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

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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 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.

Keywords: cephalopod, immune system, lysozymes, prophenoloxidase system, protease inhibitors, *Sepia officinalis*

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 (Gomez et al., 2013; Hansen and Olafsen, 1999; McFall-Ngai et al., 2010). 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 invader¹

¹**Footnote 1: Abbreviations:** APO: activated phenoloxidase; BAPNA: N α -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:

elimination (Wang et al., 2013). Among them, we chose to focus on (1) one of the most effective signaling pathway 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) (Amparyup et al., 2013; Cerenius et al., 2008; Fiolka et al., 2012; Herreweghe and Michiels, 2012; Rowley and Powell, 2007; Wang et al., 2013; Xue et al., 2009).

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. Cerenius et al., 2008; Luna-Acosta et al., 2010; Siddiqui et al., 2006). Phenoloxidases (POs), a family of copper (Cu) proteins catalyse the rate-limiting step in melanin production: oxidation or hydroxylation of phenols into quinones. Importantly, POs are synthesized as zymogenic form called prophenoloxidases (proPO) and activated by serine protease cleavage (Amparyup et al., 2013; Cerenius and Söderhäll, 2004; Masuda et al., 2012). Because of their immune functions highest PO levels are usually found in association with epithelial barriers, respiratory and 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; Herreweghe and Michiels, 2012; Park et al., 2007; Rao et al., 2010; Wang and Zhang, 2010). 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 (Herreweghe and Michiels, 2012; Saurabh and Sahoo, 2008).

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 behaviour, a deeply modified body plan organization, and highly diverse modes of life (Bassaglia et al., 2013; Hanlon and Messenger, 1988). However little is known about their immune system (Castellanos-Martínez and Gestal, 2013; Ford, 1992). 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; Kishimura et al., 2010, 2001; Sof'ina et al., 1988).

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 $\pm$ standard deviation; weight = 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-liter 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 ml hemolymph was then withdrawn from anterior mantle vein (King et al., 2005) using syringe with 18-gauge needle and kept in ice, and animals 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, N α -benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), bovin serum albumin (BSA), Bradford reagent, calcium chloride (CaCl₂), citric acid (C₆H₈O₇), dimethyl sulfoxide (DMSO), hen egg white (HEW) lysozyme, 3,4-dihydroxy-L-phenylalanine (L-DOPA), magnesium chloride (MgCl₂), freeze-dried *Micrococcus lysodeikticus*, Protease Inhibitor Cocktail, sodium chloride (NaCl), sodium phosphate dibasic dihydrate (Na₂HPO₄·2H₂O), 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 (100X) 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 homogenised 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 MgCl₂ and 10 mM CaCl₂) 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 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 experiment. PO-like activity was measured spectrophotometrically by recording the formation of *o*-quinones, as described by Luna-Acosta (2010) with slight modifications to distinguish artificially activated PO (APO) (corresponding to PO-like activity resulting from zymogenic PO (proPO) activation plus already 'active' form) and *in vivo* 'active' PO form. PO assays were conducted in triplicate in 96-well flat bottom plates (BD, USA). L-DOPA was used as substrate, at a final concentration

of 10 mM (Luna-Acosta, 2010), and prepared extemporaneously in Tris buffer pH 7 described above (section 2.3.). Tropolone (10 mM) and trypsin TPCK (1 g l^{-1}) were used respectively as PO inhibitor and elicitor as described by Lacoue-Labarthe et al. (2009) on *S. officinalis* embryo. To avoid uncontrolled proPO activation by tissue endogenous proteases, several wide spectrum PIs were tested: aprotinin (1 g l^{-1}), Protease Inhibitor Cocktail (D/100) and Halt Protease Inhibitor Cocktail, EDTA-Free (1X). For each sample, non-enzymatic sample autoxidation, ‘basal’, ‘inhibited’ and ‘activated’ PO-like activities were measured. For non-enzymatic sample autoxidation, 10 μl of sample was mixed with Tris buffer pH 7. For ‘basal’ PO-like activity, 10 μl of sample was firstly mixed during 10 min with 1.4 μl PI (100X), followed by adapted volume of Tris buffer pH 7 and 80 μl L-DOPA. Similarly, for inhibited or APO-like activity, 10 μl of sample was mixed with 10 μl of tropolone (140 mM) or trypsin TPCK (14 g l^{-1}), Tris buffer pH 7 and 80 μl L-DOPA. Each measurement was systematically controlled by replacing sample by buffer, always in a final reaction volume of 140 μl . Immediately after L-DOPA addition, PO-like activities were monitored at 25°C for 5 h using Mithras LB 940 luminometer (Berthold, Thoiry, France) at 490 nm (Luna-Acosta, 2010). When an inhibited PO-like activity was measured, this value was subtracted from APO and PO-like activities. Tropolone, with its copper chelator and peroxidase substrate properties, ensured that PO-like activity alone was detected (and not peroxidase). Results were also systematically corrected for non-enzymatic autoxidation of the substrate and were expressed in enzyme unit (1 U) per mg of total protein. One U corresponded to an increase of 0.001 in the absorbance per min at 25°C (Thomas-Guyon et al., 2009).

