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REVIEW

The ascidian prophenoloxidase activating system

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Abstract

Phenoloxidases/tyrosinases initiate melanin synthesis in almost all organisms, and are involved in different biological activities such as the colour change of human hair and the browning or blackening of fruit skin etc. In many invertebrates, defence reactions are linked to phenoloxidase activity and/or melanization. Contacts with foreign molecules are able to trigger the prophenoloxidase (proPO) system that requires serine protease cleavage for activating the zymogen to phenoloxidase (PO). It is generally accepted that the proPO system is fully expressed in arthropods, and, recently, progress in the regulation of crustacean and insect proPO activation steps have been achieved. After cells were stimulated by components of pathogen associated molecular pattern (PAMP), proPO activation takes place *via* zimogenic serine proteinase in turn activated by PAMPs followed by cascade, spatial and temporal control.

The proPO activating system plays a defensive role in arthropods, molluscs, annelids, ascidians and the cephalochordate *Branchiostoma belcheri*.

In the present paper, we report on ascidian proPO system and related molecules, with particular focus on the biochemical, cellular and molecular aspects of the *Ciona intestinalis,* proPO system of circulating hemocytes from naïve ascidians as well as of body wall following LPS inflammatory challenge.

Key words: *Ciona intestinalis*; ascidians; proPO; phenoloxidase; immune response; hemocytes

Phenoloxidases and related enzymes

Melanin is a pigment ubiquitous throughout the animal kingdom. In invertebrates melanization is related to phenoloxidase (PO) and in part to tyrosinase, both are copper-dependent enzymes (monophenol, L-dopa; oxygen oxidoreductase; EC 1.14.18.1), that share similar active sites and catalyse the O-hydroxylation of monophenols (monophenoloxidase or cresolase activity) and the subsequent oxidation of the reaction products (o-diphenols) to quinones. Thus, substances forming copper ion complexes can be enzyme inhibitors (Kahn, 1985; Sugumaran *et al.*, 1988). Tyrosine is the natural substrate of tyrosinase, which exhibits a lag–phase during the tyrosine conversion ascribed to an autocatalytic mechanism due to the production of L-dopa in the initial phase of the reaction pathway (Lerner, 1949).

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Vertebrate tyrosinases form dimers whereas POs, only found in invertebrates, form oligomers, from monomers to pentamers. Although, both present two sites containing copper vary in their remaining sequence.

In invertebrates, the prophenoloxidase (proPO) is converted to PO by proteolytic cleavage. The activation depends upon a cascade due to hemolymph proteases which are sensitive to peptidoglycans and lipopolysaccharides (LPSs) or other bacterial carbohydrates or fungal β-glucans. In crayfish hemocytes proPO is a 76 kDa glycoprotein that, after activation by β-1.3-glucans, is cleaved by specific serine proteases to produce the 60 kDa active PO (Aspan and Söderhäll, 1991). A similar cascade has been reported in other invertebrates (Beschin *et al*., 1998; Parrinello *et al*., 2001; Luna-Gonzales *et al*., 2003).

Molecular analysis of PO and related protein

POs can be sharply distinguished from tyrosinase and an independent evolution with short sequence traits conservation has been proposed.

