UDC: 595.384-15:504(496.5)
Original scientific paper

CARCINUS AESTUARII AS BIO- INDICATOR OF ESTUARINE ECOSYSTEMS HEALTH

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Abstract

Several organisms serve as biological indicators for assessing various ecosystems' health. Different chemical compounds found in the environment such as copper, chloroform, and adrenaline can induce to the crustaceans an adaptive response by producing more Crustacean Hyperglycemic Hormone (CHH) by the X organ and sinus gland (XO-SG) complex located in the eyestalks. Even though the above-mentioned chemical compounds provoke stress in the species organism, the abiotic factors have the same effects too. They both can increase the hemolymph glucose level, affect the total hemocyte count (THC), and the differential hemocyte count (DHC), and cause the destabilization of the lysosome membrane (decrease of NRRT, $p<0.05$). In the long-term, stressors will adversely impact the *C. aestuarii* health, compromise reproduction, and the general population health of this species. The Crustacean Hyperglycemic Hormone (CHH) is a proteinic hormone that intermediates the whole physiological process at the molecular level. It represents an open reading frame sequence of 429 base pairs, responsible for the coding of a 142-aminoacid protein with a signaling peptide of 26 amino acids, followed by a peptide attached to the CHH precursor of 40 amino-acids, and the mature peptide of 72 amino acids and it results by the first determination based on a high similarity (98.6%) with the CHH peptide from *C. maenas* compared with CHHs from Brachyura infraorder.

Keywords: Carcinus aestuarii, estuarine ecosystems health, Crustacean Hyperglycaemic Hormone (CHH), Haemolymph Glucose Level, Crustacean Hyperglycaemic Precursor Related Peptide (CPRP), PCR, HPLC Chromatography

Introduction

Research regarding the effects of environmental stressors on shrimp organisms began many years ago and is largely based on the role of neurohormonal factors. The basic neurohormone related to the animal reaction against stressors is the crustacean hyperglycemic hormone (CHH) which was reported by Abramowitz et al. In 1944 he was first identified as a 'diabetogenic factor' in *Callinectus sapidus* and *Uca puligator* species. Many subsequent studies have reported facts about the anatomy and role of neural structures in the crab eye stalk (Webster et al., 2012). The sinus glands (SG) located in the terminal medulla (MT) have been identified as neuroblood structures and this cluster has been termed the X organ (XO) (Webster et al., 2012). Over the past two decades, many more studies have been conducted to elucidate the metabolic function of CHH under various conditions, including efforts to identify and characterize this hormone at the molecular level (Dircksen et al., 2001; Webster et al., 2012). So far, the isolation and characterization of CHH peptides has reached approximately 400 CHH and CHH-like peptides in crustaceans (Christie et al., 2010; Yu et al., 2020). However, there remain unanswered questions about the physiological role, possible isoforms, and mechanism of action of this hormone. Due to the lack of studies to characterize and identify this species of CHH, this study is the first to identify and characterize CHH in crabs from the Mediterranean coast, the amino acid sequence,

and possible adaptive responses to multiple stressors. It is intended to determine the scenario. design. *C. aestuarii* (Nardo, 1847) is a coastal crab endemic to the Mediterranean Sea and an economically important species in the region's fish market. Although it shares some similarities with *C. maenas*, molecular studies reveal significant differences between the two taxa are sufficient to classify them as two distinct species. (Roman and Palumbi, 2004). The higher adaptive resistance of *C. aestuarii*, in addition to the lack of further data on CHH available, predicts the potential for structural diversity compared to *C. maenas* and inspires to further characterize CHH hormones.

Materials and methods

Experimental design and acclimation

Carcinus aestuarii adult male individuals (average carapace length of 4-6 cm and 1-1.7 g of body weight) were collected in Narta Lagoon (40° 32′ 0″ N, 19° 28′ 0″ E), Patoku Lagoon (41°37′44.4″N,19°35′27.6″E) and Marano Lagoon, Trieste, Italy (45°44′6″N,13°9′46.8″E). After collection, they were brought immediately into the laboratory, where have been acclimated for 3 days in 50 L aquaria filled with continuously aerated seawater (pH: 8 ± 0.1; salinity: 36 ± 1 ppt; and temperature of 17 ± 1°C). Animals were fed with mussels (Mytilus galloprovincialis) during acclimation. Animal maintenance and experimental procedures were in accordance with the Guide for Use and Care of Laboratory Animals (European Communities Council Directive 86/ 609/EEC) and national and institutional guidelines for animal welfare (Act No. 10465, 29/9/2011: "On the Veterinary Service in the Republic of Albania").

