Comparative Biochemistry and Physiology, Part C 183–184 (2016) 61–71

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Haemolymph from *Mytilus galloprovincialis*: Response to copper and temperature challenges studied by ^{1}H-NMR metabonomics

Giuseppe Digilio, Susanna Sforzini, Claudio Cassino, Elisa Robotti, Caterina Oliveri, Emilio Marengo, Davide Musso, Domenico Osella, Aldo Viarengo *

Department of Sciences and Technological Innovation (DiSIT), University of Piemonte Orientale "Amedeo Avogadro", Viale T. Michel 11, 15121 Alessandria, Italy

ARTICLE INFO

Article history:
Received 6 November 2015
Received in revised form 1 February 2016
Accepted 15 February 2016
Available online 17 February 2016

Keywords:
Copper
Temperature
Ecotoxicology
Haemolymph
^{1}H-NMR
Metabonomics
Mytilus galloprovincialis

ABSTRACT

Numerous studies on molluscs have been carried out to clarify the physiological roles of haemolymph serum proteins and haemocytes. However, little is known about the presence and functional role of the serum metabolites. In this study, Nuclear Magnetic Resonance (NMR) was used to assess whether changes of the metabolic profile of *Mytilus galloprovincialis* haemolymph may reflect alterations of the physiological status of the organisms due to environmental stressors, namely copper and temperature. Mussel haemolymph was taken from the posterior adductor muscle after a 4-day exposure to ambient (16 °C) or high temperature (24 °C) and in the absence or presence (5 μg/L, 20 μg/L, or 40 μg/L) of sublethal copper (Cu^{2+}). The total glutathione (GSH) concentration in the haemolymph of both control and treated mussels was minimal, indicating the absence of significant contaminations by muscle intracellular metabolites due to the sampling procedure. In the ^{1}H-NMR spectrum of haemolymph, 27 metabolites were identified unambiguously. The separate and combined effects of exposure to copper and temperature on the haemolymph metabolic profile were assessed by Principal Component Analysis (PCA) and Ranking-PCA multivariate analysis. Changes of the metabolomic profile due to copper exposure at 16 °C became detectable at a dose of 20 μg/L copper. Alanine, lysine, serine, glutamine, glycogen, glucose and protein aliphatics played a major role in the classification of the metabolic changes according to the level of copper exposition. High temperature (24 °C) and high copper levels caused a coherent increase of a common set of metabolites (mostly glucose, serine, and lysine), indicating that the metabolic impairment due to high temperature is enforced by the presence of copper. Overall, the results demonstrate that, as for human blood plasma, the analysis of haemolymph metabolites represents a promising tool for the diagnosis of pollutant-induced stress syndrome in marine mussels.

1. Introduction

The response of mussels and other bivalve molluscs to ecotoxicological challenges has been widely studied due to the relevance of these organisms in the intertidal coastal ecosystem (Bayne, 1976) and their use as sentinel organisms in biomonitoring programmes, such as Med Pol, UNEP Mediterranean Biomonitoring Program, the OSPAR Convention (Viarengo et al., 2007; Shaw et al., 2011; Fasulo et al., 2012; Cappello et al., 2013a; Fasulo et al., 2015). Being the invertebrate equivalent of mammalian blood, the haemolymph of bivalve molluscs is an attractive biofluid to assess biomarker responses to environmental stressors, because it can report about the functional status of the organs which are perfused but lacks the molecular complexity of whole organ tissues (Al-Subiai et al., 2009; Poynton et al., 2011). Most of the research activity on haemolymph was targeted to the biological role of the cellular and protein components. In particular, the role of the haemocytes in the mollusc immune response was investigated (Fisher, 1986; Canesi et al., 2002; Hong et al., 2006; Pezzati et al., 2015) and numerous biomarkers were developed (Viarengo et al., 2007). The evaluation of lysosomal membrane stability using the Neutral Red retention time method (Moore and Lowe, 1977; Grundy et al., 1996; Moore et al., 1996) has become a well-established assay in basic and ecotoxicological-applied research (Viarengo et al., 2007). It was determined that histidine-rich glycoprotein (HRG) is the most abundant protein in the mollusc haemolymph serum (Abebe et al., 2007; Renwrantz and Werner, 2008; Oliveri et al., 2014). The HRG is thought to be involved in ion homeostasis and calcium deposition, but more recently it was suggested