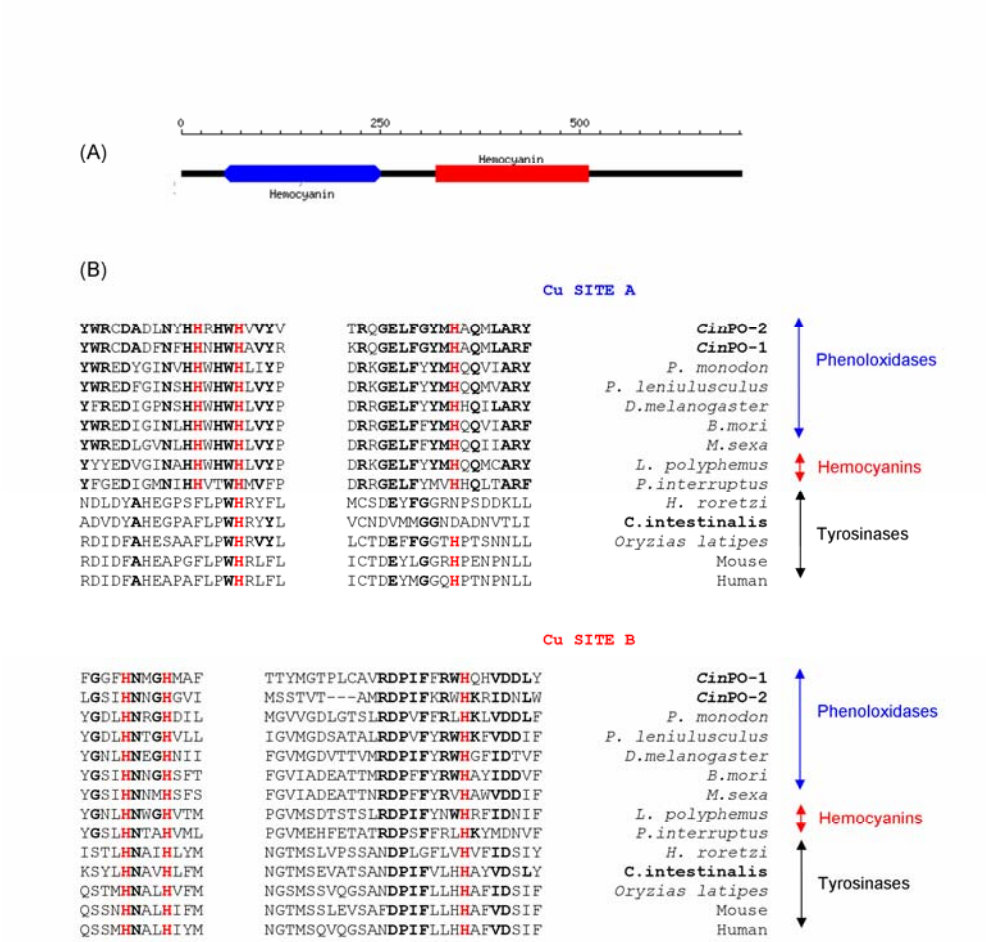

Fig. 1 A. The Conserved Domain Architecture of *C. intestinalis* phenoloxidases performed by similarity searches of the NCBI Entrez Protein Database (CDART). B. Sequence alignments of *C. intestinalis* prophenoloxidases for comparison with arthropod POs, hemocyanins and various tyrosinases. The sequences shown are segments corresponding to the copper A and B binding sites of the hemocyanins. The homologous aa are shown in boldface, the three Histidine residues (H) participating in the Cu atom are shown in red.

Conversely, a close similarity between arthropod phenoloxidases and hemocyanin, an oxygen carrier protein, has been shown (Söderhäll *et al.*, 1996). Both proteins present sequence similarity and contain two oxygen-binding sites that reversibly bind two copper atoms (Decker and Tuczek, 2000), moreover hemocyanin-like proteins can act as phenoloxidases (Immesberger and Burmester, 2004). Like proPO, hemocyanins can be activated *in vitro* by SDS, trypsin and other effectors with denaturating property (Decker *et al*., 2001; Lee *et al*., 2004), or by chitin-binding antimicrobial peptides (Nagai *et al*., 2001). This activation has been imputed to the cleavage of crayfish hemocyanin subunit 2 at the N-terminal part (Lee *et al*., 2004). Decker *et al*. (2004) described similar results on the tarantula hemocyanin, and hypothesized that after N-terminal cleavage the hemocyanin active site becomes accessible also for phenolic substances.

ProPOs from different arthropods have been cloned and sequenced (Aspan *et al*., 1995; Fujimoto *et al*., 1995; Hall *et al*., 1995; Kawabata *et al*., 1995). Comparative sequence analysis (Söderhäll *et al*., 1996), including several copper-containing proteins, showed that proPOs disclose sequence similarity to hemocyanins higher (49-59%) than to tyrosinases (33-35 %) (Parrinello *et al*., 2003).

Table 1 Possible genes involved in the *C. intestinalis* proPO activated system

	CinPO-1	**CinPO-2**	**Peroxinectin-like gene**	**Cu-Zn SOD-like gene**
mRNA	AJ7547813	AJ7547814	predicted XP_002126285	predicted XP_002122526
Protein	Complete; 794 aa	Partial at N-term; start ATG absent. 774 aa (768 aa in jgi:279870)	Complete; 960 aa	Complete; 154 aa
putative size (kDa)	92.0	86.9	105.0	15.6
Domain/Binding sites	Cu binding sites	Cu binding sites	Peroxidase/cell binding site	Cu-Zn
Reference	Burmester *et al* 2003	Burmester *et al* 2003	Present paper	Present paper

