Fish and Shellfish Immunology 86 (2019) 213–222

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

The gelatinase MMP-9*like* is involved in regulation of LPS inflammatory response in *Ciona robusta*

Patrizia Cancemi[a,b], Felicia Di Falco[a], Salvatore Feo[a,b], Vincenzo Arizza[a], Aiti Vizzini[a,*]

[a] Department of Biological Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Palermo, Italy
[b] Advanced Technologies Network Center (ATeN), University of Palermo, Palermo, Italy

ARTICLE INFO

Keywords:
Ciona robusta
Matrix metalloproteinases (MMPs)
MMP-2
MMP-9
Pharynx
Inflammation
LPS

ABSTRACT

Matrix metalloproteinases (MMPs) are a family of endopeptidases collectively able to degrade the components of the extracellular matrix (ECM), with important roles in many biological processes, such as embryogenesis, normal tissue remodelling, angiogenesis and wound healing. New views on the function of MMPs reveal that they regulate inflammatory response and therefore might represent an early step in the evolution of the immune system. MMPs can affect the activity of cytokines involved in inflammation including TGF-β and TNF-α. MMPs are widely distributed in all kingdoms of life and have likely evolved from a single-domain protein which underwent successive rounds of duplications. In this study, we focused on the *Ciona robusta* (formerly known as *Ciona intestinalis*) MMP gelatinase homologue. Gene organization, phylogenetic analysis and 3D modeling supported the closest correlation of *C. robusta* gelatinase with the human MMP-9. Real-time PCR analysis and zymographic assay showed a prompt expression induced by LPS inoculation and an upregulation of enzymatic activity. Furthermore, we showed that before of the well-known increase of TGF-β and TNF-α levels, a MMP-9like boost occurred, suggesting a possible involvement of MMP-9like in regulating inflammatory response in *C. robusta*.

1. Introduction

Matrix metalloproteinase (MMPs) are a family of proteolytic enzymes involved in ECM degradation and remodelling. MMPs regulate several physiological processes such as tissue homeostasis, host defence and tissue repair. The list of MMPs functions has been expanding rapidly, and recent findings also indicated that MMPs are immunoregulators which act on inflammatory cytokines, chemokines and other immune proteins [1]. During inflammation MMPs regulate the integrity of physical barriers, the migration of leukocytes from blood to the underlying connective tissue and the availability and activity of inflammatory mediators. Moreover, by generating chemokine gradients, they induce the recruitment and survival of the inflammatory cells to the site of injury [1,2]. Depending on the context, MMPs can activate, inactivate or antagonize the biological functions of cytokines and chemokines by proteolytic processing, so they can either promote or suppress inflammation [3–5]. Structurally, all members of the MMP family contain four distinct functional domains: the signal peptide, propeptide, catalytic domain and hemopexin-like domain. The N-terminal signal peptide is required for the secretion of enzymes. In human MMPs, the pro-peptide consists of about 80 aminoacids and keeps the enzyme in the latent form using the thiol group of a highly conserved cysteine at its carboxyl terminus. The catalytic site contains the zinc ion (Zn^{2+}) that is linked to three conserved histidine residues in the sequence HEXXHXXGXXH. The conserved cysteine in the pro-peptide acts as a fourth inactivating ligand for the catalytic zinc atom, resulting in the exclusion of water. For enzyme activation, this cysteine-zinc pairing needs to be disrupted by a conformational change or proteolysis. This system of regulation is referred to as “cysteine-switch”. Once the thiol group is replaced by water, the enzyme is able to hydrolyse the propeptide to complete the activation process and then cleave the peptide bonds of its substrates [6,7]. MMPs contain a flexible proline-rich hinge region and a carboxy (C)-terminal Hemopexin-Like Domain (with the exception of matrilysins and MMP-23), which is involved in substrate recognition [1]. Further domains can be found in specific classes: for instance MMP-2 and MMP-9 have three additional fibronectin domains located inside the catalytic domain, which allows the binding and processing of denatured collagen or gelatine [8]. MMPs expression is regulated via the modulation of gene expression, inhibition by protein inhibitors and compartmentalization. Most MMPs are not constitutively transcribed, but induced after cytokines and growth factors stimulation. In addition, most MMPs are secreted as inactive zymogens and

* Corresponding author.
E-mail address: aiti.vizzini@unipa.it (A. Vizzini).

https://doi.org/10.1016/j.fsi.2018.11.028
Received 5 August 2018; Received in revised form 4 November 2018; Accepted 13 November 2018
Available online 16 November 2018
1050-4648/

activated in the extracellular space. MMPs, in the extracellular space, can be checked by their endogenous tissue inhibitors, the TIMPs (tissue inhibitors of metalloproteinases). Four different forms (TIMP-1 to TIMP-4) have been described to date. TIMPs forming tight 1:1 complexes with MMPs can inhibit them with relatively low selectivity. Finally, MMPs can be stored in inflammatory cell granules, which restrict their compass of action.

Based on their domain organization, MMPs can be classified in four different groups: archetypal, matrilysin, gelatinases and furin-activable MMPs. The gelatinases MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) degrade a broad spectrum of ECM molecules, such as different collagen types (I, IV, V, VII, X, IX), elastin, fibronectin, aggrecan, vitronectin, laminin [9], as well as cytokines, such as pro-TNF-α [10], TGF-β [11], pro-IL-1β, pro-IL-8 and monocyte chemoattractant protein (MCP)-3 [5]. Likewise, these enzymes are able to release or generate several factors with pro- or anti-angiogenic properties [12]. MMPs are found in all kingdoms of life and belong to the metzincin superfamily of metalloproteinases. To date, at least 24 different vertebrate MMPs have been identified, which are structurally similar to each other, indicating that they evolved by duplication of a common ancestral gene followed by divergent evolution [13].

Ascidians are chordate invertebrates with an innate immune system. The ascidian *Ciona intestinalis*, recently recognized as *Ciona robusta* [14,15] occupies a key phylogenetic position in chordate evolution and is considered to be the sister group of vertebrates [16–18]. *C. robusta* is a powerful model for studying innate immunity and the evolution of immune genes [19–21]. In our previous papers [22–32], we reported that LPS induces an inflammatory reaction in the *C. robusta* pharynx, considered the immunocompetent organ, including the expression of characteristic innate immune genes. A search of *Ciona* genome revealed the presence of six MMPs: MMP-21, MMP-24, MMP-24-2 MMP-14, MMP-14-2 and only one corresponding to gelatinase MMP-9 l*ike*. In the present paper, we have highlighted the identification and the molecular characterization of *C. robusta* gelatinase, its phylogeny relationships and possible involvement in innate immune response, during LPS mediated inflammation.

