RESEARCH REPORT

Phenoloxidases of different sizes are modulated by LPS inoculation into *Ciona intestinalis* tunic and pharynx

MR Trapani[#], MA Sanfratello[#], V Mangano, D Parrinello, A Vizzini, M Cammarata

Marine Immunobiology Laboratory, Department of Biological Chemical Pharmaceutical Science and Technology, University of Palermo, Via Archirafi 18, Palermo, Italy

 # Equal contribution

Abstract

In the present study, to further characterize the pro-phenoloxidase (proPO) and active phenoloxidase (PO) involved in the *Ciona intestinalis* inflammatory response, tunic and pharynx homogenate supernatants were separated on high pressure liquid chromatography and fractions were assayed for the PO activity before and after LPS inoculation, as well as before and after trypsin treatment which activates proPO. The LPS inoculation *per se* did not significantly change the basal PO activity of the tunic homogenate supernatant (THS) and pharynx homogenate supernatant (PHS) restricted in two confluent peaks, whereas a significant enhancement was observable after the trypsin treatment. This trypsin effect suggests that proPO is the main component of the HPLC separated fractions, and indicates that LPS inoculation mainly challenges the pro-enzyme production by tunic cells and hemocytes, as well as the activation of the serine-protease pathway. The protein size analysis and DOPA-MBTH assay, disclose two active proteins of 90.0 and 170.0 kDa differently contained in the two main chromatographic peaks. Due to the SDS activating effect on the proenzyme analyzed by SDS-PAGE, the size of proPO could not be shown, whereas modulation of an oligomerization process of the 90 kDa component is suggested.

Key Words: ascidian; phenoloxidase; pro-phenoloxidase; HPLC; inflammation; LPS; *Ciona intestinalis*

Introduction

In invertebrates, phenoloxidases initiate melanin synthesis in almost all organisms, and pathway products are involved in different biological activities. The "prophenoloxidase activating system" plays a key role in humoral and cellular immune response exerting a defensive role in arthropods, molluscs, annelids and ascidians. This system comprises an enzyme cascade that activates prophenoloxidase (proPO) to phenoloxidase (PO). After cells were stimulated by components of pathogen associated molecular pattern, the zymogen is activated *via* serine proteases (Söderhäll and Cerenius, 1998; Cammarata *et al.*, 2008; Cammarata and Parrinello, 2009; Cerenius and Söderhäll, 2013), hemocytes degranulate and

release components of the inflammatory reaction (reviewed in Cerenius *et al.,* 2010).

In ascidians, POs were at first identified by histochemical reaction in the tunic hemocytes (Barrington and Thorpe, 1968), and a quinone-tanning system involved in the production of tunic scleroproteins was suggested (Chaga, 1980). These enzymes, are copper-dependent orthodiphenoloxidases (Sugumaran *et al.*, 1988) which catalyses both the ortho-hydroxylation of monophenol (*i.e.*, tyrosine) forming o-diphenol, 3,4-dihydroxy-L-phenylalanine (DOPA) and, then, the dehydrogenation of diphenol into o-quinones, which can polymerize producing insoluble melanin (Nappi and Seymur, 1991). Non-self agents activate serine proteases that, in turn, convert proPO into POs involved in tunic graft rejection (Raftos *et al.*, 1987a, b, 1988), mixed hemocyte allo- or xenogeneic reactions *in vitro* (Kelly *et al.*, 1992; Fuke, 1980), non-fusion reaction between allo- or xenogeneic colonial ascidian partners (Ballarin *et al.*, 1996, 2002), and inflammatory response to LPS (Cammarata and Parrinello, 2009). In *Ciona intestinalis* genome, two genes, referred to *Cin*PO-1

Corresponding author:
Matteo Cammarata
Marine Immunobiology Laboratory
Department of Biological Chemical
Pharmaceutical Science and Technology
University of Palermo
Via Archirafi 18, Palermo, Italy
E-mail: matteo.cammarata@unipa.it

and *Cin*PO-2 (GenBank/EMBL accession numbers AJ547813 *Cin*PO-1 and AJ547814 *Cin*PO-2), encode proteins with predicted molecular masses of 92.0 kDa and 86.9 kDa, respectively (Immesberger and Burmester, 2004). An antibody against anti-CinPO1 confirmed the predicted molecular weight and cells localization (Cammarata *et al.* 2008).