Immesberger and Burmester (2004) cloned and sequenced two *Ciona intestinalis* PO cDNAs (*Cin*PO-1 and *Cin*PO-2). *Cin*PO-1 and *Cin*PO-2, with predicted molecular masses of 92.0 kDa and 86.9 kDa respectively, showed 43.2 % identity and do not contain signal peptides indicating a non classical release. Figure 1 shows the sequence alignment of *C. intestinalis* PO sites compared to arthopod POs, hemocyanins and some tyrosinases, and displays a close similarity between POs and hemocyanins. A third putative *Cin*PO showing high similarity to *Cin*PO-2 had been identified by a search on JGI *Ciona intestinalis* V2 as *Cin*PO-3 (Cammarata *et al*., 2008). However, after a further analysis *Cin*PO-3 appeared to be a product of an uncorrect assembly of the sequences in the V2 genome version. In fact, a long stretch of undetermined nucleotides, at the scaffold end, explained the assumed high similarity of introns and esons as well as of the presumptive protein sequences.

ProPO activating system

The first defence line of the innate immunity includes PO pathway products that participate in several responses such as melanization and encapsulation, cytotoxicity, phagocytosis, clotting, microbial killing and wound repair (Söderhäll, 1982; Cammarata *et al*., 1997; Gillespie *et al*., 1997; Huang *et al*., 2000; Nagai and Kawabata, 2000; Nappi and Ottaviani, 2000; Cerenius and Söderhäll, 2004; Jiravanichpaisal *et al*., 2006; Cerenius *et al*., 2008). It has been proposed that a molecular cross-talk takes place between the proPO system and cellular defence responses which share activation by microbial products signals, such as coagulation and blood cell degranulation (Lemaitre and Hoffmann 2007; Cerenius *et al*., 2008).

Crustacean granular and semigranular hemocytes contain proPO, and, upon exposure to bacteria, they undergo degranulation *in vitro* leading to exocytosis (Johansson and Söderhäll, 1989b, 1999). In crayfish, *Pacifastacus leniusculus,* the 76-kDa peroxinectin, purified from the hemocytes (Johansson *et al*., 1995), mediates the attachment and spreading of hemocytes *in vitro* (Johansson and Söderhäll, 1988), and stimulates degranulation events when added to granular cells monolayer (Johansson and Söderhäll, 1989a, b). Peroxinectin-like is a cell adhesion protein also detected in shrimp (*Poeneus monodon*) hemocyte lysate supernatant (Sritunyalucksana *et al*., 2001). Sequence comparison shows that the shrimp protein is similar to crayfish peroxinectin (69 %) and to various peroxidases or putative peroxidases from invertebrates and vertebrates.

Since the biological effect of crayfish hemocyte peroxinectin is mimicked by the peptide Gly-Arg-Gly-Asp-Ser (G**RGD**S) (Johansson and Söderhäll, 1989c), the possibility exists that crayfish hemocyte integrin-like receptors recognize and bind RGD or KDG (Rouslahti, 1996; Holmblad *et al*., 1997). Vertebrate integrins form a family of integral membrane proteins that act in cell-cell adhesion and as receptors in trans-membrane signalling (Hines, 1992).

Peroxinectin may act as opsonin, and promote the adhesion of bacteria or other particles to the phagocyte surface, facilitating their subsequent ingestion by the cell. Peroxonectin also binds Cu-Zn-superoxide dismutase (CuZnSOD) at the surface of circulating hemocytes, and this interaction, facilitated by the close localization, modulates both the enzyme activities. The hydrogen peroxide, produced by the superoxide dismutases, can be substrate for the peroxinectin and antimicrobical substances (Johansson *et al*., 1999). Therefore, the CuZnSOD is involved in arthropod proPO activating system.

We carried out a bioinformatic analysis and, in Table 1, show for the first time that the *C. intestinalis* predicted peroxinectin-like gene contains both the active site of the peroxidase and the cell binding site (Gly-Arg-Gly-Asp-Ser, LK**KGD**R), moreover the deduced aminoacid complete sequence reveals 35 % identity with *P. leniulusculus* peroxinectin. In *C. intestinalis* genome, the presence of eleven alpha and five β chain integrin genes, suggest putative peroxinectin cell surface