2. Materials and methods

2.1. Tunicates and LPS inoculation

C. robusta ascidians were collected from Sciacca Harbor (Sicily, Italy), maintained in tanks with aerated sea water at 15 °C, and fed every second day with Coraliquid marine invertebrate food (Sera Heinsberg, Germany). The LPS solution (*Escherichia coli* 055:B5, Sigma-Aldrich, Germany) was prepared in sterile Marine Solution (MS:12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 43 mM Tris HCl, 0.4 M NaCl, pH 8.0) and inoculated into the tunic matrix close to the pharynx wall at the median body region at a concentration of 100 μg/100 μl per animal. Animals were sacrificed at different times (1, 2, 4, 8, 24, 48 and 72 h). Untreated ascidians (naive ascidians) and ascidians injected with MS (sham ascidians), were used as controls. At least three different animals were treated for each experimental condition.

2.2. Total RNA and protein extraction

Ascidian tissue fragments, (about 200 mg) explanted at different treatments times, were immediately soaked in RNAlater Tissue collection solution (Ambion, Austin, TX) and stored at −80 °C for total RNA extraction, or subjected to homogenization for protein extraction.

Total RNA extraction was performed using an RNAqueousTM-Midi Kit purification system (Ambion, Austin, TX), as previously described [33].

For protein extraction, pharynx tissues were homogenized in an ice bath with a buffer containing sodium deoxycholate 10 mM pH 8 and mixed for 2 h at 4 °C. After incubation, lysates were centrifuged at 20.000 rpm for 30 min at 4 °C, in order to remove cell debris and the supernatants containing proteins were collected. Protein concentration was quantified spectrophotometrically, by using the Bradford assay, as previously described [34,35].

Table 1
Primers used for cloning and expression.

Gene	Primer sequence (5′-3′)	Application
*Cr*MMP-9like	5′- AAGCGTAGTCTTGTCGGTGA-3′	RACE5′
	5′- GCTCTTTGTCATCGTCGGTC-3′	NESTED5′
	5′- TAACGTTGATGCTGCGATGG-3′	RACE3′
	5′- GGCCTTCAAGAGACAGAGGT -3′	NESTED3′
*Cr*MMP-9*like*	5′- GACGAGTTCCGCGTAACGTT -3′	Real-time PCR′
	5′- ATGGAATACCGTGCTCTTGTAGGA -3′	Real-time PCR
*Cr*TGFβ	5′-TTTCAGGGACCCAAAAACGA-3′	Real-time PCR
	5′-GCCAGCTATAATGACATCCAAGGT-3′	Real-time PCR
*Cr*TNF*α*	5′-GCCTCCCATAGACCGTTGTTAA-3′	Real-time PCR
	5′-CGGGACACCTTCAGCACAT-3′	Real-time PCR
Actin	5′-TGATGTTGCCGCACTCGTA-3′	Real-time PCR
	5′-TCGACAATGGATCCGGT-3′	Real-time PCR

2.3. Cloning and sequence analysis

A search in the Ensembl genome browser identified the sequence: *Gelatinase* (ENSCING00000005511). The sequence of the cDNA was obtained by using the GeneRacerTM kit (Invitrogen, USA). 5′- and 3′-RACE were performed by using the primers listed in Table 1. The overlapping RACE products were cloned into the pCR™IIvector (TA Cloning Kit, Invitrogen,USA) and sequenced. They contained the complete coding regions.

2.4. Bioinformatic analysis

The full length cDNA was analysed using the ExPASy translation tool (http://web.expasy.org/translate/). A BLAST (http://blast.ncbi.nlm.nih.gov/Blast) search allowed us to identify the known protein sequences that are homologous to *C. robusta* gelatinase. Physical and chemical parameters, such as molecular mass and theoretical isoelectric point, were computed using the Prot-Param tool on ExPASy (http://www.expasy.org/tool/protparam/). The NCBI Conserved Domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to predict domain architecture and other conserved domains based on the sequence homology. Functional motifs were determined by comparison on the Prosite database (http://prosite.expasy.org/scanprosite/). The putative cleavage site of the signal peptide was predicted by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignments of sequences were carried out using CLC (Version 7.0.0). Different 3D structures were predicted using the I-TASSER program (http://zhanglab.ccmb.med.umich.edu/I-TASSER), and the structures were validated by Ramachandran plot analysis (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). Finally, a phylogenetic tree using the Neighbour Joining method was constructed using MEGA 6.0, after 1000 bootstrap iterations.

2.5. Real-time PCR analysis

The differential expression of the *C. robusta* gelatinase, TGF-β and TNF-α cDNAs was studied by Real-time PCR using the SYBR-Green method and the specific sets of primers listed in Table 1. Real-time PCR analysis was performed using the Applied Biosystems 7500 Real-time PCR System. Differential expression was performed in a 25 μl PCR reaction containing 2 μl cDNA converted from 250 ng of total RNA, 300 nM forward and reverse primers, and 12.5 μl of Power SYBR-Green