In this ascidian, an inflammatory response after inoculation of erythrocytes (Parrinello *et al.*, 1984a, b), foreign proteins (Parrinello, 1981), or lipopolysaccharide (LPS) (Parrinello *et al.*, 2007), has been shown in tunic and pharynx where hemocytes and humoral components are involved. The inoculated LPS permeates both tunic and pharynx tissues stimulating the expression of several inflammatory factors (reviewed in Parrinello, 2009). Such a response involves hemocytes (compartment/morula cells, granular amebocytes, and unilocular refractile granulocytes URGs) recruited into the inflamed tunic (Parrinello, 1981; Parrinello and Patricolo, 1984). In the pharynx, compartment cells (hemocytes with large vacuoles) express *Ci*typeIX- collagen 1α-chain (Vizzini *et al.*, 2008), galectin-like lectins (Vizzini *et al.*, 2012), tumor necrosis factor-α-like cytokine (*Ci*TNF-α; Parrinello *et al.*, 2008, 2010), a mannose-binding lectin (Bonura *et al.*, 2009) and a cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins (Bonura *et al.*, 2010). In a previous paper (Cammarata *et al.*, 2008), the zymogram of the whole tunic homogenate supernatant from LPS inoculated ascidians, indicated that PO activity could be related to 90.0 kDa and 120.0 kDa protein bands, whereas the density of a 170.0 kDa band was weak. The hypothesis that size differences between active components, as an effect of LPS inoculation, could be due to an oligomerization process, has been proposed.

In the present study, to study the modulation of proPO/PO involved in the inflammatory response and the serine protease activation process, tunic and pharynx homogenate supernatants were separated on high pressure liquid chromatography (HPLC), and fractions were assayed for the enzyme activity before and after LPS inoculation, as well as before and after trypsin treatment. The results of chromatographic analysis, SDS-PAGE analysis, and DOPA-MBTH reaction showed that the PO activity was confined in two confluent peaks that match proteins of 90.0 and 170.0 kDa, suggesting a dimerization process. Since, in samples from LPS-treated ascidians, a significant enhancement was manifest after a trypsin treatment, proPO production appeared to be modulated and a serine-protease pathway could be involved.

Material and Methods

Ascidians, LPS inoculation and sample preparation
Ascidians were gathered from Termini Imerese marinas (Italy), maintained in aerated sea water at 15 °C and fed every second day with a marine invertebrate diet (Coraliquid, Sera Heinsberg, Germany). LPS (*Escherichia coli* 055:B5, LPS, Sigma-Aldrich, Germany) was prepared in sterile marine solution (MS: 12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 43 mM Tris, 0.4 M NaCl, pH 8.0). According to previous papers, each specimen received 100 μg of LPS in 100 μl of MS, inoculated into the median region of the body wall just under the tunic. Ascidians, either untreated (naïve) or injected with 100 μl MS (sham), were used as a control. The ascidian tunic surface was cleaned and sterilized with ethyl alcohol. For tunic homogenate supernatant (THS) and pharynx homogenate supernatant (PHS) preparation, tunic and pharynx tissues were excised in order to avoid the presence of connective tissue and the underlying epidermis in the sample, whereas the cuticle was resected by using a razor blade. Samples were homogenized with an Ultra-Turrax (IKA) homogenizer, in distilled water for 3 min on ice. Homogenates were centrifuged at 27,000*g* for 20 min at 4 °C, and the resulting supernatants were dialyzed against PBS at 4 °C, divided into aliquots, and stored at -80 °C. Since serine proteases were used to activate the proPO cascade, according to the method of Jackson *et al.* (1993), protease inhibitors were not added to the sample preparations.

Protein content determination
Protein content was estimated by the method of Bradford (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

HPLC size exclusion chromatography
THS and PHS were subjected to size exclusion chromatography using BioSuite 250, 10 μm SEC, 7.5×300 mm column (Waters) on a HPLC system (Shimadzu Scientific Instruments, North America). The column was washed with TBS (150 mM NaCl, 10 mM Tris, pH 7.4). 200 μL of each sample were injected into the column which was eluted with TBS at a flow rate of 1 mL/min for 30 min. The chromatogram was recorded with a UV detector at 280 nm (mAU). The collected fractions were concentrated by centrifugation at 500*g* with micro-concentrators (3K Omega Centrifugal Devices Nanosep), and the final concentrated samples were stored at -80 °C until use.

Assay of PO activity in THS and PHS fractions after HPLC
PO activity was measured spectrophotometrically according to Winder and Harris (1991), by using L-Dopa (3,4- dihydroxy-L-phenylalanine) as the substrate and 6 mM 3-methyl-2 benzothiazolinone hydrazone hydrochloride (MBTH) as a specific reagent. Briefly, 45 μl of sample (THS or PHS fractions) with 45 μl of trypsin from bovine pancreas (1 mg/ml) or 45 μl of distilled water as control, were incubated for 20 min at 20 °C in 45 μl reaction mixture containing 20 mM L-DOPA and MBTH in distilled water (DOPA-MBTH). After the reaction, dopaquinone was detected within 60 min at 5 min intervals by spectrophotometric measurement at 505 nm. PO activity was expressed in units (U) per minute, where 1 U was equal to 0.001 ΔA505 min-1 mg-1 protein. Since the kinetics of the reaction with DOPA-MBTH reached their highest level after 20 min incubation, the U value observed at that time was taken into account in all experimental approaches.