Table 2 Properties and modulation of the ascidian phenoloxidases

	C. intestinalis		*S. plicata*	*B.schlosseri*	*P. mamillata*	*H. roretzi*
References	(1-7)		(5,7,8)	(9,10)	(5,11)	(12)
Treatment	**HLS**	**THS**	**HLS**			
Trypsin	↑	↑	↑	↑	↑	↑
Trypsin + STI	-----		-----	-----	-----	-----
LPS	↑	↑	↑	↑	↑	↑
PO inhibitors	↓		↓	↓	↓	↓
Calcium effect	No	No	No	Yes	No	No
PO Containing cell types	URG Granular amebocytes		Morula	Morula	compartment cell granular hemocytes	ND
PO Subunit MW	74	90	ND	80	70	62
Method	Cloned		HLS	Isolated	HLS	Isolated
Biological activities	Cytotoxicity		Cytotoxicity	Non fusion reaction	ND	Antimicrobial activity

Hemocyte lysate supernatant (HLS); Tunic homogenate supernatant (THS); Unilocular refractile granulocytes(URG)

(1) Söderhäll and Smith, 1992; (2) Peddie and Smith, 1993; (3) Parrinello *et al*., 1995; (4) Cammarata *et al.,* 1996; (5) Parrinello *et al*., 2003; (6) Cammarata *et al*., 2008; (7) Arizza *et al.,* 1995; (8) Cammarata *et al*., 1997; (9) Ballarin *et al*., 1994; (10) Ballarin *et al*., 2008; (11) Cammarata *et al*., 1999; (12) Hata *et al*., 1998

adhesion receptors (Ewan *et al*., 2005). In Table 1, we also report the sequence of a *C. intestinalis* putative gene Cu-Zn SOD with 46 % identity to the predicted aminoacid sequence of the *H. roretzi* enzyme, obtained by using the aminoacid sequence of *H. roretzi* Cu-ZnSOD (Abe *et al*., 1999) as a query in an aminoacid-based BLAST search (tblastn) versus the NCBI/GenBank database.

Ascidian proPO activating system and innate immunity

Ascidians occupy a key phylogenetic position in the evolutionary line leading to vertebrates (Hori and Osawa 1987; Field *et al*., 1988; Zeng and Swalla 2005; Delsuc *et al*., 2006), therefore both solitary and colonial ascidians are of interest in studying the evolution of defence mechanisms. Phagocytosis, cytotoxicity, encapsulation and tissue damage (Wright and Cooper, 1983; Parrinello and Patricolo, 1984; Parrinello *et al.*, 1984, 2001, 2007; Ballarin *et al*., 2008) in inflammatory responses, as well as in inflammatory events linked to allorecognition responses, have been shown (Sabbadin, 1982; Rinkevich, 1992; Raftos *et al*., 1988). Both share hemocytes degranulation in tissues of solitary ascidians (Parrinello *et al.*, 1984) and in the contact area of incompatible botryllid colonies (Sabbadin, 1982; Ballarin, 2008).

Differently than arthropod POs, which are monophenoloxidases, ascidian hemocyte POs are orthodiphenoloxidases. O-diphenol oxidase activity and phenolic compounds were at first identified by histochemical reaction (Barrington and Thorpe, 1968) in the tunic hemocytes suggesting a quinone-tanning system involved in the production of tunic scleroprotein (Chaga, 1980).

Although ascidian POs show the highest activity at 6-9 pH range (Jackson *et al*., 1993; Arizza *et al*., 1995; Ballarin *et al*., 1994), they can differ in several biochemical properties. In *Botryllus schlosseri* the L-dopa oxydizing activity is enhanced by divalent cations (Ballarin *el al*., 1994), whereas the PO activity of other ascidian species does not appear to be Ca^{2+} or Mg^{2+}-dependent (Jackson *et al*., 1993; Arizza *et al*., 1995; Cammarata *et al*., 1999). In crustaceans, although calcium ions are requested, a high cation concentration exerts a suppressive effect. A further difference concerns the activating mechanism: β 1,3-glucans and oligosaccharides induce proPO-activation in arthopods (Söderhäll and Smith, 1986; Söderhäll, 1992) and in solitary ascidian *C. intestinalis* (Jackson *et al.,* 1993), whereas do not activate *Styela plicata* (Arizza *et al*., 1995) and *B. schlosseri* (Ballarin *et al*., 1994) POs.

