Fish & Shellfish Immunology 55 (2016) 452–459

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Impact of ocean acidification on antimicrobial activity in gills of the blue mussel (*Mytilus edulis*)

B. Hernroth [a, b, *], S. Baden [c], H. Tassidis [b], K. Hörnaeus [d], J. Guillemant [d], S. Bergström Lind [d], J. Bergquist [d]

[a] The Royal Swedish Academy of Sciences, Sven Lovén Center for Marine Science, Kristineberg 566, SE – 451 78 Fiskebäckskil, Sweden
[b] Dept. of Natural Science, Kristianstad University, SE – 291 88 Kristianstad, Sweden
[c] Dept. of Biological and Environmental Sciences, University of Gothenburg, Kristineberg 566, SE – 451 78 Fiskebäckskil, Sweden
[d] Dept. of Chemistry – BMC, Analytical Chemistry and SciLifeLab, Uppsala University, Box 599, SE – 75124 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 27 January 2016
Received in revised form
7 April 2016
Accepted 8 April 2016
Available online 8 June 2016

Keywords:
Ocean acidification
Mytilus edulis
Antimicrobial peptide
Gill tissue
Vibrio
LPS
Proteome analysis
LC-Orbitrap MS/MS analysis

ABSTRACT

Here, we aimed to investigate potential effects of ocean acidification on antimicrobial peptide (AMP) activity in the gills of *Mytilus edulis*, as gills are directly facing seawater and the changing pH (predicted to be reduced from ~8.1 to ~7.7 by 2100). The AMP activity of gill and haemocyte extracts was compared at pH 6.0, 7.7 and 8.1, with a radial diffusion assay against *Escherichia coli*. The activity of the gill extracts was not affected by pH, while it was significantly reduced with increasing pH in the haemocyte extracts. Gill extracts were also tested against different species of *Vibrio* (*V. parahaemolyticus*, *V. tubiashii*, *V. splendidus*, *V. alginolyticus*) at pH 7.7 and 8.1. The metabolic activity of the bacteria decreased by ~65–90%, depending on species of bacteria, but was, as in the radial diffusion assay, not affected by pH. The results indicated that AMPs from gills are efficient in a broad pH-range. However, when mussels were pre-exposed for pH 7.7 for four month the gill extracts presented significantly lower inhibit of bacterial growth. A full in-depth proteome investigation of gill extracts, using LC-Orbitrap MS/MS technique, showed that among previously described AMPs from haemocytes of *Mytilus*, myticin A was found up-regulated in response to lipopolysaccharide, 3 h post injection. Sporadic occurrence of other immune related peptides/proteins also pointed to a rapid response (0.5–3 h p.i.). Altogether, our results indicate that the gills of blue mussels constitute an important first line defence adapted to act at the pH of seawater. The antimicrobial activity of the gills is however modulated when mussels are under the pressure of ocean acidification, which may give future advantages for invading pathogens.

1. Introduction

Climate change, caused by increased levels of greenhouse gases, mainly carbon dioxide (pCO_2), in the atmosphere, now emerges as one of the most important challenges of the 21st century [1]. Surface water of the world oceans is gradually getting warmer and its uptake of CO_2 across the air-sea interface alters seawater carbon chemistry, which is acidifying the oceans. So far, pH has been reduced by 0.1 units, representing an increase in $[H^+]$ of about 30% [2]. A further reduction of approximately 0.4 units is predicted by 2100 [3]; a phenomenon termed “ocean acidification” (OA).

Our recent research has shown that marine invertebrates, such as the sea-star *Asterias rubens* [4] and the Norway lobsters *Nephrops norvegicus* [5,6] become immune suppressed when exposed to seawater, mimicking the pH conditions that are predicted to occur by the end of this century. Such OA-effects have recently been shown in the blue mussel, *Mytilus edulis*, and it was found that the mussels' bacteriostatic capacity against the bivalve pathogen *Vibrio tubiashii* was significantly reduced at pH ~7.7 [7].

