Developmental and Comparative Immunology 38 (2012) 232–242

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

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

Looking for putative phenoloxidases of compound ascidians: Haemocyanin-like proteins in *Polyandrocarpa misakiensis* and *Botryllus schlosseri*

Loriano Ballarin [a], Nicola Franchi [a], Filippo Schiavon [a], Silvio C.E. Tosatto [a], Ivan Mičetić [a], Kazuo Kawamura [b,*]

[a] Department of Biology, University of Padova, Italy
[b] Laboratory of Cellular and Molecular Biotechnology, Faculty of Science, University of Kochi, Japan

ARTICLE INFO

Article history:
Received 18 January 2012
Revised 3 May 2012
Accepted 8 May 2012
Available online 12 June 2012

Keywords:
Ascidians
Botryllus
Polyandrocarpa
Phenoloxidase
Haemocyanin-like proteins
Morula cells

ABSTRACT

Phenoloxidases (POs) and haemocyanins constitute a family of copper-containing proteins widely distributed among invertebrates. Both of them are able, under appropriate conditions, to convert polyphenols to quinones and induce cytotoxicity through the production of reactive oxygen species, a fundamental event in many immune responses. In ascidians, PO activity has been described and studied in both solitary and colonial species and the enzyme is involved in inflammatory and cytotoxic reactions against foreign cells or molecules, and in the formation of the cytotoxic foci which characterise the non-fusion reaction of botryllids. Expressed genes for two putative POs (CiPO1 and CiPO2) have been recently identified in *C. intestinalis*.

In the present study, we determined the cDNA sequences of two haemocyanin-like proteins from two colonial ascidians: *Botryllus schlosseri* from the Mediterranean Sea and *Polyandrocarpa misakiensis* from Japan. Multiple sequence alignments evidenced the similarity between the above sequences and crustacean proPOs whereas the analysis of the three-dimensional structure reveals high similarity with arthropod haemocyanins which share common precursors with arthropod proPOs. *Botryllus* HLP grouped in the same cluster with *Ciona* POs, whereas *Polyandrocarpa* HLP clustered with arthropod haemocyanins; all of them share the full conservation of the six histidines at the two copper-binding sites as well as of other motifs, also found in arthropod haemocyanin subunits, involved in the regulation of enzyme activity. *In situ* hybridisation indicated that the genes are transcribed inside morula cells, a characteristic haemocyte type in ascidians where PO activity is located, at the beginning of their differentiation. These results represent a first attempt to identify candidate molecules responsible of the PO activity in compound ascidians.

1. Introduction

Phenoloxidases (POs) are copper-containing enzymes, widely distributed in invertebrates, able to convert phenolic substrata to quinones which, then, polymerise to form melanin. They exert a pivotal role in immune responses through the induction of cytotoxicity consequent to the production of reactive oxygen species and semiquinones which can either induce oxidative stress or rapidly react with biomolecules altering their functionality. In addition, quinones themselves are cytotoxic as they can either undergo oxidation and generate reactive oxygen species (Nappi and Vass, 1993; Nappi and Ottaviani, 2000) or react with –SH groups on essential molecules (Kato et al., 1986). Melanin also has toxic properties and its formation (e.g. around nodules of non-self material enveloped by host immunocytes) helps in preventing the survival of foreign organisms (Nappi and Ottaviani, 2000). In arthropods, where the enzyme has been particularly studied, PO is usually stored, as inactive zymogen (proPO), inside specific granules of PO-containing circulating immunocytes, the morphology of which varies greatly among the different species (Aspán et al., 1995; Kawabata et al., 1995).

