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Prophenoloxidase system, lysozyme and protease inhibitor distribution in the common cuttlefish *Sepia officinalis*

Charles Le Pabic *[,1], Georges Safi [1], Antoine Serpentini, Jean-Marc Lebel, Jean-Paul Robin, Noussithé Koueta

Normandie Université, F-14032 Caen, France
UMR BOREA, MNHN, UPMC, UCBN, CNRS-7028, IRD-207, IBFA Université de Caen Basse-Normandie, Esplanade de la Paix, CS 14032, 14032 Caen Cedex, France
Centre de Recherches en Environnement Côtier, Université de Caen Basse-Normandie, 54 rue du Docteur Charcot, 14530 Luc-sur-Mer, France

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ABSTRACT

The immune system of cephalopods remains poorly understood. The aim of this study was to determine the specific activity of immune enzymes in epithelial barriers, circulatory and digestive systems of the common cuttlefish *Sepia officinalis*. Three enzyme groups with putative functions in immunity were investigated: phenoloxidases (POs), lysozymes and protease inhibitors (PIs). Consistent with a role in immunity, highest PO activities were found in the integument as well as the respiratory and circulatory organs under zymogenic (proPO) and active form. Surprisingly, high PO activities were also found in the digestive gland and its appendages. Similarly, high lysozyme activities were detected in the integument and circulatory organs, but also in the posterior salivary glands, highlighting the implication of this antibacterial enzyme group in most tissues exposed to the environment but also within the circulatory system. Albeit highest in digestive organs, the ubiquitous detection of PI activity in assayed compartments suggests immune function(s) in a wide range of tissues. Our study reports proPO/PO, lysozyme and PI distributions in *S. officinalis* body compartments for the first time, and thus provides the fundamental basis for a better understanding of the humoral immune system in cephalopods as well as invertebrates.

1. Introduction

Most aquatic organisms inhabit environments rich in bacteria and other microorganisms. Unlike air, water functions as a medium for both transport and growth of microbes (Hansen and Olafsen, 1999; McFall-Ngai et al., 2010; Gomez et al., 2013). Thus a critical function of the immune system of aquatic organisms is to provide a protection against this constant pathogenic threat (Iwanaga and Lee, 2005). What adaptations of the immune system allow aquatic species to thrive in these conditions? Unlike vertebrates, invertebrates rely on innate immunological mechanisms alone as defense against pathogens (Loker et al., 2004). Cell-mediated defense mechanisms are mainly carried out by hemocytes, which behave like macrophages (Heath-Heckman and McFall-Ngai, 2011), while humoral factors take part in various functions such as pathogen recognition, signaling pathway activation, and invader elimination (Wang et al., 2013). Among them, we chose to focus on (1) one of the most effective signaling pathways of invertebrates—the melanization cascade, (2) one well-known hydrolytic enzyme group involved in pathogen elimination—the lysozymes, and (3) components of the immune proteolytic cascade, the protease inhibitors (PIs) (Rowley and Powell, 2007; Cerenius et al., 2008; Xue et al., 2009; Fiołka et al., 2012; Herreweghe and Michiels, 2012; Amparyup et al., 2013; Wang et al., 2013).

While a major immune mechanism among many invertebrate taxa, melanization remains understudied in mollusks. It is involved in (1) non-self recognition, (2) production of toxic compounds against pathogens, (3) wound healing and (4) cellular-defense factor synthesis (e.g. Siddiqui et al., 2006; Cerenius et al., 2008; Luna-Acosta et al., 2010). Phenoloxidases (POs), a family of copper (Cu) proteins catalyze the rate-limiting step in melanin production: oxidation or hydroxylization of phenols into quinones. Importantly, POs are synthetized as zymogenic form called prophenoloxidases (proPO) and activated by serine protease cleavage (Cerenius and Söderhäll, 2004; Masuda et al., 2012; Amparyup et al., 2013). Because of their immune functions, highest PO levels are usually found in association with epithelial barriers, respiratory and

Abbreviations: APO, activated phenoloxidase; BAPNA, Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride; BH, branchial hearts; BHA, branchial heart appendages; BSA, bovin serum albumin; Cu, copper; DG, digestive gland; DGA, digestive gland appendages; DMSO, dimethyl sulfoxide; HEW, hen egg white; Hcy, hemocyanin; L-DOPA, 3,4-dihydroxy-L-phenylalanine; PI, protease inhibitor; PO, phenoloxidase; proPO, prophenoloxidase; PSG, posterior salivary glands; SE, standard error; SH, systemic heart; Stc, stomach; TI, trypsin inhibition; WB, white bodies.