A

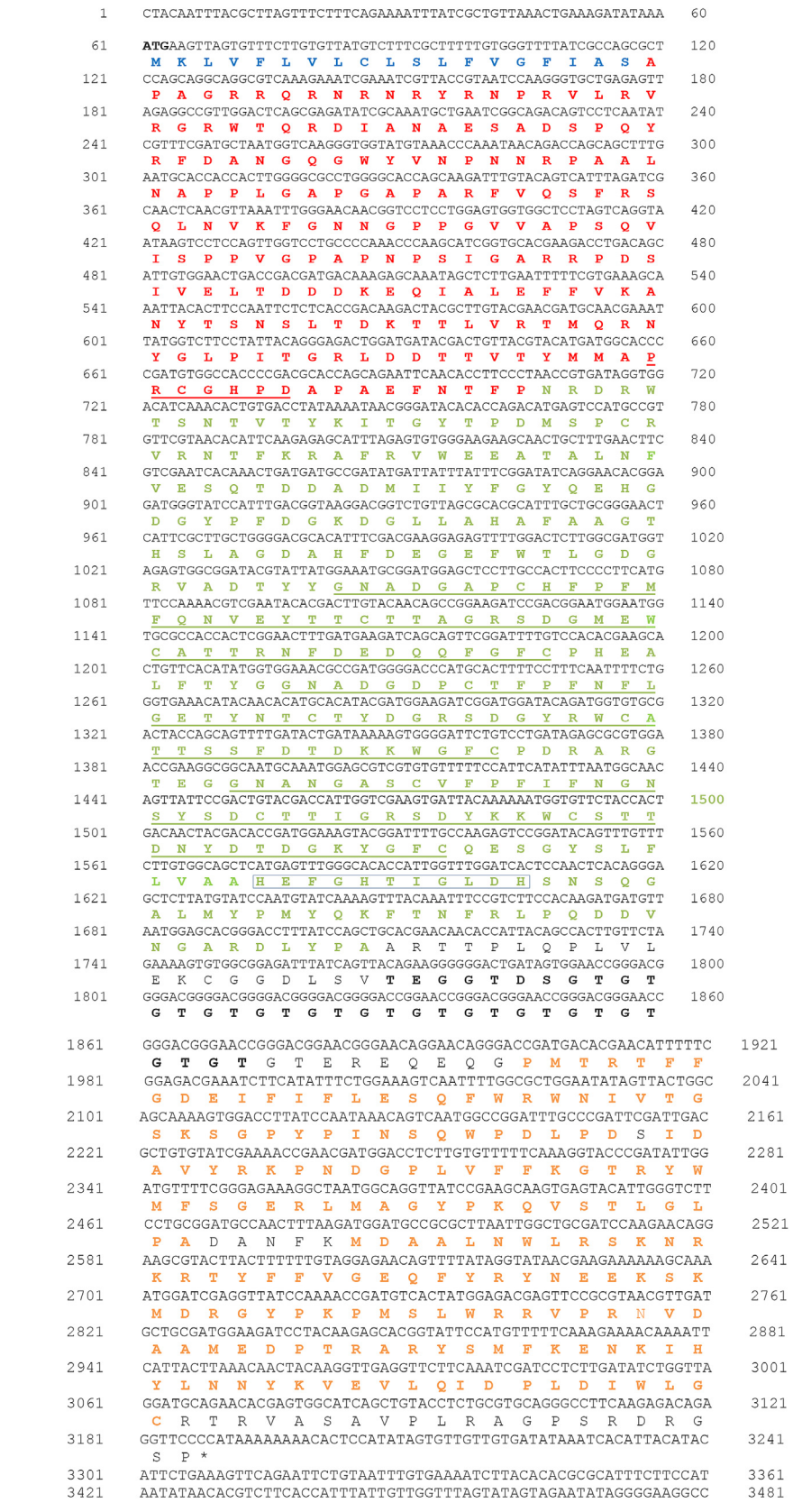

Fig. 1. *CrMMP-9like* nucleotide and amino acid sequences. The start codon is shown in bold, the stop codon is indicated by an asterisk. The signal peptide is light blue; propeptide is red and the consensus sequence is underlined; the catalytic domain is green, the consensus sequence containing the three histidine is in boldface and Fibronectin type II-like are underlined; the Hinge domain is black, while the four hemopexin-like domain are orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

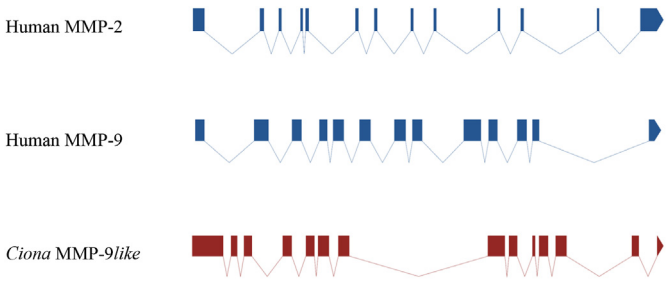

Fig. 2. Comparison of the intron/exon sizes between human MMP-2 and MMP-9 genes (in light blue) and *Ciona* gelatinase (in red). The thin line represents the introns, and the open boxes indicate the exons of the respective genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PCR MasterMix (Applied Biosystems, USA).

The 50 cycles of the two-step PCR program consisted of an initial polymerase activation for 3 min at 95 °C, followed by a denaturing step at 95 °C for 15 s, and then annealing/extension was carried out at 60 °C for 45 s when the fluorescent signal was detected. Each set of samples was run three times, and each plate contained quadruplicate cDNA samples and negative controls.

Amplification specificity was tested using Real-time PCR melting analysis. To obtain sample quantification, the $2^{-\Delta\Delta Ct}$ method was used, and the relative changes in gene expression were analysed as described in the Applied Biosystems Use Bulletin N.2 (P/N 4303859). The amount of *MMP-9like,* TGF-β and TNF-α transcripts was normalized to actin in order to compensate for variations in input RNA amounts. Relative expression was determined by dividing the normalized value of the target genes with the normalized value obtained from the untreated samples.

2.6. Gelatin zymography

Aliquots containing 30 µg of protein samples were solubilized in Laemmli buffer under nonreducing conditions and loaded onto 7.5% polyacrylamide SDS-PAGE gels co-polymerized with 0.1% gelatine [36]. Gels were run at 150 V in a Tris-glycine buffer, as previously described [37]. After electrophoresis, gels were washed for 1 h in a buffer containing 50 mM Tris-HCl pH 7.5 and 2.5% Triton X-100 to remove the SDS and then incubated with an activation buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, for 18 h at 37 °C to allow the activation of the gelatinases. Gels were stained with 0.2% Coomassie Brilliant Blue G-250 in 40% methanol and 10% acetic acid and destained in 7% methanol and 5% acetic acid. Band intensity was measured with Image J software. Protein samples extracted from human breast cancer tissues were used as reference for MMP-2 and MMP-9 activity levels [38,39]. For the inhibition assay, after electrophoresis, gels were incubated with the described incubation buffer with 500 mM of EDTA, a chemical inhibitor of MMPs.

2.7. Western blotting

Protein samples (30 µg/lane) were subjected to 8% SDS polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane (HyBond ECL, Amersham) for 1 h at 100 V and stained with Ponceau S (Sigma Aldrich). Membranes were blocked with 5% milk in T-TBS ON at 4 °C under agitation. A mouse monoclonal antibody for MMP-9(2C3): sc21733 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), raised against amino acids 603–614 of human MMP-9 was used diluted 1:1000 in milk 1% in T-TBS for 1 h a room temperature, as previously described [37]. Following incubation with the mouse peroxidase-linked antibody diluted 1:2000 in milk 1% in T-TBS, the reaction was revealed by the ECL detection system, using Versadoc by Biorad. The correct protein loading was ascertained by red Ponceau staining.

2.8. Statistical methods

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. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Sequence analysis of gelatinase in C. robusta

To investigate the possible involvement of MMPs and in particular of gelatinases in LPS induced inflammatory responses, we firstly performed a search for MMPs sequences in the *Ciona* genome. The analysis revealed the presence of six MMP genes, one of which with accession number ENSCING00000005511 contained highly conserved domains typical of human gelatinases. The full-length mRNA of *C. robusta* gelatinase was isolated by performing a 5′- and 3′-RACE strategy. The cDNA and aminoacid sequence analysis showed an ORF of 3127 bp encoding an 823 aminoacid polypeptide with a predicted molecular size of 91.724 kDa and a pI of 6.32. In silico analysis revealed a predicted signal peptide of 19 aminoacids (1-19), a pro-peptide (20–247) of 228 aminoacids containing a highly conserved cysteine residue in the consensus sequence PRCXXPD, a catalytic domain (248–658) containing three histidine residues in the consensus sequence HEXXHXXGXXH, three Fibronectin type II-like domains (328–376, 386–434, 444–492), a Hinge domain (570–606) and four hemopexin-like domains (614–657, 659–702,708-757, 759–802) (Fig. 1).

Compared with the genomic organization of *H. sapiens* MMP-2 and MMP-9 (containing thirteen exons and twelve introns) the *C. robusta* gelatinase (Fig. 2) contains an additional exon and intron (fourteen exons and thirteen introns), consistent with the presence of a longer propeptide (corresponding to 228 aminoacids) than the human propeptide (80 amino acids long).