The immune defence of mussels is mainly based on encapsulation and phagocytosis by circulating haemocytes [8] including fundamental mechanisms for cell killing, such as reactive oxygen metabolites and lysosomal enzymes [9–11]. An acidified environment generates shell dissolution in mussels causing hypercapnia, which may reduce the functional properties of haemocytes [12–14]. However, despite shell dissolution the study by Asplund et al. [7] showed that calcium haemostasis was maintained after 4 months of exposure to OA and neither haemocyte counts nor phagocytic capacity were shown affected by the OA-condition. The

* Corresponding author. The Royal Swedish Academy of Sciences, Sven Lovén Center for Marine Science, Kristineberg 566, SE – 451 78 Fiskebäckskil, Sweden.
E-mail address: bodil.hernroth@kva.se (B. Hernroth).

http://dx.doi.org/10.1016/j.fsi.2016.04.007
1050-4648/

immunomodulation behind the reduced bacteriostatic capacity may involve other mechanisms such as e.g. transduction of bacterial signals into the host cell, which so far have not been investigated in relation to OA. Neither has potential modulation of the activation of antimicrobial peptides (AMPs) been included in OA-studies, even though they constitute important components of mussel immunity [15]. Such broad-spectrum AMPs are potent against a wide range of microbes, including bacteria, fungi, viruses and protozoa and have been described for both plants and animals, as reviewed by e.g. Zasloff [16] and Yeaman and Yount [17].

The limited access to gene sequences makes proteomic studies of *Mytilidae* difficult and relatively few proteins have been identified in these non-model organisms [18–20]. In the last few decades, six groups of AMPs have been described in the Mediterranean mussel *M. galloprovincialis*; defensins [21], myticins [22,23], mytilins [24], mytimicin [25], mytimacins and big defensins [26], and an additional peptide, the myticusin, has been found in *Mytilus corusus* [27]. Among these, three have been isolated also from haemocytes of *M. edulis*. These are mytilin (isoform A and B), mytimicin and defensin (isoform A and B) [28,29], which are characterized as cationic, cysteine rich β-sheets. It has still not been clarified whether AMPs of *Mytilus* are constitutively expressed [30] or might be inducible [21,28] as in insects and vertebrates.

Blue mussels are filter-feeding bivalves, and gills are one of the major sites of interaction with the environment. By using gamma camera technique, it has been demonstrated that high concentrations of pathogens can accumulate along the ciliated filaments [31]. In coastal waters the mussels are exposed to pathogens from e.g. improper disposal of human sewage waste and leaching from agricultural activities, including cattle grazing at nearshore meadows. Thus, *Escherichia coli* are commonly used as fecal indicator bacteria in recreational waters and sea food. However, there are also resident pathogens in seawater, such as bacteria of the genus *Vibrio*, which are able to infect humans as well as shellfish. Like in other organisms there is a need of an efficient first-line defence on the mussel epithelium. Mitta et al. [32] have shown mytilin immune reactivity in infiltrating haemocytes of the gill epithelium of *M. galloprovincialis* and Balserio et al. [33] have shown expression of myticin C in granular haemocytes infiltrating this tissue. If AMPs are delivered on gill epithelium they are supposed to act at a pH level in accordance to the ambient seawater (pH ~ 8.1), compared to AMPs in haemolymph (pH ~ 7.5) [7] and the acidic vesicles of haemocytes (pH ~ 3–5 as measured in phagolysosomes of oysters by Beaven and Paynter [34]). On a short term, pH of the ambient sea water may fluctuate considerably, particularly in tidal zones, where adapted bivalves can survive for hours by closing the shells during hypoxic events. However, virtually nothing is known about functional properties of epithelium peptides and potential effects of long term exposure to OA.

The aim of this study was to investigate if peptide extracts of the gills of *M. edulis* have antimicrobial activity against the Gram-negative bacteria *E. coli* and *Vibrio* spp. We investigated if the activity was affected by lowered pH, at a level mimicking future ocean acidification (OA). This was carried out through *in vitro* studies comparing the minimal inhibitory concentration of peptide extracts, from gill tissue and haemocytes of *M. edulis*, against *E. coli* at different pH. In another experiment, metabolic activity of different marine bacterial species of the genus *Vibrio* was investigated after incubation with gill extracts at pH 8.1 (pH of seawater today) and 7.7 (pH predicted for 2100). In addition, bacteriostatic capacity of gill extracts from mussels that for four months were pre-exposed to seawater of either pH 8.1 or 7.7, was determined as growth inhibition of *V. parahaemolyticus*. Furthermore, the full proteome and especially the small proteins, including AMPs of gill extracts were investigated, using LC-Orbitrap MS/MS analysis. Quantitative peptide/protein expression was compared between different time points after immune challenge with lipopolysaccharide (LPS).