* Corresponding author at: UMR BOREA, MNHN, UPMC, UCBN, CNRS-7028, IRD-207, IBFA Université de Caen Basse-Normandie, Esplanade de la Paix, CS 14032, 14032 Caen cedex, France. Tel.: +33 231 565 102; fax: +33 231 565 346.
E-mail address: charles.lepabic@gmx.fr (C. Le Pabic).

[1] These authors contributed equally to the study.

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circulatory systems in arthropods and bivalves (Asano and Ashida, 2001; Franssens et al., 2008; Luna-Acosta et al., 2011a; Masuda et al., 2012; Zhou et al., 2012).

Lysozymes cleave the 1,4-β-glycosidic linkage between N-acetylglucosamine and N-acetylmuramic acid of bacteria cell walls in order to lyse the cell—a process used in both immune defense and gastric digestion (Herreweghe and Michiels, 2012). Apart from this antimicrobial property, lysozymes interact with immune system compounds (e.g. complement pathway, lectins, proPO) to modulate or enhance humoral immune response (Goto et al., 2007; Park et al., 2007; Rao et al., 2010; Wang and Zhang, 2010; Herreweghe and Michiels, 2012). Their chitinase activity is also used to ward off fungal infection (Herreweghe and Michiels, 2012). Consistent with these properties they are mainly associated with the epithelial barriers, respiratory and circulatory systems of numerous aquatic organisms such as bivalves and fish, and in some cases digestive tracts of bivalves because of their use of bacteria as food (Saurabh and Sahoo, 2008; Herreweghe and Michiels, 2012).

Finally, PIs aid in the defense of various organisms by regulating or inhibiting bacterial protease activities through interaction with their reaction sites or entrapment. PIs also play a central role in the regulation of a wide variety of immune processes including (1) hemolymph coagulation, (2) proPO activation and (3) synthesis of cytokines and antimicrobial peptides (Xue et al., 2009). However, PIs also take part in other processes such as digestion, where protease regulation is necessary, and are consequently not concentrated in tissues with immune functions.

Cephalopods are a highly derived group in the molluscan clade, with a complex nervous system allowing elaborate body patterning and behavior, a deeply modified body plan organization, and highly diverse modes of life (Hanlon and Messenger, 1988; Bassaglia et al., 2013). However little is known about their immune system (Ford, 1992; Castellanos-Martínez and Gestal, 2013). Furthermore, cephalopods possess anatomical peculiarities within mollusks such as a closed circulatory system with a central systemic heart (SH) and two branchial heart (BH) complexes (Schipp, 1987), as well as clearly identified hematopoietic organs—the white bodies (WB) (Claes, 1996), suggesting immune pathway modifications. Yet, immune involvement of POs has never been studied in cephalopods, only one study reported lysozyme repartition in the octopod *Eledone cirrhosa* (Malham et al., 1998), and PI activity was mostly described in the plasma (Armstrong, 1992; Thøgersen et al., 1992; Vanhoorelbeke et al., 1994), and the digestive gland of several species (Ishikawa et al., 1966; Sof'ina et al., 1988; Kishimura et al., 2001, 2010).

Based on previously described enzyme repartitions and roles, we hypothesized that (1) higher proPO/PO- and lysozyme-activities would be found in the circulatory system and tissues directly exposed to the outer environment in *Sepia officinalis*: plasma, BH and their appendages (BHA), SH, WB, integument, mantle and gill, and (2) that PIs would be ubiquitous in *S. officinalis* compartments because of the general need to regulate proteases.