Preliminary assays showed similar effects on PO-like activity with aprotinin (1 g l^{-1}), Protease Inhibitor Cocktail (D/100) and Halt Protease Inhibitor Cocktail - EDTA-Free (1X), with concentrations inhibiting 1 g l^{-1} elicitor trypsin TPCK, which is much higher than maximal tissue intrinsic serine protease concentrations found in these compartments (not

shown). Consequently, Halt Protease Inhibitor Cocktail - EDTA-Free was used in our study. The use of a PI Cocktail to avoid unwanted *in vitro* proPO activation by tissue endogenous proteases allowed to distinguish PO-like activities from already activated form when sampling occurred, and those from inactive zymogenic form proPO at the same moment. Therefore, in addition to activity levels, PO and APO-like activities informed us about proPO location and compartments where proPO activation occurs most often, i.e. compartments most submitted to uncontrolled elicitors (intern or extern).

2.4.3. Lysozyme assay

Lysozyme-like activity was quantified according to Malham et al. (1998) with slight modifications. Fifty μl of HEW lysozyme ($85 \mu\text{g ml}^{-1}$ in Tris buffer pH 8 described in section 2.3.) standard was serially diluted and placed in triplicate in 96-well flat bottom plates (BD, USA), as well as 50 μl of each sample and 50 μl of Tris buffer pH 8, as blank. One hundred and fifty μl of the substrate, *M. lysodeikticus* [0.075 g/100 ml of phosphate/citrate buffer pH 5.8 ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 4.45 g/250 ml distilled H_2O ; $\text{C}_6\text{H}_8\text{O}_7$, 2.1 g/100 ml distilled H_2O ; NaCl, 0.09 g/100 ml buffer)], was then added to each well. The reductions in turbidity in the wells were read on Mithras LB 940 luminometer (Berthold, Thoiry, France) at 25°C for 5 minutes at 10 second intervals at 450 nm using negative kinetics. Lysozyme concentrations were calculated from the standard curve ($\mu\text{g HEW lysozyme equivalent ml}^{-1}$). Final lysozyme-like activity was thus expressed as $\mu\text{g HEW lysozyme eq. mg protein}^{-1}$.

2.4.4. PI assay

As described by Malham et al. (1998) and Thompson et al. (1995), PI activity was measured by transferring 20 μl of sample and 10 μl of trypsin TPCK ($100 \mu\text{g ml}^{-1}$ of 0.05 M Tris buffer pH 8) in 96-well flat bottom plates (BD, USA), and mixed at room temperature for 5 minutes. In parallel, intrinsic trypsin activity was measured by replacing 10 μl of trypsin by

Tris buffer pH 8 described in section 2.3. A positive control was used by replacing the sample with Tris buffer pH 8. Two hundred μl of BAPNA substrate solution (5.2 mg BAPNA ml^{-1} DMSO in 10 ml of 0.01 M trizma base buffer pH 7.4) was added to each well and incubated for 15 minutes at room temperature. Absorbance was read at 405 nm using Mithras LB 940 luminometer (Berthold, Thoiry, France), and PI activity was expressed as the percentage of sample trypsin inhibition (TI) compared to the positive control. As most proteinaceous protease inhibitors specifically inhibit proteolytic enzymes of one of four mechanistic classes (i.e., aspartic proteases, cysteine proteases, metalloproteases and serine proteases) by interacting with their reactive sites, this assay allowed quantifying serine PIs (Xue et al., 2009). $\text{PI activity} = [\text{O.D.}_{405\text{nm}} (\text{sample} + \text{trypsin} + \text{S}) - \text{O.D.}_{405\text{nm}} (\text{sample} + \text{S})] / \text{O.D.}_{405\text{nm}} (\text{trypsin} + \text{S}) \times 100$. Final activity was expressed as TI % μg of protein $^{-1}$.