Ascidian orthodiphenoloxidases are copper-dependent enzymes inhibitable by copper chelating substances (Kahn, 1985; Sugumaran *et al.*, 1988). Like in arthropods, the ascidian proPO requires proteolytic cleavage for its activation. The level of PO activity is significantly higher after incubation with serin-proteases, decreases as an effect of protease inhibitors, and it is activated by LPS (Table 2). Smith and Peddie (1992) and Jackson *et al*. (1993) reported that the LPS-sensitive protease activity, contained in the *C. intestinalis* hemocyte lysate supernatants, may be associated with *in vivo* prophenoloxidase activaction. Benzamidine, soybean trypsin inhibitor (STI), phenylmethylsulphonyl fluoride, tosyl phenylalanyl

chloromethyl ketone, tosyl-L-lysin-chloromethyl ketone inhibited both protease and proPO activation, this inhibitory activity is short lived and precedes hemocyte PO activity (Jackson and Smith, 1993).

At least three proteases are contained in solitary ascidians *C. intestinalis*, *S. plicata* (unpublished data) and *Phallusia mamillata* hemocytes (Guerrieri *et al*., 2000), and could regulate the otherwise uncontrolled protease activity.

PO containing hemocytes

Phenoloxidase participates in tunic formation (Chaga, 1980), melanin production and non fusion reaction (Ballarin *et al*., 1996), and an increased PO activity can be found in *C. intestinalis* tissues inflamed by LPS inoculation (Parrinello *et al*., 2001). In *C. intestinalis* (Smith and Söderhäll, 1991) and *B. schlosseri* (Ballarin *et al*., 1993) PO oxidative activity of circulating hemocytes from naïve ascidians as well as hemocyte lysate supernatants has been revealed by L-dopa reaction. A similar activity has also been reported in *Ascidia mentula*, *Ascidia virginea*, *Ascidiella scabra*, *Ascidiella aspersa*, *Polycarpa pomaria*, *Dendrodoa grossularia* and *Morchellium argus* (Jackson *et al.*, 1993). The specificity of the PO reaction of *S. plicata, P. mamillata* and *C. intestinalis* hemocytes (Arizza *et al.*, 1995; Parrinello *et al*., 2003) has been supported by tropolone, phenylthiourea and diethyltiocarbamate usually used as inhibitors (Sugumaran *et al*., 1988; Cadenas, 1989; Kahn, 1995).

The enzymatic assay of the hemocyte lysate and the cytochemical reaction of the hemocytes revealed a limited heterogeneity in the ascidian PO-containing cell types. PO-activity as well as the possible substrates tunichrome and halocyamine-reducing polyphenol, have mainly been identified in the hemocytes called "morula cells" (Azumi *et al*., 1990; He *et al*., 1992; Ballarin *et al.,* 1996; Parrinello *et al*., 2003). Evidence of PO positive “morula cells” have also been drawn by assaying hemocyte populations enriched through a density gradient separation of the hemolymph from *C. intestinalis* (Jackson *et al*., 1993; Parrinello *et al*., 2003), *B. schosseri* (Ballarin *et al*., 1994) and *S. plicata* (Arizza *et al*., 2005). This hemocyte-type is a round globular granulocyte (berry-like under the light microscope; 5.5-11.0 μm diameter) with large granules containing material of various electron-density (De Leo, 1992). Light microscopy observations show large globular granules varying in number, shape and content feature. Although Smith and Peddie (1992) suggested that *C. intestinalis* morula cells contain PO, we found a weak reaction after the treatment with L-dopa-MBTH. On the contrary, a strong PO activity was found in the unilocular refractile granulocytes (URGs) identified in the hemolymph and characterized by a single PO-positive large granule that occupies the largest part of the cytoplasm (Parrinello *et al*., 2003). In addition PO-positive large granules were also found in granulocytes. Although a defined differentiation line was not established, the possibility exists that granulocytes with large granules, URGs and morula cells may be components of a lineage characterized by a different state of the granule content including PO activity level as well as hemocyte functions. Accordingly, URGs exert PO-dependent cytotoxic activity whereas morula cells do not show any cytotoxic activity against erythrocytes (Parrinello *et al.,* 1996) and tumour cell lines (Peddie and Smith, 1993).