Electrophoresis and PO activity

PO activity was assessed by polyacrylamide gel as described by Cardenas and Dankert (2000) with some modification. The PHS, THS and the relative fractions showing PO activity, were subjected to polyacrylamide gel performed according to the method of Laemmli (1970) using a Mini Protean II cell (Bio-Rad). The gels were calibrated with high molecular weight range standard protein (Sigma-Aldrich, USA). For the native electrophoresis SDS was omitted from all solutions. To identify the PO activity of the protein bands, the gels were washed twice with PBS-T (0.1 M NaCl; 0.02 M KCl; 0.01 M KH_2PO_4; 0.06 M Na_2HPO_4, pH 7.4, 2.5 % Triton X-100), and a final wash of 10 min in PBS (0.1 M NaCl; 0.02 M KCl; 0.01 M KH_2PO_4; 0.06 M Na_2HPO_4, pH 7.4). The gel was incubated in a solution containing 20 mM L-DOPA and 6 mM MBTH in distilled water. After 1 h of incubation, the gel was washed several times in distilled water. The molecular weights of the bands were calculated using the program AlfaEaseFC.

Statistical analysis

Student's t-test was used to estimate statistical significance. Multiple comparisons were performed with one-way analysis of variance (ANOVA), and different groups were compared by using Tukey's t-test. Standard deviations were calculated on four experiments. $p < 0.01$ was considered statistically significant.

Results

The PO activity of THS and PHS HPLC fractions is enhanced by trypsin

In Figure 1 a typical chromatographic profile, obtained by separating THS samples on a size exclusion column BioSuite 250, is shown. At 5.0 min and 5.5 min of retention time, two main confluent peaks (1, 2 respectively) were identified by spectrophotometric analysis at 280 nm. The chromatographic profiles of known proteins separated on the same column were registered and the protein sizes marked on the HPLC profile (Fig. 1). The peak 1 and 2 matched proteins of mass similar or superior than that of the phosphorylase b (97 kDa), whereas the subsequent major peaks matched proteins ranging from 13.7 to 67 kDa. Although the elution profile that characterized the chromatographic separation of PHS samples differed from that of THS, the elution volumes of two low peaks were similar to those of the THS peaks 1 and 2 (Fig. 1C inset).

Fractions from each peak were collected, pooled, tenfold concentrated and assayed for the PO activity. DOPA-MBTH reaction disclosed that, in the absence of a trypsin treatment, the THS fractions showed a low PO activity restricted to the confluent peaks 1 and 2. Values of 68 ± 1 U and 84 ± 16 U for the THS peak 1 and 2 respectively, were recorded. A lower activity was found by analyzing PHS peak 1 (5 ± 1 U) and 2 (51 ± 4 U) (Fig. 1 bars).
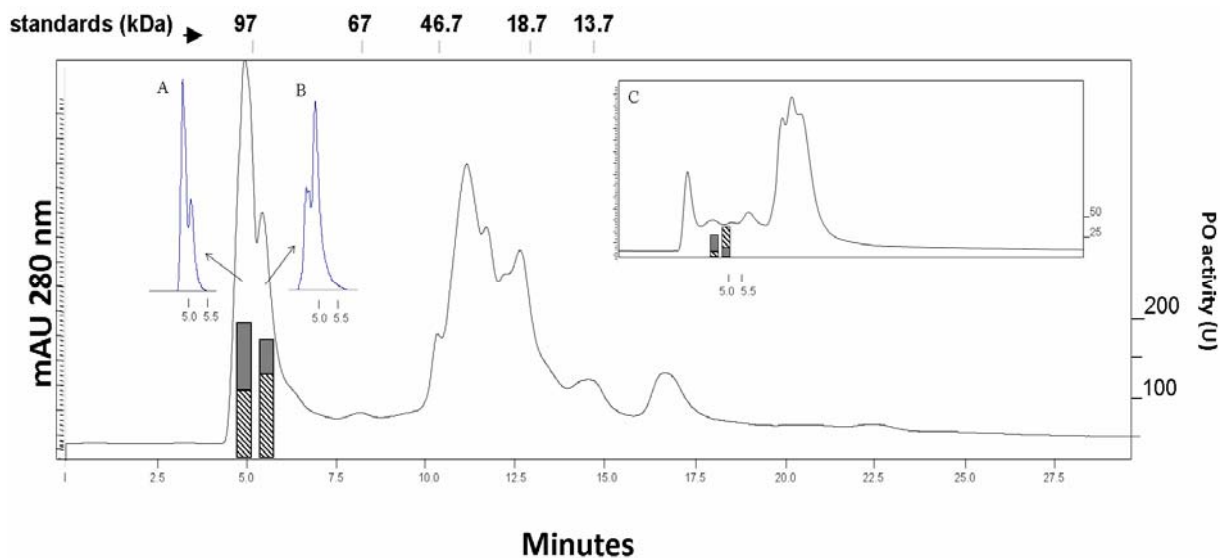