Arthropod proPOs are closely related to arthropod haemocyanins with which they share common evolutionary precursors, conservation of the organisation of the copper-binding sites and of other motifs involved in the stabilisation of the protein subunits (Van Holde et al., 2001; Immesberger and Burmester, 2004), and similar three-dimensional structure (Hazes et al., 1993; Li et al., 2009). In addition, arthropod haemocyanins, under certain conditions, can acquire PO activity (Decker and Jaenicke, 2004; Lee et al., 2004). The activation of proPO to PO has been particularly studied in crustaceans: it requires the degranulation of PO-containing cells and the release of the zymogen in the extracellular

* Corresponding author. Address: Department of Biology, University of Padova, via U. Bassi 58/B, 35121 Padova, Italy. Tel.: +39 049 8276197; fax: +39 049 8276199.
E-mail address: loriano.ballarin@unipd.it (K. Kawamura).

0145-305X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dci.2012.05.008

milieu where it is converted to active PO by extracellular serine proteases, called proPO-activating enzymes (Jiang and Kanost, 2000), which are the end product of a cascade triggered by the recognition of foreign molecules on the surface of microbial cells (Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004; Cerenius et al., 2008).

Tunicates are considered the sister group of vertebrates (Delsuc et al., 2006). Most of the literature data on this subphylum refer to ascidians which represent the richest in species and best studied class of tunicates. In ascidians, PO activity has been described and studied in both solitary (Chaga, 1980; Smith and Söderhäll, 1991; Jackson et al., 1993; Hata et al., 1998; Cammarata et al., 1997, 2008; Parrinello et al., 2003) and colonial (Ballarin et al., 1994a, 1998; Frizzo et al., 1999; Shirae and Saito, 2000; Shirae et al., 2002; Cima et al., 2004; Zaniolo et al., 2006) species; in these organisms, the enzyme is involved in inflammatory and cytotoxic reactions against foreign cells or molecules (Akita and Hoshi, 1995; Cammarata et al., 1997, 2008; Ballarin et al., 1998, 2005; Hata et al., 1998; Shirae and Saito, 2000; Shirae et al., 2002; Parrinello et al., 2003). In colonial botryllid ascidians, the enzyme mediates the formation of the cytotoxic foci along the contacting edges of genetically incompatible colonies which characterises the non-fusion reaction (Hirose et al., 1990; Ballarin et al., 1995, 1998; Shirae and Saito, 2000; Shirae et al., 2002; Cima et al., 2004; Zaniolo et al., 2006). However, despite the abundance of data on POs and their activity in ascidians, the sequences and the three-dimensional structures of the molecules responsible of the observed enzyme activity are still scarcely known. Expressed genes for two putative POs (CiPO1 and CiPO2) have been identified in *C. intestinalis* (Immesberger and Burmester, 2004) and this paper still represents the only molecular data on ascidian PO in the literature.

The present report presents the results of the first biomolecular study aimed to identify molecules with PO activity in colonial ascidians exploiting the expected similarity with arthropod POs and haemocyanins (Immesberger and Burmester, 2004). We identified and determined the cDNA sequences of putative haemocyanin-like proteins (HLPs) from two colonial ascidians: *Botryllus schlosseri* from Mediterranean (Adriatic) Sea and *Polyandrocarpa misakiensis* from Japan: *in situ* hybridisation (ISH) indicated that the genes are transcribed inside morula cells (MCs), a characteristic haemocyte type in ascidians where PO activity is located, at the beginning of their differentiation. Multiple sequence alignments confirmed the similarity between our sequences and crustacean proPOs and haemocyanins; phylogenetic analysis indicated that *Botryllus* putative HLP grouped in the same cluster with *Ciona* POs, whereas *Polyandrocarpa* putative HLP clustered with arthropod haemocyanins. Three-dimensional models of these molecules stressed the close relationship with arthropod haemocyanin subunits.

2. Materials and methods

2.1. Animals

Colonies of *B. schlosseri*, collected from the lagoon of Venice, were reared, attached to glass slides, in large, aerated aquaria with continuous seawater flow, at the Marine Station of the Department of Biology, University of Padova, in Chioggia, in the southern lagoon. Colonies of *P. misakiensis* were reared in the field, inside culture boxes set in the bay facing the Usa Marine Biological Station of the University of Kochi (Japan). Before their use, they were brought in the laboratory of Cellular and Molecular Biotechnology of the Faculty of Science and starved for few days, at room temperature, in 20-l aquaria filled with seawater.