Extract eyestalk preparation and injection

To prepare eyestalk extract, eyestalks were removed from two individuals (4 eyes), pressed into a mortar by a pestle, and mixed with water and quartz sand. The remaining eyestalk was cauterized by heating to stop the hemolymph flow. 0.5 ml distilled water was added to the extract and, thereafter was centrifuged. Hemolymph glucose level was measured in the intact animals and after eyestalks removal. Eye-stalked animals were injected with freshly-prepared extract and hemolymph glucose levels were determined using glucose oxidase assay.

Animal exposure to copper, adrenaline, chloroform, temperature and hypoxia

In order to evaluate the effect of abiotic factors (hypoxia and temperature) and xenobiotic factors (copper, adrenaline and chloroform), intact and eyestalk ablated group was tested before and after exposure copper, (70 µg/L, administered as CuCl2), adrenaline (0.1 mM) chloroform (0.005 g/L), temperatures (4, 17,22. 6°C and 32 °C) and hypoxia (4 mg O2L−1). Copper, adrenaline, and chloroform are used according to their real doses found in the animal environment (Nuro et al., 2018), while for the hypoxia and temperature we referred to values measured in the animal's natural habitat. The eyestalk ablation was done bilaterally and then that region was cauterized by heating. Reagents used were supplied by Sigma-Aldrich (St Louis, Missouri, USA). Oxygen concentration was continuously, during the whole exposure period, controlled by an OxyGuard® Mk III oxygen probe.

Hemolymph collection and measurement of hemolymph glucose levels

Before collecting the hemolymph, the animals were anesthetized for 5 min in order to avoid stress. The hemolymph was collected (about 500 μL per crab) at the base of the fourth moving leg with a sterile 1 ml hypodermic syringe fitted with a 25G needle. Animals were divided in different groups: control group, intact group after exposure, eyestalk group before and after exposure to the stressor (copper: n=20; chloroform and hypoxia: n=10; thermal exposure and adrenaline: n = 6). Glucose level was measured by using glucose oxidase assay point-of-care One touch® II GlucoMeter (Lifescan, Miltipas, CA, USA) and commercial kit test strips (precision of strips ± 3% coefficient of variation in the tested range). Total hemocyte count (THC) and differential hemocyte count (DHC) were performed according to the standard method previously described by Blaxhall and Daisley, (1973) and improved by Aliko. (2015). During hemocyte identification cell size, shape and granularity were evaluated according to criteria given by Matozzo et al., (2001) under the light microscope (KN-100TC, Kyowa, Tokyo) connected with a YCU-300F 3CCD camera.

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Lysosomal stability using neutral red retention assay

To evaluate the lysosomal membrane stability, Neutral Red Retention Assay (NRRT) was performed according to Martinez-Gómez et al. (2008) and adopted for the species under study. For each individual, NRRT was measured every 15 until 50% of the hemocytes lost the dye to the cytosol. A mean of NRRT and glucose concentrations was calculated for each group.

Tissue preparation and chromatography running

20 SG from 20 individuals were used to run chromatography. Sinus glands extracts were analyzed by liquid chromatography/mass spectrometry (LC/MS) as previously described (Sgarra et al 2009, JPR). Briefly, extracts were LC-MS analyzed with a 1200 Series Capillary HPLC (Agilent) coupled to an HCTultra ion trap (Bruker Daltonics). Proteins were separated on a mRP-C18 High-Recovery Protein Column (0,5 x 100 mm - Agilent) using a water 0.1% TFA -/ acetonitrile 0.1% TFA linear gradient. Total ion chromatogram (TIC) was elaborated using the Data Analysis software (v.3.4, Bruker Daltonics) and the m/z spectra were converted to reconstructed mass spectra (Molecular mass vs. Intensity).

