Fish and Shellfish Immunology 75 (2018) 41–47

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Alterations in haemolymph proteome of *Mytilus galloprovincialis* mussel after an induced injury

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ARTICLE INFO

Keywords:
Biomarkers
Haemolymph
Inflammation
Mussels
Proteomics
Acute phase response

ABSTRACT

A proteomic and biochemical approach was performed to assess the effects of an induced muscle injury on the haemolymph of bivalve molluscs. For this purpose, *Mytilus galloprovincialis* were exposed to puncture of adductor muscle for three consecutive days, and their haemolymph proteome was then compared to healthy animals using 2-dimensional electrophoresis (2-DE) to identify proteins that differed significantly in abundance. Those proteins were then subjected to tandem mass spectrometry and 6 proteins, namely myosin, tropomyosin, CuZn superoxide dismutase (SOD), triosephosphate isomerase, EP protein and small heat shock protein were identified. SOD and tropomyosin changes were verified by spectrophotometric measurements and western blotting, respectively. As some of the proteins identified are related to muscular damage and oxidative stress, other biomarkers associated with these processes that can be evaluated by automatic biochemical assays were measured including troponin, creatine kinase (CK), and aspartate aminotransferase (AST) for muscle damage, and SOD, trolox equivalent antioxidant capacity (TEAC) and esterase activity (EA) for oxidative stress. Significantly higher concentrations of troponin, CK, AST, and TEAC were observed in mussels after puncture, being also possible biomarkers of non-specific induced damage.

Biological significance

Mussels are worldwide employed as sentinels of marine environment through national and internationals biomonitoring programs; however, the immune acute phase response in mussels is poorly known to date. The lack of information makes it difficult to understand vital processes like response to tissue damage at molecular level. The study herein presented is focused to the comprehension of the response of *M. galloprovincialis* to muscular damage, with the aim to identify possible new biomarkers of acute phase response.

1. Introduction

Marine bivalves are worldwide employed as sentinels of marine environment through national and international biomonitoring programs because they are sedentary, filter-feeding, economic and easy to sample [1–6]. Since invertebrates lack an adaptive immune system, they rely on innate immunity to provide defence against pathogens and inflamation [7–11]. Haemolymph is the circulatory fluid of invertebrates with analogous function to vertebrate's blood [8]. The haemolymph haemocytes play a major role in cellular defence [12], pathogen recognition and agglutination [13] while the proteins present in the serum of mussels' haemolymph are further known to be involved in the immune response and to bind metals [14–17]. However, the understanding of the molecular mechanisms related to haemolymph response to tissue damage is still scarce and mostly restricted to the study of individual proteins [8,18]. This lack of information makes it difficult to understand vital processes like response to tissue damage at molecular level.

Evaluation of protein expression is dependent on different parameters such as developmental stage and physiological status of the animal as well as the selected tissue for the analysis [19] or the

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https://doi.org/10.1016/j.fsi.2018.01.038
Received 13 November 2017; Received in revised form 15 January 2018; Accepted 25 January 2018
Available online 31 January 2018
1050-4648/

exposure to environmental conditions [20]. There are several studies in bivalves describing the alteration in expression of various proteins considered initially as biomarkers of pollution as well as in response to temperature changes [21]. In some investigations shifts in protein expression were observed during the reproductive cycle [22], in response to food availability [23] or salinity [24], and among other conditions generally considered as confounding factors. Nevertheless, it is likely that there are other specific proteins that may be involved in the biological response to induced damages [25]. In this context, proteomic studies already provided useful information about the identification of key elements of immune response to pathogens [7] or pollution [26] which could be used as biomarkers in biomonitoring programs. A proteomic approach has been used previously in bivalves for preliminary screenings of changes in protein expression caused by pollutants [27].

In mammals, the acute phase response (APR) is a key component of the innate immune response to infection, tissue injury, immunological disorders or pollution [28,29]. The main functions of APR are to restore homeostasis and to remove the cause of its disturbance [30] and it is usually measured in serum or plasma since these biofluids reflect the overall picture of the biochemical changes occurring in the organism [31] and are relatively easy to sample. In the last decades, increasing number of data about the APR become available in a wide range of species; however, little information regarding APR in bivalves is currently available. Therefore, we hypothesized that the induction of mechanical trauma in mussels could induce an APR, and will allow the detection of new biomarkers of APR in haemolymph that could be useful in other fields such as pollution biomonitoring.

The aim of this study was to identify possible new biomarkers of APR response in mussels after an experimental muscle injury. For this purpose, abductor muscle puncture was performed in a group of mussels (test group) and their haemolymph proteome was compared to that of control animals. After proteomic analysis, two of the identified proteins with differential expression were selected among the two studied groups and verified as possible biomarkers of non-specific response. In addition, since the proteomic study revealed significant differences in abundance of proteins related to muscular and oxidative stress, a battery of additional biomarkers linked to these processes were evaluated by high-throughput automated assays in the present study.