3.2. Alignment and structural analysis

The deduced amino acid sequence of *C. robusta* gelatinase showed significant homologies with human gelatinases (MMP-2 and MMP-9). We found: an identity of 46% and a similarity of 58% with the *H. sapiens* MMP-2 and an identity of 53% and a similarity of 64% with the *H. sapiens* MMP-9. Multiple alignments analyses of the *C. robusta* gelatinase sequence and the *H. sapiens* MMP-2 and MMP-9 revealed that *C. robusta* gelatinase MMP-9 shows the highest homology with human MMP-9, having a greater similarity in terms of the amino acid sequence of peptide signal, pro-peptide and catalytic domain, and the hinge domain present in human MMP-9 and absent in human MMP-2 sequence was also found (Fig. 3A). Furthermore, the structural homology modeling analysis (Fig. 4) performed on the basis of the known crystal structure of Human MMP-9 (c1l6j) and resulting from the super-imposition of the 149–548 residue sequence, showed 48% identity with the template, and the amino acids in the catalytic site (His^{505}, Glu^{506}, His^{509}, Gly^{512}, His^{515}) were found to be highly conserved. Based on these experimental observations regarding chimico-physical parameters (molecular weight), domain structures (Fig. 3B) and structural homology (Fig. 4), we propose to name the *C. robusta* gelatinase as MMP-9*like*.

3.3. Phylogenetic analysis

A phylogenetic tree based on the amino acid sequence was generated to demonstrate the relationship of *C. robusta* MMP-9*like* with vertebrate and invertebrate members. The tree shows three main

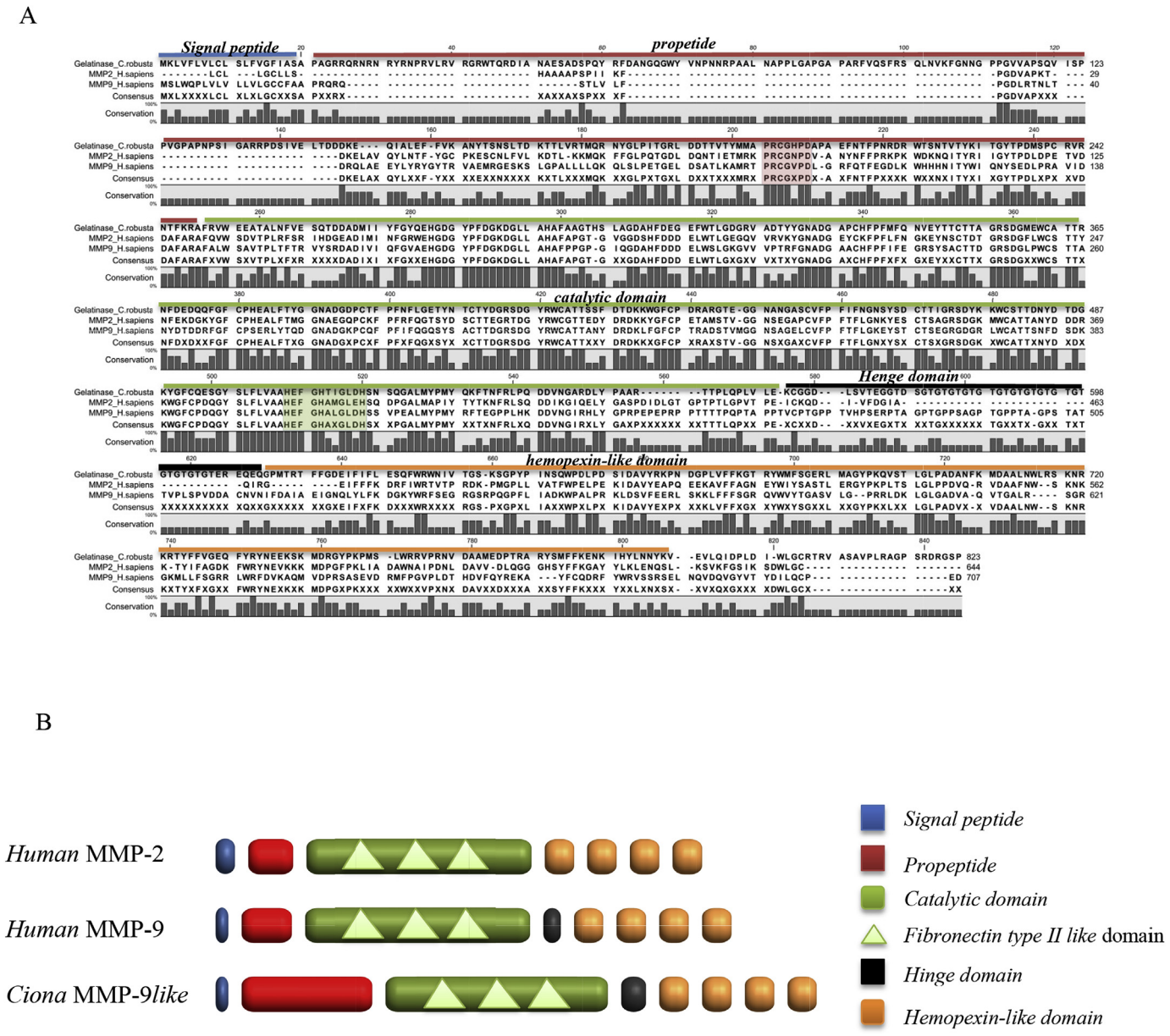

Fig. 3. Multiple amino acid sequences alignment of human MMP-9 and MMP-2 and *C. robusta* MMP-9*like* (A). Human MMP-9 and 2, *C. robusta* MMP-9*like* domain organization (B).

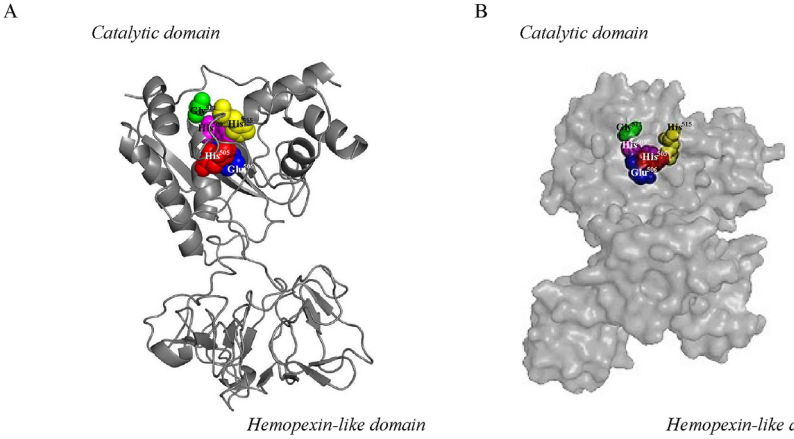

Fig. 4. Three-dimensional structure of *Cr*MMP-*9like.* The conserved amino acids are highlighted as coloured spheres, with numbers representing their location.