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Abbreviations: VB, antimicrobial peptides, Cu^{2+}, copper; EDTA, ethylenediaminetetraacetic acid; FT, Fourier transformation; GSH, glutathione; HRG, histidine-rich glycoprotein; MOPS, (3-(N-morpholino)propanesulfonic acid; TSP, (3-trimethylsilyl)-2,2,3,3-tetradeuteropropionate); PCA, Principal Component Analysis; MVA, multivariate analysis.

* Corresponding author at: Department of Sciences and Technological Innovation (DiSIT), University of Piemonte Orientale, "A. Avogadro", V.le T. Michel 11, 15121 Alessandria, Italy.

E-mail address: viarengo@uniupo.it (A. Viarengo).

http://dx.doi.org/10.1016/j.cbpc.2016.02.003
1532-0456/

that, together with antimicrobial peptides (AMPs), it may participate in modulating immune response (Charlet et al., 1996; Yin et al., 2005; Li et al., 2009; Venier et al., 2011; Gerdol et al., 2012; Xue et al., 2012).

The low molecular weight compounds of haemolymph serum have comparably received much less attention, both in terms of metabolite characterisation/quantitation and the study of their biological functions. Catechols were found in scallop haemolymph and their role was suggested to be related to the activity of the nervous system, as their serum levels were correlated with muscular and ciliary activity (Pani and Croll, 2000). Lactic acid was detected in mussel haemolymph and its presence was thought be related to the anaerobic metabolism of these bivalve intertidal molluscs (Wu and Wang, 2010). A decrease in the carbohydrate concentration in starved molluscs and a circadian variation of glucose content was also reported (Gabbott, 1976). Changes in the concentration of amino acids and of the taurine/glycine ratio in mussel tissues and haemolymph in response to environmental stress were also demonstrated (Feng et al., 1970; Jeffries, 1972; Zhang et al., 2011b).

To unravel the biochemical response of organisms to external stimuli, exposure to xenobiotics, or environmental challenges, untargeted analytical techniques such as Nuclear Magnetic Resonance (NMR) or mass spectrometry (MS) based metabolomics (or metabonomics) represent an innovative approach (Lindon et al., 2000; Griffin, 2006; Simpson and Bearden, 2013). Metabolomics allows the identification and quantitation in a single analysis of a large number of low molecular weight compounds, i.e. the metabolites (Dunn and Ellis, 2005). Moreover, the global metabolic response can be evaluated without any a priori mechanistic hypothesis about the number, identity, and biochemical significance of the metabolites involved. In humans, metabolomics has been successfully employed to study the changes in the metabolite profile of blood plasma (Fossel et al., 1986; Lindon and Sweatman, 1996), urine (Lehnert and Hunkler, 1986; Nicholson and Wilson, 1989), and cerebrospinal fluid (Lindon et al., 1999), in response to pathological conditions (Lindon et al., 2000), to develop new biomarkers suitable to identify and stage important pathologies. In environmental sciences, several studies use metabolomics to reveal the response of aquatic organisms to environmental stressors or when the level of stressors/toxicants is below those required to give clear answers in traditional end-point assays (Viant et al., 2003; Samuelsson et al., 2006; Tuffnail et al., 2009; Sogin et al., 2014; Brandão et al., 2015). Among aquatic organisms, molluscs have been involved in metabolic phenotyping of organs and tissue extracts due to their prominent role as sentinel organisms in ecotoxicology (Hines et al., 2007; Zhang et al., 2011a; Zhang et al., 2011b; Cappello et al., 2013a, 2013b; Lankadurai et al., 2013; Cappello et al., 2015). However, metabolomic studies of marine invertebrates haemolymph are very scarce and limited, to the best of our knowledge, to *Haliotis rufescens* (Viant et al., 2003) and *Daphnia magna* (Poynton et al., 2011).