RNA extraction and 5', 3' Rapid Amplification of cDNA ends (RACE)

The RNA from the medullae, was extracted and quantified by Nanodrop 2000 (ThermoFisher). The synthesis of both 5' and 3' RACE cDNA was carried out using SMART cDNA synthesis kit (BD Biosciences), using specific CHH primers drawn with Primer3Plus v. 4.0.0 (Untergasser et al. 2012) and OligoCalc v. 3.27

(Kibbe 2007). A CHH from Carcinus maenas was used as a template (GenBank: X17596) and the resulting primers were further compared with additional CHH XO-type to verify correspondence with the sequences. PCR amplification was run on a qRT-PCR CFX96 (Bio-Rad). PCR products were then Sanger sequenced (Macrogen, Rotterdam). The Carcinus aestuarii CHH sequence (526 nt encoding a pre-pro-peptide putative of 142 aa, was deposited to GenBank (Accession number: MW246807).

Statistical analysis

One-Way ANOVA, amino-acidic sequences database from infraorder achyura (NCBI) and CLC Genomics Workbench v12 (Qiagen)were used to test significance and to compare possible similarities. ModelTest-NG was used to evaluate the best molecular evolution model based on the Phylogenetic tree created with MrBayes 3.2.7a (Ronquist et al. 2012).

Results and discussions

Basal glucose level and variation in control and exposed groups the basal glucose level in C. aestuarii resulted to variate from 34.5 to 41mg/dl hemolymph. Glucose levels decreased drastically into the eyestalk ablated animals
($p<0.05$) because of the CHH missing. Glucose levels increase significantly even after the extract and hormone (obtained by HPLC) injection ($p<0.05$). The same thing was observed even when the animals was exposed to abiotic factors such are temperature and hypoxia and xenobiotic factors such are copper, adrenaline and chloroform ($p<0.05$), Figure 1.

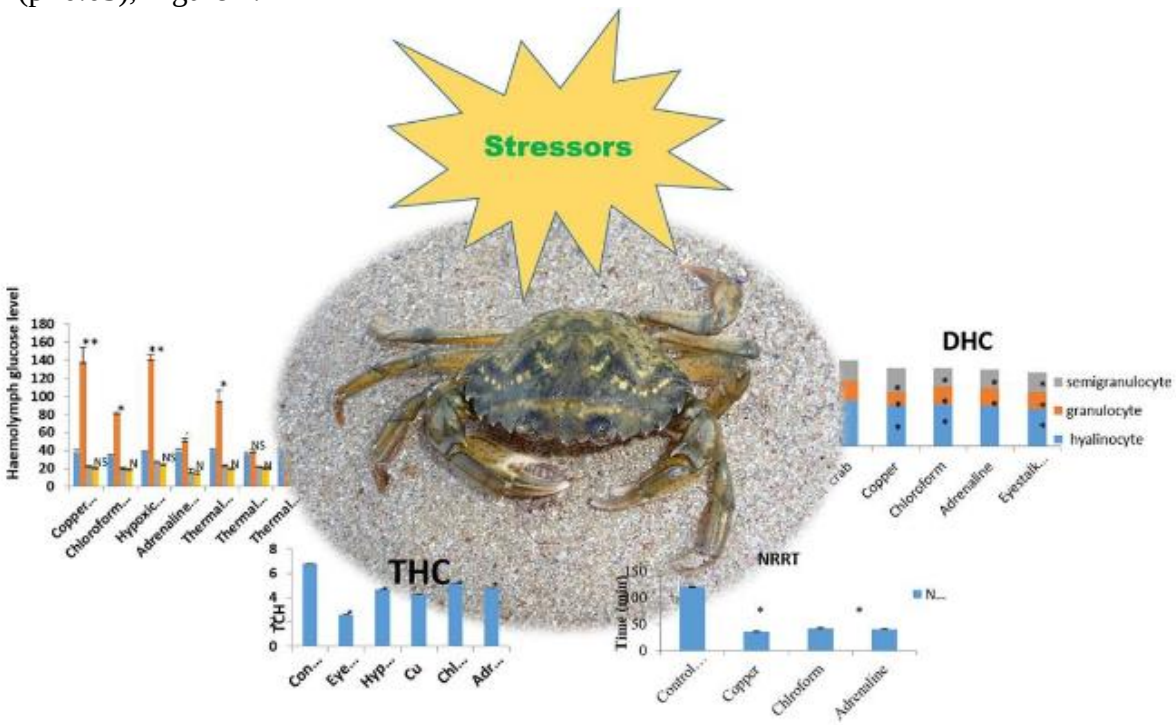

Figure 1. Hemolymph glucose levels in control and exposed groups to different stressors