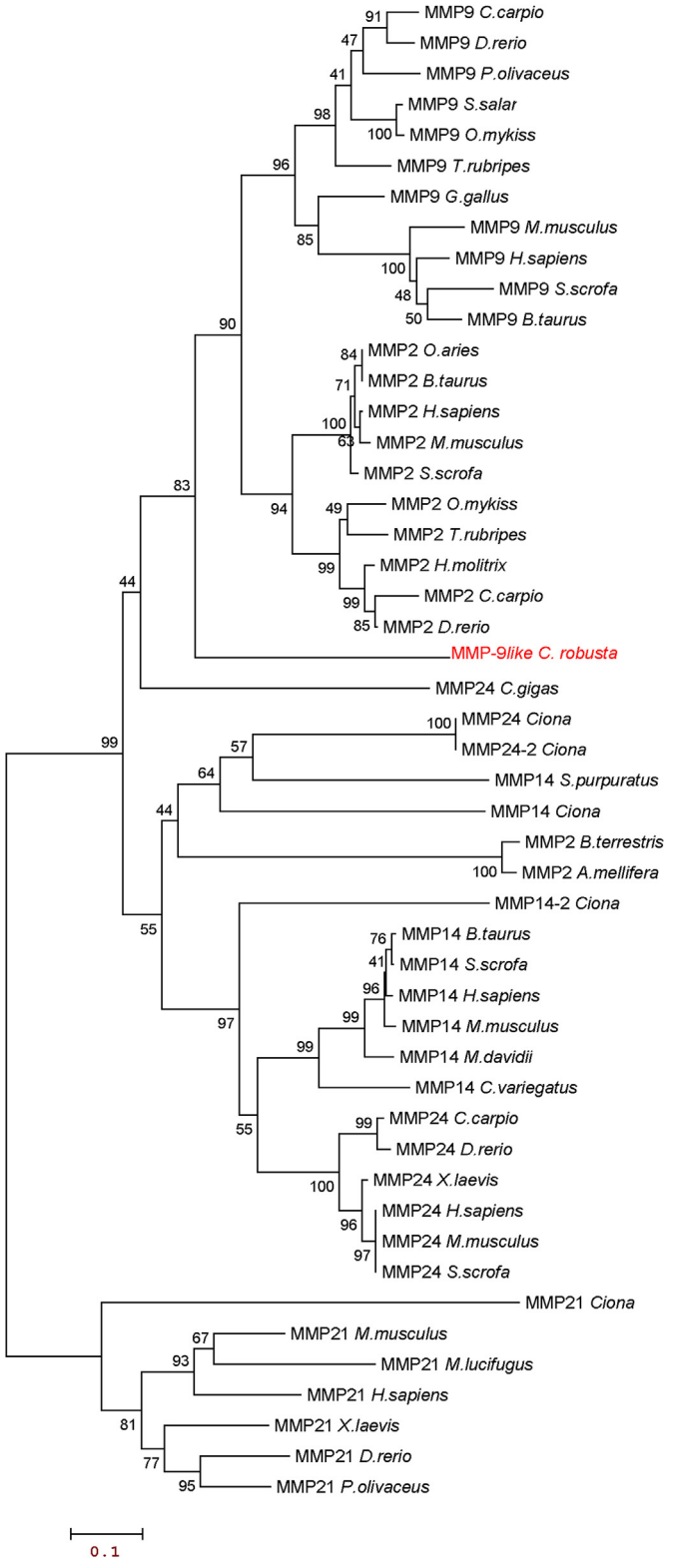

Fig. 5. Phylogenetic tree of MMPs family members of vertebrates and invertebrates. The tree was constructed using the Neighbour Joining method and bootstrap analysis. Bootstrap value indicates the number of particular node occurrences per 1000 trees, generated by bootstrapping the sequences. The bar indicates the number of amino acid residue substitutions per site. The accession numbers were as follows: NP_001159652.1 (*Ovis aries* MMP-2), NP_999357.2 (*Sus scrofa* MMP-2), NP_004521.1 (*Homo sapiens* MMP-2), DAA20015.1 (*Bos taurus* MMP-2), BAA78479.1 (*Oncorhynchus mykiss* MMP-2), XP_020720421.1 (*Bombus terrestris* MMP-2), XP_006563337.2 (*Apis mellifera* MMP-2), ALL25777.1 (*Hypophthalmichthys molitrix* MMP-2), AGI02587.1 (*Cyprinus carpio* MMP-2), AAH76545.1 (*Danio rerio* MMP-2), BAE06265.1 (*Takifugu rubripes* MMP-2), NP_032636.1 (*Mus musculus* MMP-2), NP_001033093.1 (*S. scrofa* MMP-9), NP_777169.1 (*B. taurus* MMP-9), NP_001133929.1 (*Salmo salar* MMP-9), CAC85923.1 (*O. mykiss* MMP-9), NP_989998.1 (*Gallus gallus* MMP-9), BAB39390.1 (*C. carpio* MMP-9), NP_998288.1 (*D. rerio* MMP-9), NP_001032959.1 (*T. rubripes* MMP-9), XP_019945088.1 (*Paralichthys olivaceus* MMP-9), NP_038627.1 (*M. musculus* MMP-9), NP_004985.2 (*H. sapiens* MMP-9), AAX59992.1 (*Strongylocentrotus purpuratus* MMP-14), NP_776815.1 (*B. Taurus* MMP-14), NP_004986.1 (*H. sapiens* MMP-14), NP_032634.3 (*M. musculus* MMP-14), NP_999404.2 (*S. scrofa* MMP-14), XP_015427968.1 (*Myotis davidii* MMP-14), XP_015255890.1 (*Cyprinodon variegatus* MMP-14), NP_006681.1 (*H. sapiens* MMP-24), NP_034938.3 (*M. musculus* MMP-24), XP_020933352.1 (*S. scrofa* MMP-24), XP_011450367.1 (*Crassostrea gigas* MMP-24), XP_018927930.1 (*C. carpio* MMP-24), XP_018093960.1 (*Xenopus laevis* MMP-24), XP_699803.4 (*D. rerio* MMP-24), AAN09805.1 (*M. musculus* MMP-21), AAI70298.1 (*X. laevis* MMP-21), AAW62254.1 (*H. sapiens* MMP-21), NP_001304682.1 (*D. rerio* MMP-21), XP_019938805.1 (*P. olivaceus* MMP-21), XP_006104048.1 (*Myotis lucifugus* MMP-21), XP_002120500.1 (*Ciona* MMP-21), XM_009864054.2 (*Ciona* MMP-14), XP_009862356.1 (*Ciona* MMP-14-2), XM_002125266.3 (*Ciona* MMP-24), XP_002125302.1 (*Ciona* MMP-24-2).

clusters, and the branching pattern essentially corresponds with to the evolutionary relationships among the species (Fig. 5). The first cluster includes vertebrate MMP-2, MMP-9 and *Cr*MMP-9*like*, the second cluster includes invertebrate MMP-2 (*B. terrestris* and *A. mellifera*) and vertebrate and invertebrate MMP-14 and MMP-24, while the third cluster includes vertebrate and *C. robusta* MMP-21. This analysis suggests that the MMP family proteins share a common ancestor and that human MMP-2 and MMP-9 probably arose due to the duplication and diversification of an ancestor gene.