2. Material and methods

2.1. Animals and experimental set-up

Wild mussels *Mytilus galloprovincialis* (3–5 cm length, 9–10 g weight) from El Gorguel (geographic reference 37° 34′ 39,243" N 0° 52′ 35,893" W), an unpolluted site of Murcia (SE Spain), were collected and transported to the University of Murcia toxicology laboratory.

Acclimation period and experimental set-up are shown in Fig. 1. During 2 days, mussels were acclimated in a 12 L tank under controlled conditions of water pH (8.03 ± 0.07), osmolarity (1086.3 ± 28.39 mmol kg^{-1}) and temperature (24 °C), with continuous aeration, and natural photoperiod. During this period, mussels were daily fed with microalgae *Isochrysis galbana*, clon t-ISO (0.1% of microalgal organic matter per mussel live weight).

The experimental set-up was performed in duplicate. The proteomic analysis was performed in the first run (n = 6 controls and tests) while animals from the second run (n = 10 controls and test) were employed for proteomic data verification and biochemical analysis. Animals (n = 16) were punctured with 23G x 1″ syringe in the adductor muscle on days 1, 2 and 3 (test group). The rest of the animals (n = 16) were kept as control group.

The present study complied with all relevant regulations.

2.2. Sample collection and processing

Mussel haemolymph was collected on the fifth day after experiment initiation by a gentle aspiration with a 23G x 1″ syringe. Samples were centrifuged (4000 g, 10 min, 4 °C) and subsequently stored at −80 °C until the proteomic experiment. Biochemical measurements were performed immediately after haemolymph collection and centrifugation.

2.3. Two-dimensional electrophoresis (2-DE)

Total protein content of each sample was quantified following Bradford determination [32] and an equal protein amount of each sample were pooled in control (n = 6) and test group (n = 6), respectively. 2-DE of each pooled sample was run in triplicate. Briefly, in the first dimension isoelectric focusing (IEF) was carried out by running 38 micrograms of haemolymph proteins in 3–11 NL pH IPG strips of 11 cm length (GE Healthcare Life Sciences). The second dimension was performed by subjecting reduced and alkylated IPG strips to SDS-PAGE in homemade 12% polyacrylamide gels of 140 mm × 140 mm x 1.5 mm. Analytical gels were silver-stained using a protocol compatible with MS analysis [33].

2.4. Computational image and statistical analysis

2-DE gels were digitalized using a flatbed scanner in transmission mode and 2-DE proteome maps were analysed to identify protein spots which differed significantly in abundance between control and test gels by specific 2D-software (ImageMaster™ 2D platinum 7.0, GE Healthcare Life Sciences, Munich, Germany).

Analysis included manual aligned by defining landmarks distributed

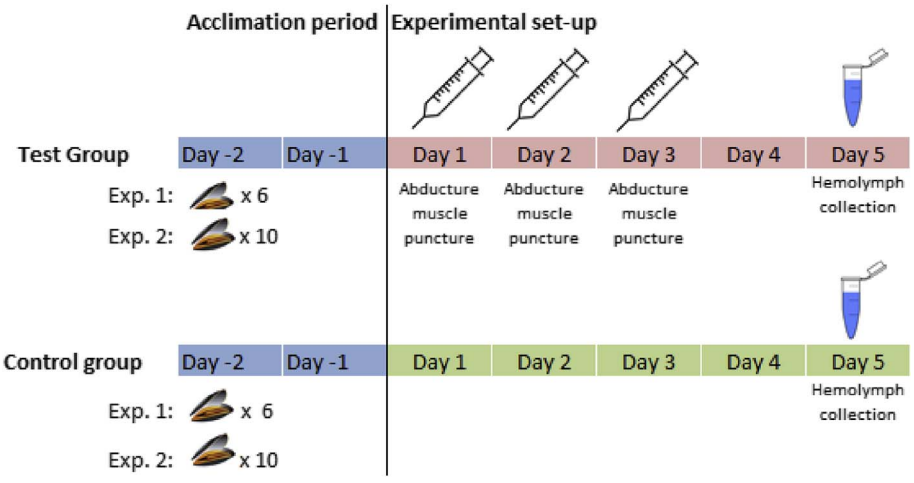

Fig. 1. Acclimation period and experimental set-up in test and control mussels. Mussels from test groups were punctured in the adductor muscle with a 23G syringe at days one, two and three after acclimation period. Controls were kept under the same conditions than animals of the test group, excluding the puncturing of adductor muscle. Experimental set-up 1 (Exp. 1) employed 6 animals for each group, while experimental set-up 2 (Exp.2) consisted of 10 test and 10 control animals.