Our data corroborate with studies from other authors (Powell and Rowly, 2008). This might be related to the ability of the animal to use glucose for energy to set up the homeostasis (Jacobo et al., 2016). On the other hand, immobilization of glucose from the crab hemocytes is possible since a high amount of glucose is stored in *C. aestuarii* hemocyte (Matozzo and Marin, 2010). During thermal exposure must be used another strategy which is based on the immobilisation of glucose from hepatopancreas and/ or muscles, based on immunology pathways (Lorenzon et al., 2005). Our results regarding the decrease in THC levels support this hypothesis. The same trend was observed during the exposure to hypoxia and xenobiotic factors. A significant glucose level was measured after 2h exposure, while in eyestalk-ablated animals this process does not take place, which suggests an immediate response by CHH (Aliko et al., 2018; Jacobo et al., 2016). After adrenaline treatments, animals increase their glucose level (Model et al., 2019) but not as high as chloroform and copper exposure. Chloroform also provoked a hyperglycaemia response into intact animals. Similar findings are reported from studies in Cancer pagurus (Webster, 1996), lobsters Jasus edwardsii (Morris dhe Oliver 1999; Speed et al., 2001) and Nephrops norvegicus, (Spicer et al., 1990), crabs, Eriocheir sinensis (Zou et al., 1996), and Maia squinado (Durand et al., 2000).

Total and differential haemocyte count

In all variants of exposure, total haemocyte count decreases significantly ($p<0.05$), from $2.61 \pm 0.09 \times 106$ (eyestalk-ablated) to $4.7\pm 0.16 \times 106$ (hypoxia), $4.3 \pm 0.1 \times 106$ (copper), $4.8 \pm 0.2 \times 106$ (adrenaline), and $4.1 \pm 1.2 \times 106$, $6.4 \pm 2.3 \times 106$, $3.9 \pm 1.3 \times 106$ respectively at 4°C, 17°C and 32°C, concerning control ($6.8 \pm 0.23 \times 106$cell/mL). Three hemolytic types: hyalinocytes ($51.6 \pm 0.5\%$), granulocytes, and semi granulocytes which were similar in number ($23.8 \pm 0.5\%$ and $24.7 \pm 0.06\%$ respectively), were found a higher proportion of semi-granulocytes were found in animals exposed to copper, while granulocytes were lower concerning the control ($p<0.05$). During the thermal exposure, granulocytes were affected mostly by thermal stress. This may occur as a result of cell death, or hemocyte immobilization to gills or other tissues (Matozzo et al., 2010; 2012; Lorenzon et al., 2001; Johnson et al., 2011).

Furthermore, a new hemocyte type was reported, whose function needs to be further elucidated. Lysosomal membrane stability Crabs exposed to abiotic and xenobiotic factors reported a significant reduction of Neutral Red Retention Time (NRRT) in all cases by a factor of 3–4, ranging from 36.4 ± 2.3 to 42.3 ± 2.6 min ($p<0.05$). This a bio-indicator of reduced membrane stability and damage to the membranous level will affect immunological competence such as phagocytosis and overall fitness (Anguirre-Martinez et al., 2013; Martinez-Gómez et al., 2008; Matozzo and Bailo, 2015). Xenobiotics are fused with lysosomes and form the phagolysosome. The lysosomal membrane destabilization may affect the Mg2+ATPase dependent H+ion proton pumps which may result in a leakage of lysosomal enzymes into the cytosol and induce apoptosis. A proposed mechanism of CHH-induced adaptive response of Mediterranean shore crabs to different stressors is given in Figure 2.

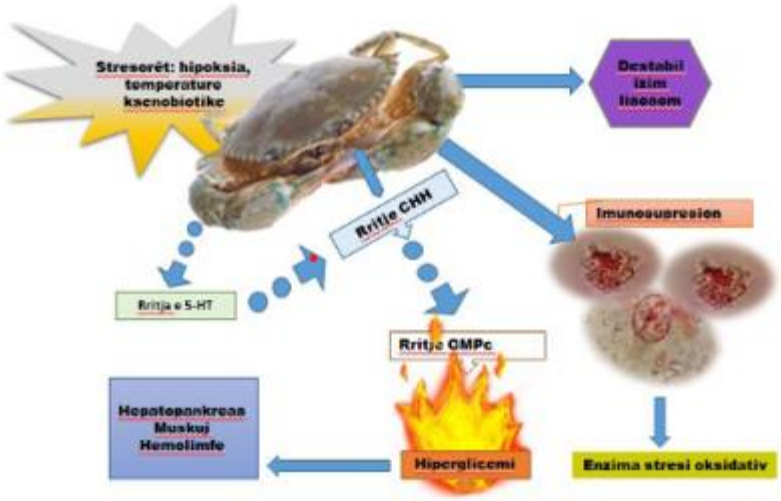

Figure 2. Mechanism of CHH induced adaptive response of *C. aestuarii* exposed to stressors