Botryllus colonies alternate cyclical (weekly at 20 °C) changes of generation, lasting 24–36 h, during which adult zooid tissues undergo apoptosis and are replaced by palleal buds which grow to functional maturity (Manni et al., 2007). The phase of generation change is also known as take-over, whereas phases more than one day from the preceding or following take-over have been collectively called mid-cycle (Manni et al., 2007). In *Polyandrocarpa*, adult zooids form continuously new palleal buds which, soon, loose any connection with the parent zooid and complete their development as isolated buds (Kawamura and Nakauchi, 1986). The term "developing buds" refers to the isolated buds growing to maturity.

2.2. Haemocyte collection and short-term cultures

B. schlosseri haemolymph was collected with a glass micropipette after puncturing, with a fine tungsten needle, the colonial marginal vessel whereas *P. misakiensis* haemolymph was collected from developing buds punctured with a fine tungsten needle. In both cases, colonies were previously immersed for few min in 0.01 M Na-citrate in artificial seawater (ASW; 0.45 M NaCl, 0.03 M $MgCl_2$, 0.02 M $MgSO_4$, 0.01 M $CaSO_4$, 0.01 M KCl, 0.0008 M NaBr; Tropic Marine Neu, Euraquarium, Bologna, Italy), in order to prevent cell clotting, and then blotted dry. Haemolymph was then centrifuged at 700*g* for 10 min at 4 °C and the pellet was re-suspended in ASW to a final concentration of 10^6 cells/ml. Sixty microliters of haemocyte suspension were placed in the centre of culture chambers, prepared as described elsewhere (Ballarin et al., 1994b), and left to adhere to coverslips for 30 min at room temperature.

2.3. Histological sections

B. schlosseri colonies were anesthetized with MS222 (Sigma) and fixed for 2 h in Bouin's solution, rinsed in PBS, dehydrated, and embedded in Paraplast X-TRA (Oxford Labware). Sections (7 μm thick) were cut with a Leitz 1212 microtome, stained with Mayer's hematoxylin (Fluka) and observed under a Leitz Dialux 22 light microscope.

Fragments of *P. misakiensis* zooids were fixed for 1 h in 4% paraformaldehyde, dehydrated with ethanol, included in JB4 resin (Polyscience Inc.) as described below, and serially sectioned at a thickness of 2 μm.

2.4. Cytoenzymatic assay for PO

Fixed haemocyte were incubated for 60 min in a saturated solution of L-DOPA (Fluka) or 2 mM catechol in phosphate-buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l Na_2HPO_4, pH 7.2). After a final washing in PBS, coverslips were mounted on glass slides with 80% glycerol. Positive sites appeared dark brown (Hose et al., 1987). Phenylthiourea (PTU; 1 mM) was added to the incubation mixture as a control for specificity. Control (untreated) haemocytes were stained with haematoxylin.

2.5. PO activity assay

B. schlosseri and *P. misakiensis* haemolymph was collected as described above in the absence of anti-clotting agents. Half of the collected volume was subjected to sonication with a Braun Labsonic U sonifier (50% duty cycle) for 1 min and centrifuged at 10,000 rpm for 10 min to obtain whole blood lysate (WBL); the remaining haemolymph was centrifuged at 780*g* for 10 min. The supernatant (blood plasma; BP) was collected, whereas the pellet was resuspended in ASW, sonicated and subsequently centrifuged at 10,000*g* for 10 min to collect the supernatant (haemocyte lysate; HL). Fifty microliters of WBL, BP or HL were incubated with 950 μl of a saturated solution of L-DOPA in PBS and the reaction was followed for 5 min at 490 nm. One unit (U) of phenoloxidase activity determines an increase of 0.001 absorbance units/min at