In *P. mamillata,* “hemocytes with large granules” show a low PO activity whereas the morula cells do not show any positive reaction with L-dopa-MBTH. The activity of the hemocyte lysate supernatant of enriched populations, obtained through a Percoll density gradient, can be enhanced by trypsin that presumably activates the proPO remaining after cell lysis. Usually, hemocytes preparation and further treatments may cause the activation of a part of proPO content by endogeneous proteases. In this ascidian, another hemocyte type named compartment cells characterized by few large vacuoles show spontaneous proPO activation, and vacuoles appear to be PO-positive. However, the lysate supernatant from enriched populations are less sensitive to trypsin proteolysis activation indicating that these cells may be more reactive to handling which can activate proPO. Proteolytic activation of proPO is sustained by electrophoresis and L-dopa-MBTH stain of trypsin-treated and untreated hemocytes with large granule lysate supernatants; the electropherograms show an increased mobility of the enzyme after proteolysis and, accordingly, reveal an additional protein fragment (Parrinello *et al*., 2003).

Tunic POs and inflammatory response

Recently, the PO activity of tunic homogenate supernatant (THS) from naïve *C. intestinalis* has been assayed (Cammarata *et al*., 2008). As already reported upon the hemocyte lysate supernatant (HLS), the PO activity of THS is Ca^{2+}-independent, but, unlike HLS (pH 6-9) requires a lower pH (7-8) and is more thermo-stable. The THS activity is lost after two days at 0 °C and after about one year at -80 °C, whereas the HLS activity disappears after 2-3 h at 0 °C and 3-4 week at -80 °C. Likewise the HLS-proPO, the treatment with exogenous trypsin enhances the activity and STI inhibits it, whereas a further difference resulted from a more effective activation due to LPS acting at a lower concentration than that needed for activating HLS-proPO. In addition, since LPS inoculation enhances the THS-PO activity of samples assayed in the absence of trypsin the possibility exists that in the tunic several serine proteases or different proteases could be involved in the activation phase (Cammarata *et al.*, 2008).

To check for tunic matrix and tunic cells PO activity in the inflammatory response, an *in vitro* enzyme assay of the inflamed tunic tissue excised at 24 or 48 h after LPS inoculation has been performed. The PO activity appears to be distributed throughout the tunic matrix, as well as inside the large granule of URGs and hemocytes with large granules. These observations are in accordance with the report on circulating hemocytes from naïve ascidians, whereas it is of interest that morula cells in the inflamed tunic disclose an evident PO activity suggesting a distinct step of the cell lineage not found in circulating hemolymph from naïve ascidians

and presumably due to the LPS challenge. Microscope observations of wet inflamed tunic fragments after L-dopa-MBTH treatment, showed both a strong PO reaction and a high density of PO-positive hemocyte populations (Fig. 2).

Since the deduced aminoacid sequence of a *C. intestinalis Cin*PO-2 isoform has been reported (GenBank Accession n. AJ547814) by Immersberg and Burmester (2004), a peptide (11-aa, EFHNDRRNRGF) has been selected through an antigen-prediction program, and anti-*CinPO*-2-peptide specific antibodies has been raised in rabbits (Cammarata *et al*., 2008). The immunohistochemistry reaction shows an intense dye of the inflammatory cells supporting that proPO-2 synthesis could be enhanced by LPS inoculation (Cammarata *et al*., 2008). The same assay revealed that, after LPS inoculation, the enzyme is distributed in the tunic matrix, mainly in the outer layer, in addition anti-*Cin*PO-2 antibodies also marked the pharynx vessel epithelia.

Immesberger and Burmester (2004) reported that 86.9 kDa is the predicted molecular size of *Cin*PO-2, and this value fits with the 90kDa revealed by the L-dopa-MBTH reaction and immunoblotting analysis of THS from naïve ascidians (Cammarata *et al*., 2008). After the LPS inoculation, an additional 120 kDa band reacts with L-dopa-MBTH and anti-*Cin*PO2 antibodies, whereas a 170 kDa L-dopa-MBTH positive band does not react with the antibodies. Presumably, this band could be considered as a dimeric form of *Cin*PO-1 (92.0 kDa) which cannot be identified by antibodies directed to a *Cin*PO-2 peptide. Anyway, an oligomerisation process may be responsible of *Cin*POs higher in size (120 and 170 kDa). A similar process may be taken in account for the differences observed in the THS- and HLS-PO (Parrinello *et al*. 2003) molecular sizes, and suggests that further analyses are needed.