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Identification and characterization of CHH by HPLC Chromatography and LC-MT

Results of the chromatography show the presence of two protein piques: component A and B with a mass of 13120 Da and 9524.6 respectively. Since component B resulted in a bigger mass than expected (8-9.5 kDa, Jia et al., 2012) we can speculate that: 1) it might represent CHH and MIH together; 2) more than one type of CHH could be present in our species; or 3) a new cluster of CHH peptides specific for this species appears. Nucleotide Characterisation and deduced peptide sequences Sanger sequencing provided the nucleotide sequences of the CHH prepropeptide of *C.*
aestuarii (Figure 3), which resulted in an open reading frame sequence of 429 nt.

MYSKTIPAMLAIITVAYLCALPHAHARSTPGYGRMDRILAALKTSPMEPSAALAVEHGT
THPLEKRQIYDTSCKGVYDRALFNDLEHVCDDCYNLYRTSYVASACRSNCYSNLVFRQC
MDDLLMMDEFDQYARKVQMV*GRKK*

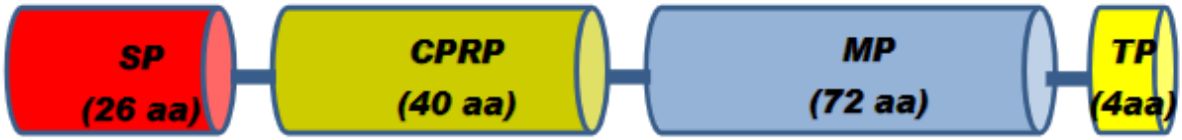

Figure 3. Amino-acid sequence of the CHH pre-pro-peptide

The deduced protein consisted of 142 aa with a signal peptide (SP) of 26 aa, followed by a CPRP of 40 aa and a mature peptide (MP) of 72 aa (Qyli et al., 2020). The presence of CPRP classifies CHH found in C. aestuariiin Type I (Webster et al., 2012). Furthermore, the CHH transcript keeps the same organization as the arthropodial tandem sequence: [LIVM]-x(3)-C-[KR]-x-[DENGRH]-C-[FY]-x-[STN]-x (2)-F-x (2)-C and also classifies this hormone as Type I (DeKlejn and Van Herp, 1995). The presence of 6 cysteine's supports the hyperglycaemic activity of the hormone (Giulianini and Edomi, 2006). Comparison of CHH sequence revealed a high similarity of 98.6% between C. aestuariiand C. maenas, Figure 4.

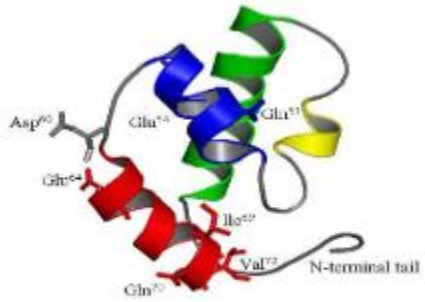

Figure 4. 3D structure of mature CHH peptide

Conclusions

C. aestuarii plays a key role as a bio-indicator of estuarine ecosystem health by changing the glucose levels, THC, and DHC depending on the stressor's exposure. This work represents the first study characterizing and sequencing the crustacean hyperglycemic hormone in *Carcinus aestuarii.* Transcripts discovered an open reading frame (ORF) of 429nt, coding for a prepropetide made up of a signal peptide (26 aa), a CPRP (40 aa), and a mature peptide (72aa) classifying it
into the Type I CHH. It has a similarity of 98.6% with *C. maenas* and relies on GenBank, in addition to the complete CHHcharacterization of *C. maenas,* this was the second complete CHH

sequence given for a Carcinus species. Bayesian analysis positioned *C.aestuarii* in Brachiura suborder, near *C. maenas*, holding an accession number: MW246807.

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