Here, we report the distribution of proPO/PO, lysozymes and PIs in 13 body compartments selected for their functions in circulation, respiration, immunity, digestion or as physical barriers in adult common cuttlefish: respiratory and circulatory compartments (gill, plasma, SH, BH, BHA, and WB), digestive organs (posterior salivary glands (PSG), stomach (Stc), cecum, digestive gland (DG) and its appendages (DGA)), and integument and mantle, as first epithelial barrier and underlying muscle tissue, respectively. Higher activated PO (APO)- and lysozyme-activities were mainly found in the integument, circulatory and respiratory organs consistent with a role in immunity. In addition, we found high PO-activities in the DG, potentially highlighting its role(s) in protection of the digestive tract against infections and/or hemocyanin (Hcy) metabolism. Lastly, higher PI-activities were mostly found in digestive system organs despite their presupposed ubiquitous repartition.

2. Material and methods

2.1. Animals and tissue samples

Ten adult common cuttlefish *S. officinalis* (mean ± standard deviation; mass = 1.03 ± 0.38 kg; dorsal mantle length = 21.3 ± 3.5 cm) were obtained from traps deployed along the Calvados coast (Basse-Normandie, France) during summer 2012. Prior to experimentation, the animals were maintained in 4500-L tanks in an open seawater circuit and starved for 24 h at 15 °C at the Centre de Recherches en Environnement Côtier (C.R.E.C., Luc-sur-Mer, Basse-Normandie, France).

In order to study basal enzymatic activities in cuttlefish tissue, we used animals without visible wounds, with normal swimming behavior and predatory behavior display upon prey presentation. We also carefully visually checked for the absence of macroscopic parasites in animals and organs sampled. Following ethical procedures (Directive 2010/63/EU), cuttlefish were anesthetized as described by Andrews et al. (2013) through placement for 10 min in seawater containing 2% ethanol. Five milliliter hemolymph was then withdrawn from anterior mantle vein (King et al., 2005) using syringe with 18-gauge needle and kept in ice, and animals were killed by rapid decapitation. Digestive gland, DGA, PSG, Stc, cecum, WB, SH, gill, BHs and BHA were then harvested and placed on ice. In addition, pieces of mantle and integument (with associated mucus) were sampled (Fig. S1). At the end of the dissection, tissues were rinsed in cold extraction buffers and kept at −80 °C until enzyme extraction. Hemolymph samples were centrifugated at 500 *g* to separate plasma and hemocyte fractions, and cell-free plasma was stored at −80 °C until analysis.

2.2. Chemicals

Aprotinin, Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), bovin serum albumin (BSA), Bradford reagent, calcium chloride ($CaCl_2$), citric acid ($C_6H_8O_7$), dimethyl sulfoxide (DMSO), hen egg white (HEW) lysozyme, 3,4-dihydroxy-L-phenylalanine (L-DOPA), magnesium chloride ($MgCl_2$), freeze-dried *Micrococcus lysodeikticus*, Protease Inhibitor Cocktail, sodium chloride (NaCl), sodium phosphate dibasic dihydrate ($Na_2HPO_4{\cdot}2H_2O$), trizma base, trizma hydrochloride (Tris–HCl), tropolone and trypsin TPCK (N-tosyl-L-phenylalanyl chloromethyl ketone) were obtained from Sigma-Aldrich (France). Halt Protease Inhibitor Cocktail, EDTA-Free (100×) was obtained from Thermo Fisher Scientific (Waltham, USA).

2.3. Enzyme extraction

Tissue samples were weighed before to be ground in liquid nitrogen. Once a fine powder was obtained, the sample was homogenized in a known amount (10 mL to 1 g) of cold Tris buffer pH 8 (10 mM Tris–HCl and 150 mM NaCl) for lysozyme and PI assays (Safi, 2013) or Tris buffer pH 7 (0.1 M Tris–HCl, 0.45 M NaCl, 26 mM $MgCl_2$ and 10 mM $CaCl_2$) for PO assay (Luna-Acosta, 2010). The mixture was homogenized, stored at 4 °C for 1 h, and then centrifuged for 10 min at 15,000 *g* and 4 °C. The resulting supernatant containing Tris soluble proteins was collected for enzymatic studies.

2.4. Biochemical analysis

2.4.1. Protein assays

All activities were expressed in relation to protein concentration measured according to the Bradford method (1976) using bovine serum albumin (BSA) as standard.

2.4.2. PO assays

In order to partly discriminate PO synthesis and activation site, we took care to avoid unwanted activation of proPO during each step of