2.5. Statistical analysis

All results are given as mean $\pm$ standard error (SE) for adult organs. Residual distribution was tested for normality (Shapiro test) and homogeneity of variances (Levene test). In some cases, logarithmic (Log_{10}) or reciprocal transformations were used to meet the underlying assumptions of normality and homogeneity of variances. For normal values, one-way analysis of variance (ANOVA) were used followed by non parametric pairwise permutational t -tests ($n < 30$). Significant differences between PO-like and APO-like activities were tested for each organ with a Student's t -test. Statistical significance threshold was set at $p < 0.05$. R software was used for statistics and graphics.

3. Results and discussion

3.1. APO- and PO-like activity distribution and potential functions

Based on previously described enzyme repartitions and roles, we hypothesized that higher proPO/PO-activity would be found in the circulatory system and tissues directly exposed to the outer environment in *S. officinalis*.

The highest APO-like activity was measured in the integument (12.2 ± 1.5 U mg prot⁻¹), consistent with its role as first epithelial barrier (Figure 1). A role of POs in cuttlefish wound healing was suggested by tissue thickening and darkening (Harms et al., 2006) similar to the proPO induced sclerotization and pigmentation process of the insect cuticle (Arakane et al., 2005; Pryor, 1940). Apart from their roles in immunity, POs may be involved in cuttlefish body patterning by chromatophore melanogenesis although melanin presence as pigment in the cephalopod integument remains controversial (see Messenger, 2001). Future studies will have to determine PO implication in one or both processes in *S. officinalis* integument as already shown in vertebrates (Haffner and Wieser, 1981; Mackintosh, 2001; Zanna et al., 2009). It is also noteworthy that the mantle, the main body part in direct contact with the integument did not present any PO activity, suggesting local proPO synthesis. Similar PO repartition was recently reported in bivalve mollusks with high and low activities in mantle and muscle, respectively (Luna-Acosta et al., 2011b; Zhou et al., 2012).

In the plasma, POs and Hcy share a structurally and functionally equivalent active site (Campello et al., 2008), yet POs are synthesized as inactive zymogenic form while Hcy is not. In addition, Hcy constitutes more than 90% of the total plasma protein content (D'Aniello et al., 1986; Ghiretti, 1966; Mangold and Bidder, 1989). Consequently, parallel assays of APO- and PO-activities allowed to distinguish between Hcy- and non-Hcy-associated PO activities, as PO-activity mostly resulted from Hcy oxidation (Siddiqui et al., 2006). The plasma was indeed the only compartment where PO- and APO-like activities were similar (2.6 ± 0.3 U mg

prot⁻¹), strongly suggesting that (1) plasma PO-activity was mostly Hcy-associated and that (2) PO-activity from other compartments is mostly associated with PO enzymes. It is thus interesting to note that, except for the mantle, all studied compartments presented some APO-like activity, consistent with a wide role of POs in cuttlefish immunity (Figure 1). The previously suggested lectin role of Hcy and the Hcy-derived PO activity found in our study, as well as in other cephalopods, are consistent with recently reviewed roles of Hcy as an integral component of invertebrate innate immunity and suggest a function in immune response trigger (Alpuche et al., 2010; Amparyup et al., 2013; Campello et al., 2008; Coates and Nairn, 2014; Rögener et al., 1985; Siddiqui et al., 2006; Wang and Wang, 2013).