3.4. Gene expression of CrMMP-9like in different tissues

The spatial expression pattern of *Cr*MMP-9like mRNA in adult *C. robusta* was investigate by quantitative Real-time PCR analysis of total RNA samples from different tissues (pharynx, ovary, stomach, intestine). As shown in Fig. 6 *Cr*MMP-9like expression was detected in all tissues.

3.5. In pharynx gene expression of CrMMP-9like and the cytokines TNFα and TGFβ are upregulated by LPS

To verify the involvement of *C. robusta* MMP-9*like* gene in the inflammatory response induced after LPS challenge, mRNA expression patterns in naive, sham and LPS-challenged ascidians pharynx were

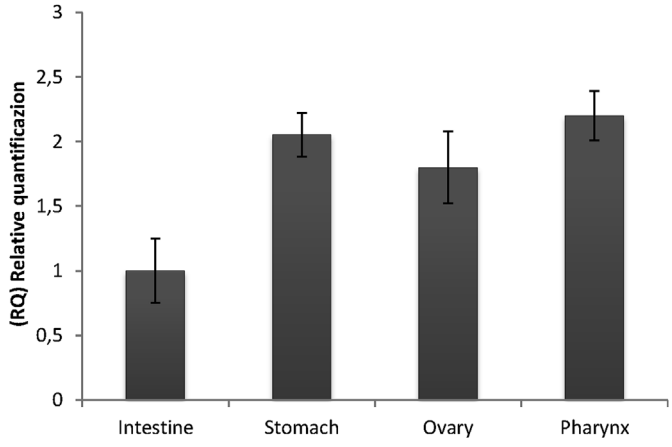

Fig. 6. Tissue expression of *Cr*MMP-9*like*. The mRNA expression level is calculated relative to actin expression and shown as mean ± SD (N = 4).

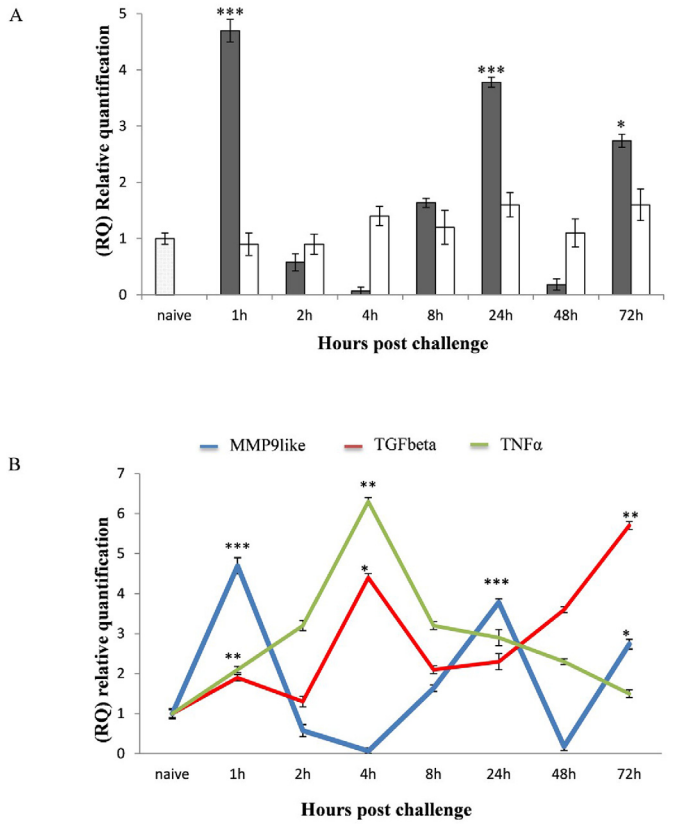

Fig. 7. Time-course Real-time PCR analysis of *CrMMP-9like* gene expression (A) in *C. robusta* pharynx after inoculation of 100 µg bacterial LPS in 100 µl MS (in gray) into the body wall, compared with the gene expression in ascidians injected with 100 µl MS (in white). Values, plotted as mean ± SD, were inferred from four ascidians examined in three distinct experiments performed in triplicate. Time-course Real-time PCR analysis of *Cr*MMP-9*like*, TGF-β and TNF-α gene expression (B). Asterisks indicate significant differences, at each time point, between LPS and MS inoculation (post hoc Tukey' *t*-test). $^{**}P < 0.01$; $^{***}P < 0.001$; ($n = 4$).

examined by Real-time PCR analysis. To examine the time course of the response, four ascidians in three distinct experiments were evaluated at increasing post inoculation time points (1, 2, 4, 8, 24, 48, 72 h). At each time point, four sham ascidians were used as controls (Fig. 7A). *Cr*MMP-9*like* gene expression was significantly boosted at 1 h, 24 h and 72 h. The sham ascidian response indicates that the inoculation procedure did not significantly modulate mRNA expression.

Furthermore a comparative analysis of TGF-β, TNF-α and *Cr*MMP-9*like* mRNA expression, was performed using Real-time PCR. The well-known trends of TGF-β and TNF-α mRNA expression [22,29] at different time points was compared with the results of *Ciona* MMP-9*like* mRNA expression with the same time course.

Fig. 7B shows that MMP-9*like* is the first gene upregulated after LPS injection as well as the most upregulated in the late phase of inflammation. Interestingly after the MMP-9*like* boost at 1 h, an upregulation of TGF-β and TNF-α was detected at 4 h. After the second wave of upregulation of MMP-9*like* at 24 h, an increase of TGF-β and a decrease of TNF-α expression were detected at 48 h.