Biological activity of PO and related molecules

In invertebrates, substances with cytotoxic activity include molecular derivates of oxygen and nitrogen, antimicrobial peptides, lectins and quinoid intermediates of melanin (Parrinello 1996; Nappi and Ottaviani 2000). The oxygen reactive forms, such as superoxide anions, hydroxyl radicals, and hydrogen peroxide anions have been implicated as components of the cytotoxic mechanisms of vertebrates (Cadenas, 1989) and invertebrates (Bell and Smith, 1993; Anderson, 1994; Valembois and Lassegues, 1995; Nappi and Ottaviani 2000). The propensity of quinones for redox-cycling makes these eumelanin precursors potential sources of reactive forms of oxygen (Riley, 1988; O'Brien, 1991). Indeed, it has been demonstrated (Smit *et al*, 1996) that phenolic compounds, converted into toxic products by tyrosinase, exhibit cytotoxic activity towards human melanoma cells.

We reported that the morula cells form *S. plicata* (Cammarata *et al*., 1997) and URGs from *C. intestinalis* (Parrinello *et al.,* 1995) display PO-dependent cytotoxic activity against tumour cell lines (unpublished data) and rabbit erythrocytes due to quinones which are known to be cytotoxic (Pawelek and Lerner, 1978; Cotelle *et al.,* 1991; Fu *et al.*, 1994; Parrinello *et al*., 2003).The presence of quinones, possibly originated by PO-driven tunichrome oxidation, has been reported in ascidians. Differently, ROI derived from the PO pathway are cytotoxic factors in *B. schosseri* non-fusion alloreaction (Ballarin *et al.,* 2002, 2008). Indeed, *in vivo,* quinones could be activated by enzymatic reduction as, under aerobic conditions, the semiquinone radical autoxidizes and forms superoxide anion radicals (Nappi and Vass, 1993). The superoxide anion, hydrogen peroxide and trace amounts of transitional-metal ions form a hydroxyl radical (Fenton reaction), which is the most toxic of all oxygen products.

There is only indirect evidence that, in *C. intestinalis,* proPO-activation products have a stimulatory influence on hemocyte behaviour *in vitro*. In this ascidian, the crude hemocyte lysate supernatant, which contains active PO and LPS-sensitive proteases, has a marked opsonic influence on the uptake of bacteria by phagocytic amoebocytes (Smith and Peddie, 1992). Such opsonic potential match to the degree of the enzyme activity; in addition, PO and proteases inhibition with benzamidine or STI precludes the opsonic effect, while pre-treatment of the lysate supernatants with LPS produces a greatly elevated phagocytic response (Smith and Peddie, 1992). Figure 3 shows a tentative model of the *C. intestinalis* proPO activating system, based on present knowledge.

Finally, morula cells can release proPO-activation products for tunic formation and regeneration in *Goniocarpa*

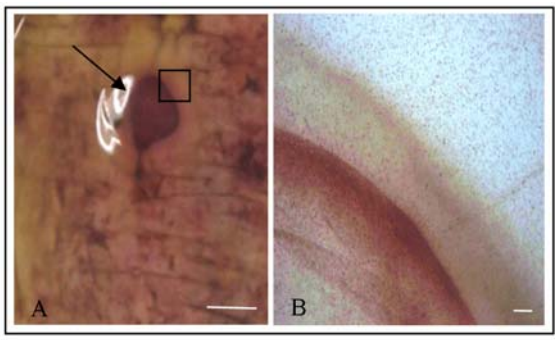

Fig. 2 Tunic fragment of *C. intestinalis* 24 h treated with L-dopa-MBTH after the inoculation of LPS. Arrowhead indicates the PO reaction at the injection site. Bar (A) = 1 cm; (B) = 200 µm.