Fig. 1 Size exclusion HPLC separation of the *C. intestinalis* tunic homogenate supernatant. The THS was analyzed by size exclusion chromatography using BioSuite 250, 10 μm SEC, 7.5 × 300 mm column on a liquid chromatography HPLC. Elution was performed with TBS over 30 min at a flow rate of 1 ml/min. The pooled fractions from each THS peak (1, 2) were further analyzed trough the same chromatographic column (insets A and B). On the top, the molecular weights from HPLC separation profile of proteins used as molecular weight standards: phosporylase b (97 kDa); bovine serum albumin (BSA, 67 kDa), enolase (46.7 kDa), myoglobin A (18.7 kDa) and RNaseA (13.7 kDa*). Inset (C)*: The active material extracted from pharynx (PHS) was analyzed trough the same HPLC column. Absorbance peaks were monitored at 280 nm and PO activity of the collected peaks was evaluated in the presence (gray column) and absence of trypsin (hatched column).

The activities of the whole THS was 59 ± 7 U respect to PHS (22 ± 2 U) samples. As shown by the higher bars (Fig. 1 bars), the trypsin treatment of each THS sample enhanced the PO activity of THS peak 1 and 2 (213 ± 21 U and 194 ± 20 U respectively). Likewise, after the trypsin treatment of the PHS peak 1 fractions, the value of PO activity was 51 ± 8 U, whereas a lower activity of the peak 2 was recorded (13 ± 4 U) (Fig.1 inset C bars).

Due to the low absorbance at 280 nm and the low PO activity of the PHS chromatographic fractions, only THS was used for further analyses.

The pooled fractions from each THS peak (1, 2) were further analyzed trough the same chromatographic column. The elution profiles, shown in Figure 1 (A and B), revealed that each of them presented two confluent peaks with 280 nm absorbance patterns enriched than that corresponding to the first separation step. The absorbance of the peak 1 (inset A) is mainly enriched of 5 min peak (elution time correspondent to that of the peak 1) conversely the graph of the peak 2 (inset B) is enriched of 5.5 peak (elution time correspondent to the peak 2).

SDS pattern and zymograms

The fractions from the THS peaks 1 and 2 were analyzed by SDS-PAGE analysis, and the DOPA-MBTH reaction on gel electrophoresis was carried out in the absence of mercaptoethanol and denaturing temperature. The protein patterns of the peak 1 and 2 disclosed the same active components (Figs 2A, B). Comparison of these patterns with standard proteins separated in the same chemical-physical condition, showed a smaller active component of 90 kDa and a greater one of 170 kDa (Figs 2A, B). The gels obtained from a same electrophoresis procedure and stained with Coomassie blue showed that each peak contained 2 additional inactive components of intermediate sizes, and showed that the 90 kDa protein was mainly represented in the peak 2 and the enzymatic reaction was more intense for the 170 kDa band (Figs 2A, B).

To avoid the possibility of proPO activation by SDS and check for the effect of trypsin, an electrophoresis was carried out in native conditions (in the absence of SDS and denaturing factors) of whole THS sample. An unique band could be found only when the samples were treated with trypsin (Fig. 2C)

LPS inoculation enhanced the PO activity of trypsin-treated THS and PHS HPLC fractions

THS and PHS samples from ascidians inoculated with LPS, prepared at 4 and 24 h post injection, were separated on the chromatographic column. The fractions from peak 1 and 2, separately pooled, were assayed with DOPA-MBTH before and after a trypsin treatment. Tunic and pharynx samples from ascidians inoculated with MS were the controls and significance values were calculated with ANOVA. In all THS (T) and PHS (P) fractions, prepared at both 4 h p.i. and 24 h p.i., the PO activity (from 50 to 84 U for THS and 22 to 41 U for
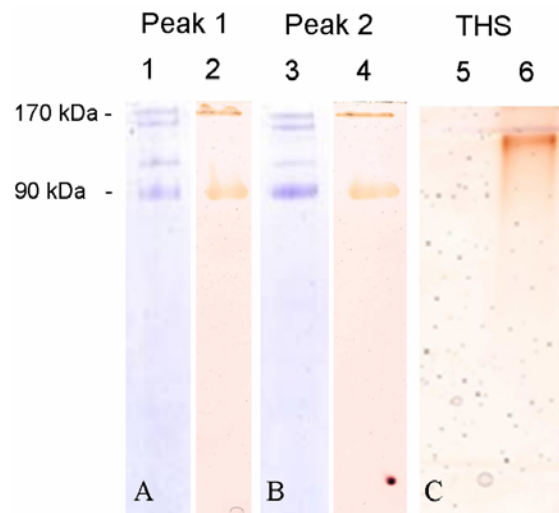