3.6. *CrMMP-9like protein expression and activity levels are induced by LPS in pharynx inflammatory response*

After that CrMMP-9like expression was detected in all tested tissues, enzymatic activity of CrMMP-9like was evaluate and found on the same different tissues (ovary, stomach, intestine, pharynx) using zymographic assays (Fig. 1S). Since the major upregulation of gene transcription in pharynx was detected in the first phase of LPS simulation, the enzymatic activity levels of CrMMP-9*like* were monitored after 1 h of LPS injection (Fig. 8). To this purpose a zymographic assay was performed using protein extracted from the *C. robusta* pharynx. Fig. 8 A shows the zymograms obtained from pharynx protein extracts of three different animals, run in parallel with a human breast cancer tissue extract used as a standard, for to test the activity level of human MMP-9 and MMP-2. In the human breast cancer tissue extract gelatinolytic bands corresponding to the activity of proMMP-9 (92 kDa), proMMP-2 (72 kDa) and MMP-2 (62 kDa) were detected. Moreover, two additional bands of 200 kDa and 116 kDa, corresponding to MMP-9 dimers and MMP-9/TIMP-1 complex were identified, respectively [40,41]. In the *C. robusta* extracts, gelatynolitic bands with about 91 kDa, corresponding to the proenzymatic activity of *Ciona* MMP-9*like* and two different bands of MW about 180 kDa, probably corresponding to MMP-9*like* dimers, were detected. After 1 h of LPS stimulation, the activity levels of proMMP-9*like* increased. Moreover at 1 h of LPS stimulation a faint gelatinolytic band with MW of about 70 kDa was detected, probably corresponding to the activated form of MMP-9*like*. Densitometric analysis performed on zymograms obtained from pharynx protein extracts after 1 h post LPS challenge of three different animals (Fig. 8B) on the proMMP-9*like* showed its significant increase during 1 h of LPS stimulation. The nature of the lytic bands was further confirmed by both inhibition and western blot analyses. Incubation of zymograms with 500 mM of EDTA, a selective inhibitor of MMPs, inhibited all the gelatinolytic activities (Fig. 8C). The western blot analysis was performed using a monoclonal antibody against human MMP-9 raised against amino acids 603–614 (DKLGLGADVAQV) highly homologous between human MMP-9 and *Cr*MMP-9*like*. In the breast cancer tissue extract, used as a standard, the antibody recognized the pro-MMP-9 form and the form of 116 kDa, corresponding to the proMMP-9/TIMP-1 complex. In *C. robusta* extracts, the antibody recognized the proMMP-9*like* and the band at higher MW, confirming the zymography results.

4. Discussion

MMPs are a large family of zinc-endopeptidases involved in multiple physiological and pathological processes. MMPs are widely distributed in all kingdoms of life, and at least 24 members have been identified in human. MMPs have likely evolved from a single-domain protein which underwent successive rounds of duplication, gene fusion and exon shuffling events to generate the multidomain architecture and functional diversity typical of the family members [42]. Their proteolytic activities influence cell proliferation, migration and adhesion, as well as tissues remodelling, such as angiogenesis, bone development, wound healing and inflammation [43,44]. Generally the constitutively expressed enzymes fulfil more homeostatic functions, whereas the induced members are linked to regulatory processes and pathological conditions. Among MMPs, the gelatinases members are involved in different aspects of inflammation. Several studies have reported that stimulation with LPS, a surface component of Gram-negative bacteria, induces MMP-9 (also named gelatinase B; 92-kDa type IV collagenase) [45,46].

Here we have identified and characterized the MMP-9*like* of *C. robusta* and studied its involvement during LPS-inflammation response in the pharynx. The *Ciona* genome search analysis revealed the presence of six MMPs of which only one corresponded to gelatinase. Analysis of domain organization and 3D modeling showed significant homology with human MMP-9, so we propose to name the *C. robusta* gelatinase as MMP-9 *like*. We found a high degree of similarity in the catalytic domain, with three histidine residues involved in catalytic activity contained in the highly conserved consensus sequence HEXXHXXGXXH. Signal peptide and pro-peptide were more similar to human MMP-9 than MMP-2. Furthermore, *C. robusta* contains a hinge domain typical of MMP-9 and not of MMP-2. The hemopexin domains (PEX) are instead

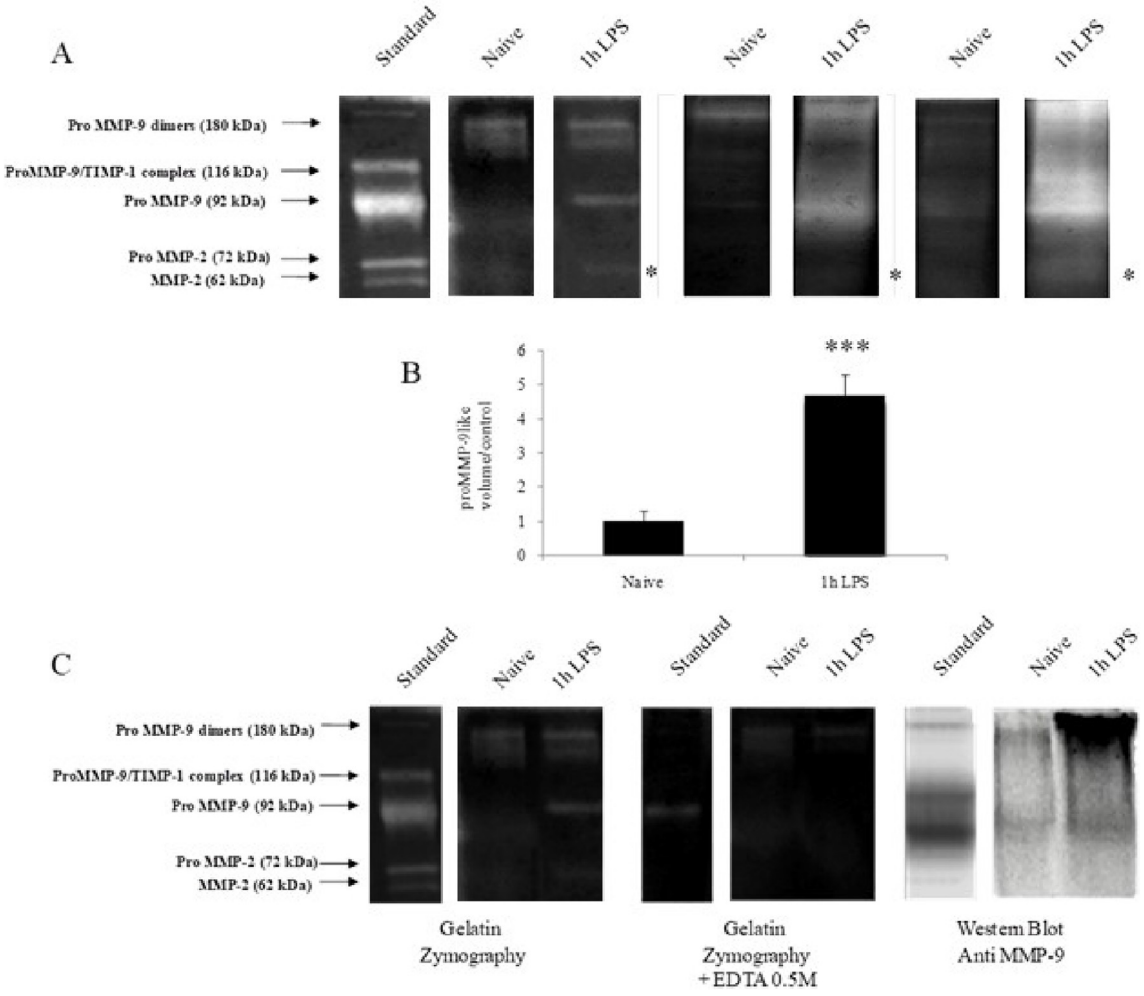

Fig. 8. Panel of gelatin zymograms (A) of protein extracted from pharynx of three naïve *C. robusta* and inoculated with LPS. The standard is represented by a human breast cancer tissue extract. B) Densitometric analysis of pro-MMP-9 activity levels, expressed as mean of the band-intensity ± SD. Statistical significance was assessed by Student's t-test: ***$p < 0.001$. C) Inhibition assay and western blot validation of MMP-9*like* expression.

more variable, with only some regions conserved. For example, the hemopexin-like domain involved in substrate binding and interaction with the tissue inhibitors of metalloproteinase (TIMPs) resulted highly conserved. The PEX domain of human MMP-9, consisting of ~210 amino acids, is involved in dimerization, cleavage of substrates, substrate specificity and interacts with inhibitors cell surface receptors [47]. It has been recognized that the region of exon 13, coding for the 4th repeat of PEX domain (658–704 aa) may evolve faster than others because of positive selection. This rapid evolution of exon 13 may have an influence on the function change of human MMP-9 and MMP-2 [48].