Organs of the respiratory and circulatory systems presented similar APO-like activities around 7 U mg prot⁻¹ (except WB) with only PO-like activity found in BH (1.0 ± 0.3 U mg prot⁻¹). Such results are consistent with PO role(s) associated with the immune system: non-self recognition, antibacterial compound synthesis and production of factors stimulating cellular defense (Cerenius et al., 2008; Söderhäll and Cerenius, 1998). The low PO-like activity measured in BH could not be ascribed to either Hcy or POs, because of the role of BH cells (i.e. rhogocytes and adhesive hemocytes) in hemolymph epuration of xenobiotics and allogenic substances (Beuerlein and Schipp, 1998; Beuerlein et al., 2002), and their involvement in Hcy catabolism suggested by Beuerlein et al. (1998). Finally, high APO-activity with lack of 'basal' PO-like activity in BHA was consistent with the Hcy extracellular storage suggested by Beuerlein et al. (2000). WB APO-like activity (2.4 ± 1.0 U mg prot⁻¹) suggested proPO presence in hemocytes before their release in cuttlefish plasma.

As expected, APO- and PO-like activities measured in the digestive system were lower than in other systems, except for the DG-DGA complex (9.8 ± 1.0 and 5.5 ± 1.2 U mg prot⁻¹, respectively), which presented higher APO-like activities than Stc, cecum and PSG (2.0 ± 0.7 ,

284 1.8 ± 0.6 and 1.5 ± 0.5 U mg prot⁻¹, respectively). DGA are known to carry out 2 main roles
285 in *S. officinalis*; absorption of small molecules from digestion (Boucaud-Camou and Boucher-
286 Rodoni, 1983; Boucaud-Camou and Pequignat, 1973), and osmoregulation via salts and water
287 absorption to maintaining the internal hyperosmotic status of cephalopods (Schippe and
288 Boletzky, 1976; Wells and Wells, 1989). Since no known link exists between PO function and
289 digestion, DGA-associated proPO may reflect a role in the immune response, perhaps by
290 providing protection against pathogen exposure from oral or rectal uptake of seawater, which
291 occurs without exposure to digestive enzymes. In the case of the DG, PO-like activities were
292 previously detected in mollusk DGs but no tissue-specific function was proposed (Blaschko
293 and Hawkins, 1952; Blaschko, 1941; Luna-Acosta et al., 2011a, 2011b). In cephalopods, most
294 of reported parasite infections (i.e. coccidians of the genus *Aggregata*, cestodes, trematodes,
295 nematodes) were found in the digestive tract (reviewed in Castellanos-Martínez and Gestal,
296 2013; Hochberg, 1990). Because of (1) the high efficiency of melanization against parasites
297 (Rao et al., 2010), (2) the important secretory ability of the DG and (3) its central position in
298 the digestive tract, we propose that the DG may serve as proPO reservoir to fight this type of
299 infection. In addition, the ability of some POs (i.e. laccases) to catalyze aromatic oxidation
300 (Dodor et al., 2004; Luna-Acosta et al., 2011a) and the high PO-like activities found in the
301 DG may also be linked to the detoxification of these compounds known to be highly
302 accumulated in cephalopods (e.g. Ansari et al., 2012; Won et al., 2009; Yamada et al., 1997).
303 In contrast, high PO-like activity (5.7 ± 1.7 U mg prot⁻¹) reflects a potential role in Hcy Cu-
304 metabolism. Because of its high Cu content (Bustamante et al., 2006; Miramand and Bentley,
305 1992; Miramand and Guary, 1980; Schippe and Hevert, 1978), many studies suggested a DG
306 implication in Hcy metabolism in *S. officinalis* (Costa et al., 2014; Decleir et al., 1978;
307 Lemaire et al., 1977; Martoja and Marcaillou, 1993; Schippe and Hevert, 1978), as
308 demonstrated in DG relatives (i.e. hepatopancreas and midgut gland) of arthropods,

gastropods, and cephalopods (Burmester, 2002; Manubens et al., 2010; Ruth et al., 1996). In *S. officinalis*, Cu-free Hcy synthesis takes place mainly in branchial glands (Schippe and Hevert, 1978; Schippe et al., 1973), but the organ where Cu-binding takes place has not been clearly determined (Decleir et al., 1976; Schippe and Hevert, 1978). Based on (1) low free form Cu concentration found in the hemolymph (D'Aniello et al., 1986), (2) the association of Cu with Hcy-size proteins in the DG (Bustamante et al., 2006; Rocca, 1969), and (3) the Hcy exchanges taking place across the DG basal membrane (Martoja and Marcaillou, 1993), our data (PO-like activity) add up to this mounting evidence that the DG may function as Cu-binding site for Hcy.