Fig. 2 Electrophoretic patterns of the HPLC separated fractions. SDS-PAGE and PAGE-DOPA-MBTH in the absence of SDS and denaturing temperature. A: Electrophoretic pattern of THS peak 1 (300 µg/ml) stained with Coomassie blue (lane 1) and after treatment with DOPA-MBTH (lane 2). B: Electrophoretic pattern of THS peak 2 (300 µg/ml) stained with Coomassie blue (lane 3) and after treatment with DOPA-MBTH (lane 4). C: Electrophoretic pattern of THS (300 µg/ml) under native conditions (7.5 % no SDS), in the absence of trypsin (lane 5) and after treatment with 1 µg/µl of trypsin (lane 6).

the PHS) was at control levels (Fig. 3). By treating samples from the peaks 1 and 2 with trypsin, the activity of treated THS 1, THS 2 and PHS 1 was significantly increased to different levels, whereas no changes were recorded in the activity of treated PHS 2 (53 ± 8 U similar to controls). In treated THS 1 the highest activation level was found at 4 h p.i. (408 ± 102 U), and it lowered to control level at 24 h p.i. In treated THS 2, the trypsin treatment enhanced the activity to a level below that of treated THS 1 (Fig. 3). In spite of an increase after the trypsin-treatment of PHS 1 at 4 h p.i. (153 ± 11 U), the enzymatic activity of PHS 2 was at control level (Fig. 3). A lower and not significant increase of PO activity, with respect to the treated THS 1 and THS 2 samples at 4 h p.i., was recorded for THS samples injected with MS and treated with trypsin (Fig. 3).

Discussion

In a previous paper (Cammarata *et al.,* 2008), it has been shown that LPS inoculation challenges inflammatory hemocytes (unilocular refractile granulocytes and granulocytes containing few large granules) which show PO activity. The enhanced PO activity found in THS has been related to the numerous inflammatory PO-positive hemocytes which populated the tissue.
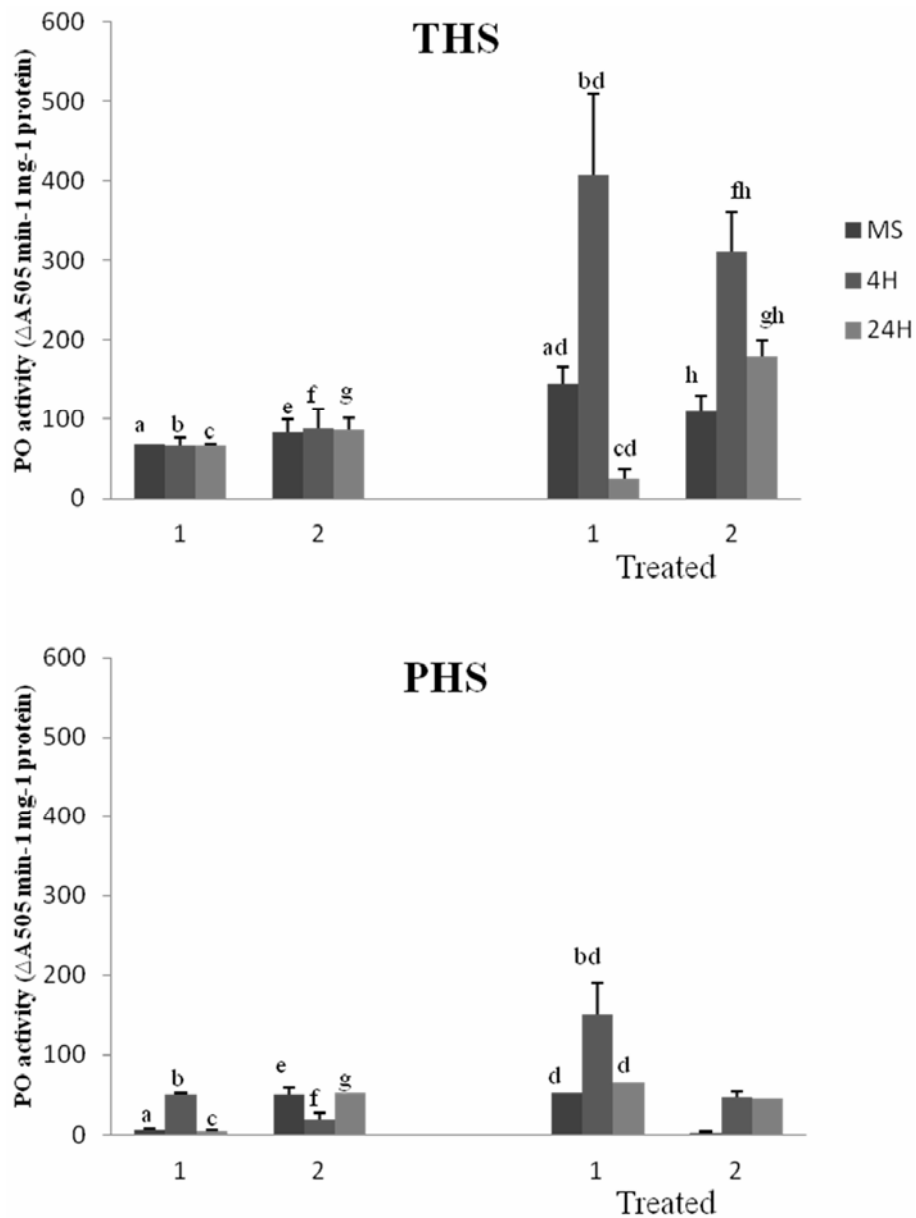
Fig. 3 ProPO/PO activity of *Ciona intestinalis* THS and PHS after inoculation of artificial sea water (MS) and after 4 or 24 h LPS injection (100 µg/ascidian). The obtained samples were assayed before and after treatment with 1 µg/µl of trypsin (treated). Multiple comparisons were performed with one-way analysis of variance (ANOVA), and different groups were compared by using Tukey's t-test. Standard deviations were calculated on four experiments. Same letters indicate statistical significant differences. $p < 0.01$ was considered statistically significant.

