue specific role. Hexamerins may also bind to small organic metabolites like riboflavin (Magee et al., 1994) and biliverdin (Miura et al., 1994) with high affinity.

Five distinct types of hexamerins have been identified in lepidopterans, which differ in terms of amino acid composition and evolutionary history: i) the arylphorins, which are rich in aromatic amino acids (~20% phenylalanine and tyrosine), ii) the distantly related arylphorin-like hexamerins, iii) the methionine-rich hexamerins, iv) the moderately methionine-rich hexamerins, and v) the riboflavin-binding hexamerins (RbHex) (Burmester, 2015). While the specific role of other hexamerin types can be easily associated with the accumulation of certain amino acids, the function of RbHex is obscure. So far, RbHex has only been identified and characterized in the giant saturniid silk moth, *Hyalophora cecropia*, as a protein that binds to riboflavin (vitamin B2) (Magee et al., 1994).

The present study is the first to clone and characterize a riboflavin binding hexamerin from the fat body of last instar larvae of a lepidopteran stored grain pest, *Corcyra cephalonica*. Further, RbHex being lepidopteran-specific (Burmester, 2015), and most major stored grain and agricultural pests belonging to the same order, its molecular elucidation could provide cues for exploitation as a potential target for pest management.

* Corresponding author at: Department of Animal Biology, School of Life Sciences, University of Hyderabad, P O Central University, Hyderabad 500046, India.
E-mail addresses: apdgsl@uohyd.ernet.in, aparnaduttagupta@gmail.com (A. Dutta-Gupta).

http://dx.doi.org/10.1016/j.cbpb.2016.01.008
1096-4959/

2. Materials and methods

2.1. Insect rearing and maintenance

The eggs of *C. cephalonica* were procured from National Bureau of Agriculturally Important Insects (NBAII, ICAR), Bengaluru. The eggs were allowed to hatch in culture troughs containing coarsely crushed sorghum seeds with multivitamin tablets. The cultures were maintained at 26 ± 1 °C, 60 ± 5% relative humidity and 14:10 h light:dark (L:D) photoperiod. The larval development proceeds through five instars and is completed in about 45–50 days followed by a short prepupal stage (4–5 days), and a pupal stage, which lasts for 7–8 days. The adult moths normally survive for 8–10 days. For the present study, all the stages of insect i.e. embryo, larvae (1st, 2nd, 3rd, 4th and 5th instar), prepupa, pupa, and adult were used. Further, 5th instar larva were classified as early-late (ELI), mid-late (MLI) and late-last (LLI) instar larvae. These larval instars were distinguished based on their body weight and the size of the head capsule (Lakshmi and Dutta-Gupta, 1990).

2.2. Full-length cDNA cloning of RbHex 82 using rapid amplification of cDNA ends (RACE) strategy

Total RNA was isolated from the fat body using Trizol reagent (Sigma, USA). Five micrograms of total RNA was used for cDNA preparation using Superscript™ III first strand synthesis system (Life Technologies, USA). Degenerate primers were designed based on the alignment of known lepidopteran hexamerin sequences (which were neither arylphorins nor methionine-rich) from GenBank, (AF032397.1, EF646282.1, AY661710.1, M57443.1, L21997.1, EU366905.1). A partial clone of 800 bp corresponding to Rb*Hex 82* was obtained. The partial fragment obtained was further confirmed using gene specific primers. For obtaining the full-length cDNA of RbHex 82, 5′ and 3′ RACE reactions were carried out using RACE kit (Clontech Laboratories Inc., USA) according to the manufacturer's protocol. The 5′ RACE was carried out with gene specific reverse primer (GSP) and universal primer A mix (UPM), while 3′ RACE was performed with gene specific forward primer and universal primer A mix. All the PCR conditions were programmed as specified in the manufacturer's protocol. The amplified products were cloned into p-GEM-T easy vector (Promega, USA) and sequenced. All the primers used in the study are listed in Supplementary Table 1.