2. Material and methods

2.1. Experiment 1: In vitro comparison of antimicrobial activity of peptide extracts at different pH

2.1.1. Sampling

Blue mussels, *M. edulis*, (length ~5–7 cm) were collected at ~2 m depth in the vicinity of Sven Lovén Centre for Marine Science, Kristineberg, (SLC-Kristineberg) at the Swedish west coast, where the tidal amplitude is only ~0.2 m. The mussels were until used, kept in containers supplied with running seawater from the water system at SLC-Kristineberg in a thermo-constant room (~32 PSU, ~14 °C).

For each sample, haemolymph from ten mussels were pooled. Two mL haemolymph per mussel was squirted into an equal volume of anti-coagulant buffer (0.05 M Tris-HCl pH 7.6, 2% glucose, 2% NaCl, 0.5% EDTA) in a 50 mL centrifuge tube and kept on ice. It was centrifuged (Beckman J-18) at 1200 × *g* at 4 °C for 15 min and the pellet was used for extraction of peptides.

The gills were dissected and pooled in the same way as for haemocytes, then frozen in −80 °C and lyophilised for about 20 h (Lyovac GT 2, Leybold-Heraus) before peptide extraction.

2.1.2. Peptide extractions

The method for extracting peptides from haemocytes was slightly modified after Mitta et al. [22]. Briefly, the haemocyte pellet was homogenized in 50 mM Tris buffer with 50 mM NaCl, pH 8.7. Organelles were collected through centrifugation before sonicated in 2 M ice cold acetic acid. After centrifugation at 10,000 × *g* at 4 °C for 20 min the supernatant was loaded on a Sep-Pak C_{18} Vac cartridge (Waters Associates) which had been equilibrated with acidified (0.05% triflouroacetic acid (TFA); 99%, Acros organics) sterile water, also used to wash out unbound fragments. For eluting the peptides acidified (0.05% TFA) 40 and 80% acetonitrile (CH_3CN) were used. Acetonitrile was evaporated in vacuum (SpeedVac Concentrator) before storing the tubes in a freezer until used for the antimicrobial assay.

Gills were homogenized in 10 vol of 50 mM phosphate buffer, then placed horizontally on an orbital shaker over night at 6 °C before centrifuged (Beckman J-18) at 5320 × *g* at 4 °C for 30 min. The supernatant was loaded on a Sep-Pak C-18 cartridge, following the same procedure as described for haemocytes.

Before testing the antimicrobial activity, as described in Section 2.1.3, the dry Sep-Pak C_{18} fractions were reconstituted in 100 μL of sterile water and the protein concentrations were determined using Coomassie assay, with bovine serum albumin (BSA) as standard [35].

2.1.3. Antimicrobial assay: MIC μg mL^{-1}

Minimal inhibitory concentration (MIC μg mL^{-1}) of the peptide fractions, used for the *in vitro* study, was determined with a radial diffusion assay with double layer agar [36]. The gram-negative bacterium *E. coli* O14 was cultured in LB-broth (20 g L^{-1} LB base) adjusted to pH 6.0, 7.7 and 8.1, respectively, to mid-logarithmic phase. Hundred μL of bacterial suspensions, at a concentration of 6 × 10^4 colony forming units (CFU) μL^{-1}, was added to agarose [0.11 g agarose (DNA-grade), 10 mL 10 mM sodium phosphate buffer, 20 μL LB-broth and 2 μL Tween-20]. The pH was set to 6.0, 7.7 and 8.1, respectively, before used as an under layer in a petri dish (90 mm in diameter). Wells, 3 mm in diameter, were punched in the agar and 5 μL of serial doubling dilutions of the peptide extraction in sterile water were added to the wells. The agar plates