Since tunic and pharynx have been examined to study the immune-related responses, both tissues were analyzed. The chromatographic profiles of THS and PHS from naïve ascidians, and the DOPA-MBTH reaction disclosed that two POs of distinct sizes (about 90 kDa and > 90 kDa) could be separated. The PO activity of each THS peak was significantly enhanced by trypsin indicating that they contained both activated PO and proPO very similar in size. These findings agree with the idea that the proenzyme is produced by the tunic cells that populated the inflamed tissue and it is present in a major and minor form. The basal activity found in untreated samples could be dependent on artificial activation of proPO due to the experimental procedures. Two lower PHS peaks overlapped elution volumes similar to those of THS separation and disclosed a low PO activity enhanced by trypsin. PHS preparations mainly contained extracts from vessel epithelia and hemocyte populations, and it is reasonable that these cells, although at a lesser extent, were engaged in proPO production.

Due to the low protein content and PO activity of PHS fractions, THS fractions were used for

further analyses. The two main chromatographic peaks of THS were confluent indicating that proteins of intermediate size could be contained. In an attempt to further separate the proteins, the isolated fractions were subjected to a second chromatographic analysis. However, only an enrichment of the component that mainly characterized each peak was obtained.

The SDS activation effect on proPO and hemocyanins is well know (Decker *et al.* 2001), therefore the precise proPO size, checked by SDS-PAGE, remains unrevealed, whereas the sizes (90 and 170 kDa) of the active forms could be revealed. In this respect, to assay the PO activity of the protein bands, the DOPA-MBTH reaction on PAGE gel was carried out in the absence of SDS and denaturing condition, and the pattern was compared with standard proteins separated in the same conditions. The PO activity of the whole tissue supernatant as well as the DOPA-MBTH on PAGE gel before and after trypsin treatment, supported the view that proPO was mainly contained in the tunic fractions. This analysis, in accordance with the HPLC profile, revealed that the PO activity was exerted by the 170 and 90 kDa proteins. Analysis of the deduced amino acid sequences of the CinPO1 and CinPO2, identified in the *C. intestinalis* genome (Immesberger and Burmester, 2004), provided predicted molecular masses of 92 kDa and 89 kDa, respectively which can be reasonably referred to the 90 kDa POs separated by HPLC, whereas, according to Cammarata *et al.* (2008), the 170kDa PO may be referred to a dimerization process. Likewise, results obtained by examining POs from circulating hemocytes, showed two active forms of different size: a greater one (175 - 188 kDa) and a smaller one (74 kDa) (Parrinello *et al.*, 2003). We do not know the functional differences between the two forms. However the possibility exists that the dimeric one is an artifact due to sample preparation and biochemical methods. The 120 kDa active form, reported by Cammarata *et al.* (2008), remains to be explained, whereas the inactive proteins of intermediate sizes found in the SDS-PAGE pattern of the fractions could be additional components separated by the chromatography. Although PHS samples were not analyzed by SDS-PAGE, a similar protein pattern can be assumed on the basis of the chromatographic profile and PO activity of the fractions a similar protein pattern may be assumed.