2.3. Quantitative RT-PCR to study tissue specificity, developmental expression and hormonal regulation of RbHex 82

The purity and quantity of RNA were assessed using Nanodrop spectrophotometer (ND-1000). Three micrograms of total RNA was converted to cDNA using Superscript III™ first strand synthesis kit. All the RNA samples were treated with DNase I prior to cDNA synthesis to eliminate any possible genomic DNA contamination. Real-time PCR was performed on an ABI Prism® 7500 fast thermal cycler (Applied Biosystems, USA). Each sample was run in triplicate in a final volume of 20 µl containing 0.3 µl of cDNA (1:10 dilution), 10 pmol of each primer and 10 µl of Power SYBR® Green PCR master mix (Applied Biosystems, USA). PCR conditions were optimized to generate >95% PCR efficiency. Dissociation curve analysis was performed after the last cycle to confirm amplification of a single product. Quantitative RT-PCR results were expressed as change in expression relative to control using target gene. C_t values were normalized to that of internal control gene C_t values (mention internal control gene) based on the $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001). All the quantitative RT-PCR primers used are listed in Supplementary Table. 1.

2.4. Homology modeling of RbHex 82

The 3D model of the RbHex 82 was built by homology modeling based on high-resolution crystal structure of homologous proteins. The crystal structure of the closest homolog of *C. cephalonica* RbHex 82 available in the Brookhaven Protein Data Bank was searched by determining the sequence similarity aided by a BLAST (NCBI) search. The results pointed to the crystal structure of *Antheraea pernyi* arylphorin (APA) with a resolution of 2.43 Å as a suitable template (PDB ID: 3GWJ). The identity score was 29% and E value was 2e − 79. Hence, the coordinates of crystal structure of the APA were used as a template to build the model by multiple sequence alignment using Clustal W software. The 3D model of *C. cephalonica* RbHex 82 was generated by the homology modeling tool Modeller (Marti-Renom et al., 2000). The steepest descent energy minimization using the Gromos96 43a1 force field was performed to regularize the protein structure geometry.

2.5. Molecular docking studies

Automated docking analysis was carried by using Autodock 4.0 (Morris et al., 2009). The three-dimensional structure of riboflavin was built and its geometry was optimized through Discovery Studio 3.1 software package (accelrys). To recognize the binding sites in RbHex 82, blind docking was carried out essentially, the grid size set to 126, 126 and 126 along X-, Y-, and Z-axis with 0.525 Å grid spacing. The docking parameters used were GA population size: 150; maximum number of energy evolutions: 250,000. During docking, a maximum number of 10 conformers were considered, and the rms (root-mean-square) cluster tolerance was set to 2.0 Å. One of the lowest energy conformations was used for further analysis.

2.6. Sequence and phylogenetic analysis

Homology search of sequences was carried out by BLAST to get the putative ortholog sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ClustalW was used for multiple alignment of the RbHex 82 sequence with the other similar lepidopteran hexamerins to show the residual similarity (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Expasy (http://www.expasy.org/) tools were used for *in silico* characterization of deduced amino acid sequence, such as signal peptide prediction, putative N- and O-glycosylation sites, putative phosphorylation sites detection, amino acid composition, theoretical pI and molecular weight determination. Phylogenetic analysis and tree rendering were done using PhyML and TreeDyn available at Phylogeny.fr.

2.7. Purification of hexamerins

The hexamerins were purified from the hemolymph of 4th and 5th instar larvae of *C. cephalonica.* The hemocyte-free diluted hemolymph (1 mg protein/50 µl) was passed through a Sephadex G-100 column (1.5 × 60 cm) equilibrated with 10 mM Tris–HCl (pH 7.4) at room temperature. The protein was eluted with the same buffer at a flow rate of 1 ml/2 min till the absorbance of the elutants at 280 nm reached a value of 0.002. Fractions containing protein were checked by SDS-PAGE. The fractions that contained hexamerins were pooled and loaded on to ion exchange DEAE Sephacel column (1.25 × 25 cm) pre-equilibrated with 10 mM Tris–HCl (pH 7.4). The bound hexamerins were eluted with a linear gradient of 0–0.5 M NaCl. The peak fractions were pooled and analyzed on 7.5% resolving SDS-PAGE for purity and probed with hexamerin antibody for confirmation (Bradford, 1976; Laemmli, 1970; Towbin et al., 1979).

2.8. Spectrofluorimetric analysis to determine the release of bound riboflavin

Equal concentrations of hexamerin fraction (in 50 mM Tris–Cl, pH 7.4) from 4th and 5th instar larvae (4th instar hexamerin fraction contains two hexamerins [except RbHex82] and 5th instar hexamerin fraction contains all the three hexamerins [includes RbHex 82]) were subjected to heat treatment at 85 °C for 5 min that resulted in unfolding