Our results are consistent with role(s) of POs in cuttlefish immunity with high activities found in circulatory system organs and integument as tissue directly exposed to environment. High activities found in DG complex organs will need further investigations to better define PO role(s) at this location, with potential links with detoxification and Hcy metabolism.

3.2. Lysozyme distribution

As with PO/proPO, we hypothesized that higher lysozyme-activities would be found in the circulatory system and tissues directly exposed to the outer environment in *S. officinalis*: plasma, BH and their appendages (BHA), SH, WB, integument, mantle and gill. All compartment samples indicated some lysozyme-like activities, except for the DGA (Figure 2). Our results support our hypothesis and highlight the importance of lysozymes in circulatory system organs and epithelial barriers, but also in one organ involved in digestive processes, the PSG. As with APO activity, one of the highest levels was found in the integument with lower activity in the mantle (2.2 ± 0.9 and 0.9 ± 0.3 μg HEW lysozyme eq. mg protein^{-1} , respectively). This high level is likely linked to the constant integument exposure to high numbers of opportunistic pathogens in water as described in fish skin (Ghafoori et al., 2014;

Hikima et al., 2001; Saurabh and Sahoo, 2008). Similarly, important lysozyme activity or mRNA expression have been detected in mantle of gastropod *Haliotis discus discus* (Bathige et al., 2013) and bivalve mollusks *Venerupis philippinarum* (Zhao et al., 2010), *Crassostrea virginica* (Itoh et al., 2007) and *C. gigas* (Itoh et al., 2010). This localization is thus consistent with previous descriptions in fish and bivalve mollusks and may reflect lysozyme activity in neutralizing pathogens after injuries or bacterial infection.

In the circulatory system, lysozyme-like activity was found in SH, WB, BHA and BH (1.7 ± 0.5 , 1.4 ± 0.4 , 1.3 ± 0.5 and 0.8 ± 0.2 μg HEW lysozyme eq. mg protein^{-1} , respectively), but not in the plasma (< 0.1). These results parallel lysozyme-like activities found in the BH complex of the cephalopod *E. cirrhosa* (Malham et al., 1998) and the role of endocytotic-active cells made up BH complex in purifying the hemolymph from allogenic compounds (Beuerlein and Schipp, 1998; Beuerlein et al., 2002; Schipp and Hevert, 1981). The absence of lysozyme-like activity found in the plasma supports our previous finding of hemolymph lysozyme in hemocytes alone (Le Pabic et al., 2012), but contrast with those found in the octopods *E. cirrhosa* and *Octopus vulgaris* (Grimaldi et al., 2013; Malham, 1996). The high activity found in WB – the site of hemocyte formation, is consistent with the previously suggested association of mature hemocytes (80% of adult cuttlefish WB) with lysozyme-like enzymes (Claes, 1996). Globally, the high lysozyme activities found in cuttlefish circulatory organs suggested not only intracellular action after endocytosis but also secretion in plasma as previously described in bivalves (Cheng et al., 1977, 1975). Apart from its bactericidal activity, some studies highlighted the lysozyme property as modulator of the immune response by interacting with compounds such as the complement pathway or lectins (e.g. Park et al., 2007; Rao et al., 2010; Wang and Zhang, 2010; Wilson and Ratcliffe, 2000). In order to investigate more deeply lysozyme roles in cuttlefish immunity, it will be

interesting to confirm such secretion in plasma and to determine its triggering parameters (e.g. infection, stress).

Surprisingly gill activity ($0.3 \pm 0.2 \mu\text{g HEW lysozyme eq. mg protein}^{-1}$) was among the lowest activity found whereas it is an organ (1) constantly exposed to water and therefore potential pathogens and (2) with inner folded epithelia suggesting some excretory function (Schippe and Boletzky, 1975; Schippe et al., 1979, 1971). This low activity may result from the healthy state of sampled cuttlefish. It could be thus interesting to follow potential increase of lysozyme-like activity in cuttlefish gill following an exposure to bacterial contamination, as shown in fish (Larsen et al., 2009; Mai et al., 2014) and abalone (Bathige et al., 2013).