In ascidians, the inoculated LPS modulates the proPO expression and activates cell proteases that in turn activates the proPO. In fact, in both in the tunic and pharynx preparations, the PO activity of the peak 1 and 2 significantly increased within few hours after LPS inoculation, and the effect of trypsin treatment indicates that the challenged tissues mainly expressed proPO. The LPS inoculation modulated the activity of the two peaks, it was higher for the peak 1 at 4 h p.i. and very low at 24 h. Conversely, at the latter time the activity of the peak 2 was lower but significantly higher than the control. The analysis of PHS fractions supported the idea that a low effect was exerted by LPS inoculation on PO activity of the pharynx tissue. A low activity was mainly bound to the peak 1 at 4 h p.i., whereas it was insignificant for the peak 2.

Differences between THS and PHS activities at the same p.i. time instead of supports the main role of tunic cells in the PO activity as a response to LPS stimulation, whereas the lower PHS activity may be imputable to a lower amount of proPO released by a minor number of PO expressing hemocytes (*i.e.,* univacuolar refractile granulocytes and morula cells). In addition, the possibility exists that the LPS modulating effect could be related to proPO releasing and activating steps or to the modulation of a presumptive dimerization process.

References
Ballarin L, Cima F, Floreani M, Sabbadin A. Oxidative stress induces cytotoxicity during rejection reaction in the compound ascidian *Botryllus schlosseri*. Comp. Biochem. Physiol. 133C: 411-418, 2002.

Ballarin L, Cima F, Sabbadin A. Morula cells and histocompatibility in the colonial ascidian *Botryllus schlosseri*. Zool. Sci. 12: 757-764, 1996.

Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72: 248-254, 1976.

Barrington EJW, Thorpe A. Histochemical and bio chemical aspects of iodine binding in the tunic of ascidian *Dendrodoa grossularia*. Proc. Roy. Soc. B 171: 91-109, 1968.

Bonura A, Vizzini A, Salerno G, Parrinello N, Longo V, Colombo P, Isolation and expression of a novel MBL-like collectin cDNA enhanced by LPS injection in the body wall of the ascidian *Ciona intestinalis*. Mol. Immunol. 46: 2389-2394, 2009.

Bonura A, Vizzini A, Salerno G, Parrinello D, Parrinello N, Longo V, *et al.* Cloning and expression of a novel component of the CAP superfamily enhanced in the inflammatory response to LPS of the ascidian *Ciona intestinalis*. Cell Tissue Res. 342: 411-421, 2010.

Cammarata M, Arizza V, Cianciolo C, Parrinello D, Vazzana M, Vizzini A, *et al.* The prophenoloxidase system is activated during the tunic inflammatory reaction of *Ciona intestinalis*. Cell Tissue Res. 333: 481-492, 2008.

Cammarata M, Parrinello N. The ascidian prophenoloxidase activating system. Inv. Surv. J. 6: 67-76, 2009.

Càrdenas W, Dankert J. Cresolase, catecholase and laccase activities in haemocytes of the red swamp crayfish. Fish Shellfish Immunol. 10: 33-46, 2000.

Cerenius L, Kawabata S, Lee BL, Nonaka M, Söderhäll K. Proteolytic cascades and their involvement in invertebrate immunity. Trends Biochem. Sci. 35: 575-83, 2010.

Cerenius L, Söderhäll K. Variable immune molecules in invertebrates. J. Exp. Biol. 216: 4313-4319, 2013.

Chaga OY. Ortho-diphenoloxidase system of Ascidians. Tsitologia 22: 619-625, 1980.

Decker H, Ryan M, Jaenicke E, Terwilliger N, SDS-induced Phenoloxidase activity of hemocyanins from *Limulus polyphemus,Eurypelma californicum*, and *Cancer magister*. J. Biol. Chem. 276: 17796-17799, 2001.

Fuke MT. 'Contact reactions' between xenogeneic or allogeneic coelomic cells of solitary ascidians. Biol. Bull. 158: 304-315, 1980.

Immesberger A, Burmester T. Putative phenoloxidases in the tunicate *Ciona intestinalis* and the origin of the arthropod hemocyanin superfamily. J. Comp. Physiol. B 174: 169-180, 2004.