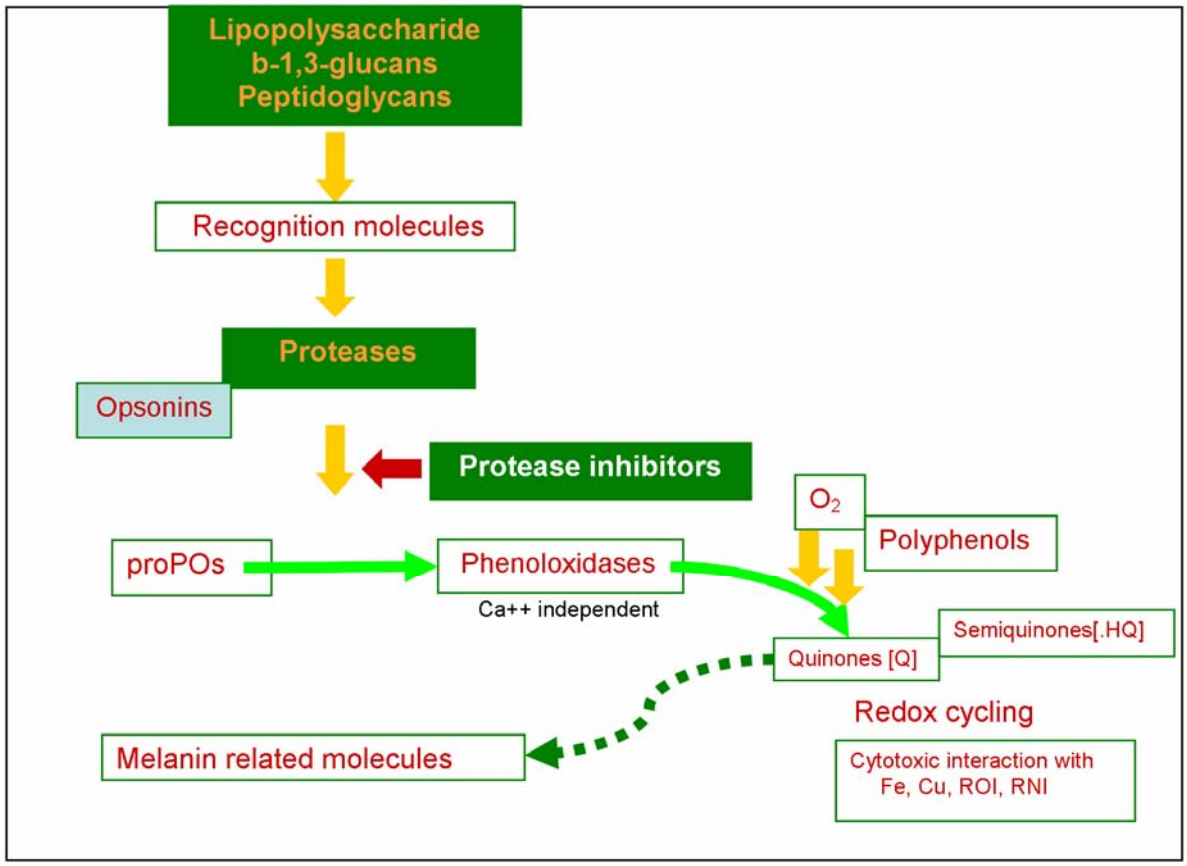

Fig. 3 A model for the activation and modulation of the pro-PO system in *C. intestinalis,* based on the following reference results: Söderhäll and Smith, 1992; Peddie and Smith, 1993; Parrinello *et al*., 1995; Cammarata *et al*., 1996, 1997; Parrinello *et al*., 2003; Cammarata *et al*., 2008.

rustica, Halocynthia aurantium (Chaga, 1980) and *B. schlosseri* (Zaniolo, 1981), and can be involved in the formation of clotting (Wright, 1981), and in the encapsulation of foreign particles (Anderson, 1971; Parrinello, 1996).

Conclusions

The melanogenetic pathway can be dependent on tyrosinase or phenoloxidase activity, the proPO activating system appears to be a sophisticated system which represents an evolutionary independent defence mechanism characteristic of invertebrates. Apparently protostomes and deuterostomes share proPO and PO-related factors and activities. Phenoloxidase activity and proPO-like components have been reported in the hemolymph of arthropods, annelids, molluscs, echinoderms and cephalocordata (Roch *et al*., 1992; Nappi and Vass, 1993; Beschin *et al*., 1998; Luna-Gonzales *et al*., 2003; Pang *et al*., 2005). Preliminary sequence analysis suggest that hemocyanin could be the evolutionary ancestor of PO and a parallel molecular evolution cannot be excluded.

Taking in account the *C. intestinalis* inflammatory response challenged by LPS, the proPO system appears to be a component (Cammarata *et al.,* 2008) of a very complex reaction that presumably involves several interacting cell types and factors, namely inducible cytokine-like molecules (Parrinello *et al*., 2007), *Ci*FACIT- collagen (Vizzini *et al*., 2007), and *Ci*TNFα (Parrinello *et al*., 2008).

The LPS or glucans activating effect could be associated with one or more serine proteases modulated by trypsin inhibitors, Cu-ZnSOD and peroxinectin-like molecules. Therefore, LPS- and glucan-binding proteins appear to be suitable candidates as recognition molecules central to several defence reactions.

Although some insight has been gained into the invertebrate proPO system, host recognition proteins, biological activities, molecular interaction, and signal transduction pathways, little is known on the ascidian cascade.

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