Among digestive system organs, highest activity was found in the PSG ($1.7 \pm 0.3 \mu\text{g HEW lysozyme eq. mg protein}^{-1}$), which was significantly higher than in cecum, DG (0.4 ± 0.1 and $0.2 \pm 0.1 \mu\text{g HEW lysozyme eq. mg protein}^{-1}$, respectively) and DGA. Such results are consistent with lytic lysosomal enzyme activities reported in the cephalopod *E. cirrhosa* anterior salivary glands (Malham et al., 1998), and octopod and oegopsid squid paralarvae PSG (Boucaud-Camou and Roper, 1995). PSG-associated lysozyme-like enzymes may be involved in (1) venom diffusion, (2) “external” digestive processes (Boucaud-Camou and Roper, 1995), (3) plasma depuration (Stuart, 1968), and/or (4) to prevent digestive duct infection. This last possibility is consistent with the decreasing lysozyme-like activity found between the PSG and the cecum, which may result from PSG excretions from uncompleted digestion due to the short time period between cuttlefish fishing and dissection, the low holding temperature (15°C), and the few secretion ability of Stc (Boucaud-Camou, 1973). Moreover, due to their known chitinase property in certain invertebrates, lysozymes from PSG could also contribute to shell digestion of crustaceans –the most important cuttlefish meal (Goto et al., 2007; Herreweghe and Michiels, 2012).

3.3. PI distribution

Because of the general need to regulate proteases, we hypothesized a ubiquitous distribution of PIs in *S. officinalis* compartments. Our results support this hypothesis as PI activity was found in all studied compartments, albeit significant differences were found (Figure 3). As previously described in the squid *Loligo vulgaris* (Tschesche and von Rücker, 1973), PI activity was highest in digestive system organs – DG, DGA, Stc and cecum, where proteolytic enzymes (as serine proteases) play a major role in *S. officinalis* (Perrin, 2004; Balti et al., 2012, 2009; Boucaud-Camou, 1974). Consistent with its role in digestive enzyme synthesis (proteases)(Boucaud-Camou, 1974), highest PI activity was found in the DG (2.2 ± 0.2 TI % $\mu\text{g prot}^{-1}$), as previously shown in cephalopods (Ishikawa et al., 1966; Kishimura et al., 2010, 2001; Sof'ina et al., 1988). PI activities found in the DGA, Stc and cecum (1.8 ± 0.3 , 1.7 ± 0.4 , 1.5 ± 0.4 TI % $\mu\text{g prot}^{-1}$) might mostly result mainly from DG enzymatic excretions. Indeed, among these three organs, protease synthesis was clearly highlighted in cecum epithelial cells alone, but in much lower amounts than in the DG (Boucaud-Camou, 1974). The absorption function of the cecum implies a prolonged contact with digestive fluids enriched in DG proteases (Boucaud-Camou and Pequignat, 1973). Therefore, a need to control and/or protect cecum epithelia against proteases may constitute a role for associated PI-activity. Lastly, it was previously argued that DGA-associated proteolytic activity is likely to result from extrinsic sources because to its function (1) as excretory route for enzymes and digestive fluids between DG, Stc and cecum (Boucaud-Camou, 1974; Boucaud-Camou and Boucher-Rodoni, 1983), but also (2) as main absorption site for small molecules from protease rich digestive fluid (Boucaud-Camou and Pequignat, 1973). Finally, no digestive enzymatic activity has been found in Stc epithelium by Boucaud-Camou (1973), but this organ involved in mechanical digestion collects enzyme excretion from DG and probably from PSG (Boucaud-Camou and Boucher-Rodoni, 1983). Concerning PSG, the detected

activity may be involved in the regulation of proteases present in the cephalopod saliva (Malham et al., 1998) such as chymotrypsin, which is concentrated in this tissue (Perrin, 2004). However cephalopod salivary glands are also known to contain biologically active amines and toxins (Boucaud-Camou and Boucher-Rodoni, 1983; Boyle and Rodhouse, 2005; Fry et al., 2009; Nagai, 2012; Undheim et al., 2010), and PIs may have a synergistic effect with toxins as described for proteases in *E. cirrhosa* saliva (Grisley and Boyle, 1987). This suggests the presence of different PIs in this tissue than in other digestive organs.