Jackson AD, Smith VJ, Peddie CM. In vitro phenoloxidase activity in the blood of *Ciona intestinalis* and other ascidians. Dev. Comp. Immunol. 17: 97-108, 1993.

Kelly K, Cooper EL, Raftos DA. Purification and characterization of a humoral opsonin from the solitary urochordate *Styela clava*. Comp. Biochem. Physiol. 103B: 749-753, 1992.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature (London) 227: 680-685, 1970.

Nappi AJ, Seymur J. Hemolyph phenoloxidases in *Drosophila melanogaster*, *Locusta migratoria* and *Austropotamobius pallipes*. Biochem. Biophys. Res. Commun. 180: 748-754, 1991.

Parrinello N and Patricolo E. Inflammatory-like reaction in the tunic of *Ciona intestinalis* (Tunicata). II. Capsule components. Biol. Bull. 167: 238-250, 1984.

Parrinello N, Patricolo E, Canicattı´ C. Inflammatory-like reaction in the tunic of *Ciona intestinalis* (Tunicata). Encapsulation and tissue injury I. Biol. Bull. 167: 229-237, 1984a.

Parrinello N, Patricolo E, Canicattı´ C. Inflammatory-like reaction in the tunic of *Ciona intestinalis* (Tunicata). Encapsulation tissue injury II. Biol. Bull. 167: 238-250, 1984b.

Parrinello N. The reaction of *Ciona intestinalis* L. To subcuticular erythrocyte and protein injection. Dev. Comp. Immunol. 5: 105-110, 1981.

Parrinello N, Arizza V, Chinnici C, Parrinello D, Cammarata M. Phenoloxidases in ascidian hemocytes: characterization of the prophenoloxidase activating system. Comp. Biochem. Physiol. 135B: 583-591, 2003.

Parrinello N, Arizza V, Cammarata M, Giaramita FT, Pergolizzi M, Vazzana M, *et al.* Inducible lectins with galectin properties and human IL1α epitopes opsonize yeasts in the ascidian *Ciona intestinalis* inflammatory response. Cell Tissue Res. 329: 79-90, 2007.

Parrinello N, Vizzini A, Arizza V, Salerno G, Parrinello D, Cammarata M, *et al.* Enhanced expression of a cloned and sequenced *Ciona intestinalis* TNF alpha-like (CiTNF alpha) gene during the LPS induced inflammatory response. Cell Tissue Res. 334: 305-317, 2008.

Parrinello N. Focusing on *Ciona intestinalis* (Tunicata) innate immune system. Evolutionary implications. Inv. Surv. J. 6: S46-S57, 2009.

Parrinello N, Vizzini A, Salerno G, Sanfratello MA, Cammarata M, Arizza V, *et al.* Inflamed adult pharynx tissues and swimming larva of *Ciona intestinalis* share CiTNF alpha-producing cells. Cell Tissue Res. 341: 299-311, 2010.

Raftos DA, Tait NN, Briscoe DA. Allograft rejection and alloimmune memory in the solitary urochordate, *Styela plicata*. Dev. Comp. Immunol. 11: 343-351, 1987a.

Raftos DA, Tait NN, Briscoe DA. Cellular basis of allograft rejection in the solitary urochordate, *Styela plicata*. Dev. Comp. Immunol. 11: 713-725, 1987b.

Raftos DA, Briscoe DA, Tait NN. The mode of recognition of allogeneic tissue in the solitary urochordate *Styela plicata*. Transplantation 45: 1123-1126, 1988.

Söderhäll K, Cerenius L. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol. 10: 23-28, 1998.

Sugumaran M, Hennigan B, Semensi V, Mitchell M, Rivera T. Differential mechanism of oxidation of N acetyldopamine and N-acetylnorepinephrine by cuticular phenoloxidase from *Sarcophaga bullata*. Arch. Insect Biochem. Physiol. 8: 229-241, 1988.

Vizzini A, Pergolizzi M, Vazzana M, Salerno G, Di Sano C, Macaluso P, *et al.* FACIT collagen (1 alpha-chain) is expressed by hemocytes and epidermis during the inflammatory response of the ascidian *Ciona intestinalis*. Dev. Comp. Immunol. 32: 682-692, 2008.

Vizzini A, Parrinello D, Sanfratello MA, Salerno G, Cammarata M, Parrinello N. Inducible galectins are expressed in the inflamed pharynx of the ascidian *Ciona intestinalis*. Fish Shellfish Immunol. 32: 101-109, 2012.

Winder J, Harris H. New assays for tyrosine hydroxylase and dopa oxidase activities of tyrosinase. Eur. J. Biochem. 198: 317-326, 1991.