The aim of this study was i) to characterise the metabolic profile of *Mytilus galloprovincialis* haemolymph by NMR spectroscopy (to date, no specific study on the haemolymph of *M. galloprovincialis* is available) and ii) to assess whether mussel haemolymph metabolomic changes could report about alterations of the physiological status of the organisms due to environmental stressors, namely copper and temperature. Copper was chosen as a chemical toxicant because it is one of the most important inorganic chemical contaminants in the marine coastal environment (Wu and Wang, 2010; Zhang et al., 2011b; Negri et al., 2013). Temperature has been selected as a source of environmental stress due to the rise of seawater temperature arising from global climate changes. Mussels were treated with sublethal copper concentrations at 16 °C or 24 °C. These temperatures represent the seasonal temperature fluctuation in the Mediterranean coastal waters. In addition, the 8 °C temperature increase has been indicated by several modelling studies to represent climate changes at the end of the next century (MEECE, project no. 212085; Negri et al., 2013).

2. Materials and methods

2.1. Animals and treatments

Specimens of *M. galloprovincialis* (Lam.), 5–6 cm shell length, were purchased from an aquaculture mussel farm in Arborea (Sardinia, Italy). Animals were transferred to aquaria at a density of 1 animal/L in clean, aerated seawater collected offshore. Experiments were carried out at two temperatures (16 and 24 °C). After an acclimation of 6 days, 40 mussels were kept in polypropylene plastic vessels (four replicates per treatment) and underwent semi-static exposure to 5 μg/L, 20 μg/L, or 40 μg/L copper for 4 days. During the experiment, seawater of the desired temperature was renewed every day, and copper was added together with a commercial algal preparation (30 mg animal^{-1} day^{-1}; Liquifry; Interpret Ltd., Dorking, Surrey, UK). Control animals were maintained in the same conditions with no addition of copper. Taking into account the physiological differences between male and female, only prespawning female individuals (scored by microscopic inspection of gonad biopsies) were selected for the metabonomic study; this was made to reduce the biological inter-subject variability as previously shown by Banni et al. (2011) in relation to mussel transcriptomic data.

2.2. Haemolymph collection

After exposure to heat and copper, haemolymph was sampled from the posterior adductor muscle using a 1 mL sterile syringe with an injection needle 21G. Serum was obtained after centrifugation at 200 *g* for 10 min at 4 °C, the supernatant was immediately frozen in liquid nitrogen, and stored at −80 °C.

2.3. Analysis of copper in haemolymph

Haemolymph samples were acid-digested. An aliquot of haemolymph (typically 750 μL) was treated in a glass test tube with nitric acid (69% *w*/*v*) and hydrogen peroxide (30% *w*/*v*) in the 1:1:1.3 *v*/*v* mixing ratio. Open test tubes were put in a sand bath and heated to 90 °C on a hotplate for 2 h. The digested solutions were then diluted to 25 mL in a flask with ultrapure water. Quantitative analysis of copper was performed by inductively coupled plasma mass spectrometry (ICP-MS) with a Thermo X Series II ICP-MS instrument (Thermo Fisher Scientific, Rodano, MI, Italy). Copper and its isotopes are less affected by interference from isobar adducts that could take place during the atomisation/ionisation process. To further minimise such interferences, Cu^{2+} was analysed using the CCT-KED mode. The instrumental response was the average of three scans performed on the same solution. The calibration curve was generated in the 0.01–100 μg/L linear range of Cu^{2+}. The stability of the instrumental performances was controlled using an internal standard solution of 1.0 μg/L indium in 1% nitric acid. The data presented are the average of the analyses of at least three independent biological samples ± SD%.

2.4. Total glutathione assay

Haemolymph serum was mixed with two volumes of 1 N perchloric acid/2 mM EDTA (ethylenediaminetetraacetic acid) and then neutralised with 2 M KOH/0.3 M MOPS (3-(N-morpholino)propanesulfonic acid). Neutralised sera were used for the evaluation of the glutathione (GSH) concentration using the modified enzymatic assay described by Canesi and Viarengo (1997) in which 0.1 M K–phosphate buffer/1 mM EDTA pH 7, 0.2 mM NADPH, 0.06 mM dithionitrobenzoic acid, and 0.6 Units of GSSG reductase were added to 1 mL of a reaction mixture. Calibration was performed with known GSH concentrations.