PI activities were lower in remaining compartments. Similar PI activities averaging at 0.5 TI % $\mu\text{g prot}^{-1}$ were found in all respiratory and circulatory compartments as previously reported in *E. cirrhosa* (Malham et al., 1998), except in the plasma. However, a PI – α_2 -macroglobulin, was described as the second-most abundant protein found in the *S. officinalis* plasma (Armstrong, 1992; Thøgersen et al., 1992; Vanhoorelbeke et al., 1994). α_2 -macroglobulin acts by surrounding a wide range of protease molecules – including trypsin, to prevent enzyme-substrate reaction, but does not prevent reaction with low molecular weight substrates such as the BAPNA used in our study. Therefore, the low PI activity found in our plasma assays (<0.1 TI % $\mu\text{g prot}^{-1}$) did not reflect α_2 -macroglobulin activity. Other studies on tissues of terrestrial gastropod *Helix pomatia* and cephalopod *L. vulgaris* evidenced at least two different groups of PIs. A heat- and acid-stable group (I) and a group sensitive to denaturation (II) differently located in both mollusks. Whereas *H. pomatia* presented group I PIs only in its external organs, almost all sampled tissues (organ of the digestive tract, gills, epithelial barriers) of the squid presented PIs of this group, except sexual organs which contained group II PIs in both mollusks (Tschesche and Dietl, 1972; Tschesche and von Rücker, 1973). Each group was able to inhibit a wide range of serine proteases involved in digestive and immune processes (trypsin, chymotrypsin, plasmin, kallikrein) with different yields. These observations suggested that PIs assessed in our study belong to the same PI

group (I), to the exception of the plasma. Thus, while our results highlight the presence of PI activity in cuttlefish respiratory and circulatory compartments, they also demonstrate the need for more detailed approaches to dissect out which specific PIs are associated with individual organs.

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Legends

Figure 1: PO-like and APO-like activities (U mg prot^{-1}) in 13 body compartments of cuttlefish *Sepia officinalis* L. BH: branchial hearts, BHA: branchial heart appendages, SH: systemic heart, DG: digestive gland, DGA: digestive gland appendages, PSG: posterior salivary glands, Stc: stomach, WB: white bodies. The bars represent the means ($\pm$ SE) of 10 animals. Subscript letters indicate one-way ANOVA results within each activity group (PO- or APO-like): bars with same subscript letter are not significantly different ($p < 0.05$). *, ** and ***, indicate significant PO- and APO-like activity differences in each organs for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Figure 2: Lysozyme-like activity ($\mu\text{g HEW Lysozyme eq. mg prot}^{-1}$) in 13 body compartments of cuttlefish *Sepia officinalis* L. BH: branchial hearts, BHA: branchial heart appendages, SH: systemic heart, DG: digestive gland, DGA: digestive gland appendages, PSG: posterior salivary glands, Stc: stomach, WB: white bodies. The bars represent the means ($\pm$ SE) of 10 animals. Bars with same subscript letter are not significantly different ($p < 0.05$).

Figure 3: PI activity ($\text{TI \% } \mu\text{g prot}^{-1}$) in 13 body compartments of cuttlefish *Sepia officinalis* L. BH: branchial hearts, BHA: branchial heart appendages, SH: systemic heart, DG: digestive gland, DGA: digestive gland appendages, PSG: posterior salivary glands, Stc: stomach, WB: white bodies. The bars represent the means ($\pm$ SE) of 10 animals. Bars with same subscript letter are not significantly different ($p < 0.05$).

Legends to supplementary files

Figure 1S: Internal anatomy of cuttlefish *Sepia officinalis* L., with studied tissues. BH: branchial hearts, BHA: branchial heart appendages, SH: systemic heart, DG: digestive gland, DGA: digestive gland appendages, PSG: posterior salivary glands, Stc: stomach, WB: white bodies (modified after Boyle and Rodhouse, 2005).