In *C. robusta*, LPS challenge, induces inflammatory responses in the pharynx, which is considered the major immunocompetent organ. These responses induce several innate immune genes as TNF-α, IL-17, TGF-β, Type IX collagen [49]. Real-time PCR analysis of the inflamed ascidian pharynx showed that *Cr*MMP-9*like* was also involved in LPS-inflammatory response. In particular, *Cr*MMP-9*like* gene expression was boosted mainly at 1 h, at 24 h and 72 h after LPS stimulation, suggesting that it is involved in both the early and late phase of inflammation. Our data are in agreement with other results showing the upregulation of MMP-9 during inflammation: for example, the mRNA levels and enzymatic activity of MMP-9 are elevated in the synovial fluid of patients with rheumatoid arthritis or in osteoclasts after skeletal injury [50], while MMP-9 inhibition within the intestinal muscularis reduces inflammation in mouse and rat models [51]. Moreover, while MMP-9 can be induced by various pro-inflammatory factors [52], MMP-2 is usually constitutively produced and not inducible. This evidence strengthens our observation that the *C. robusta* gelatinase is mainly related to human MMP-9. Moreover for the first time we have shown that MMP-9*like* is one of the earliest activated genes during *C. robusta* inflammatory response. Interestingly, after the *Cr*MMP-9*like* boost at 1 h, an upregulation of TGF-β and TNF-α was detected at 4 h, suggesting that *Cr*MMP-9*like* could play a role as a modulators of inflammatory processes. In mammals TNF-α is a potent pro-inflammatory cytokine regulated by MMPs, and several MMPs can cleave and activate TNF-α [3,53,54] and TGF-β [55–58]. Several studies have indicated that MMPs can directly or indirectly affect the activity of various cytokines that function in inflammation and repair processes, including transforming growth factor-β1 (TGF-β1). In *Ciona* after the second wave of upregulation of MMP-9*like* at 24 h, a further increase in the expression of TGF-β was detected at 72 h, while TNF-α decreased. We suppose that, while *Ciona* TNF-α is involved in the pro-inflammatory response, the late activation of *Cr*MMP-9*like* and *Ciona* TGF-β could be related to the anti-inflammatory response, contributing to the restoration of homeostasis.

In *C. robusta* after 1 h of LPS stimulation, the activity levels of proMMP-9*like* increased, consistent with the upregulation of gene transcription. The increase of the activity levels was further confirmed by both inhibition and western blot analyses. Moreover, at 1 h of LPS a faint gelatinolytic band with MW of about 70 kDa was also detected, probably corresponding to the activated form of *Cr*MMP-9*like*. It is

known that MMP-9 cleaves extracellular structural proteins, such as collagen types I, IV, V, VII, X, IX, elastin, fibronectin, aggrecan, vitronectin, laminin [9]. Accordingly, in *Ciona* Vizzini et al., 2008 showed that a FACIT-collagen type IX could participate in inflammation and wound healing and Ci-type IX-Col could be involved in a network of non-fibril-forming collagens that participates in the organization of the extracellular matrix and defense responses. More recently, MMPs have also been implicated in more sophisticated processes than mere ECM turnover [59]. These include the activation or inactivation of proteases, protease inhibitors, clotting factors, antimicrobial peptides, chemotactic and adhesion molecules, growth factors, hormones, cytokines, as well as the shedding of membrane-anchored forms into circulation. For these reasons, MMPs are stringently regulated at multiple levels, including transcription, activation of the zymogen forms, extracellular inhibitors, location inside or outside the cell and internalization by endocytosis [60].

In conclusion the increased expression of *Ciona* MMP-9*like* during inflammatory response to LPS challenge can be related to several functions of inflammation including bioavailability and the activity of inflammatory cytokines such as TNF-α, TGF-β [22,28,29]. Collectively, our data suggest that in *C. robusta* MMP-9*like* is a potential molecule involved in the modulation of immune response against bacterial infection and a mediator of inflammation in innate immunity.

Acknowledgements

This work was supported by a research grant from the Italian Ministry of Education (PRIN 2006 and 2010–2011 n. 20109XZEPR_007), co-funded by the University of Palermo. We thank M. Guarcello for collecting ascidians and maintaining of the aquarium.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.028.

References

[1] W.C. Parks, C.W. Wilson, Y.S. Lopez-Boado, Matrix metalloproteinases as modulators of inflammation and innate immunity, Nat. Rev. Immunol. 4 (2004) 617–629.
[2] G.S. Butler, C.M. Overall, Matrix metalloproteinase processing of signaling molecules to regulate inflammation, Periodontol 63 (2013) 123–148.
[3] R.E. Vandenbroucke, E. Dejonckheere, F. Van Hauwermeiren, S. Lodens, R. De Rycke, E. Van Wonterghem, A. Staes, K. Gevaert, C. López-Otin, C. Libert, Matrix metalloproteinase 13 modulates intestinal epithelial barrier integrity in inflammatory diseases by activating TNF, EMBO Mol. Med. 5 (2013) 932–948.
[4] P.E. Van Den Steen, A. Wuyts, S.J. Husson, P. Proost, J. Van Damme, G. Opdenakker, Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities, Eur. J. Biochem. 270 (2003) 3739–3749.
[5] G.A. McQuibban, J.H. Gong, J.P. Wong, J.L. Wallace, I. Clark-Lewis, C.M. Overall, Matrix metalloproteinase processing of oocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo, Blood 100 (2002) 1160–1167.
[6] F.X. Gomis-Rüth, Structural aspects of the metzincin clan of metalloendopeptidases, Mol. Biotechnol. 24 (2003) 157–202.
[7] T. Klein, R. Bischoff, Physiology and pathophysiology of matrix metalloproteases, Amino Acids 41 (2011) 271–290.
[8] F.X. Gomis-Ruth, Catalytic domain architecture of metzincin metalloproteases, J. Biol. Chem. 284 (2009) 15353–15357.
[9] C.M. Overall, Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites, Mol. Biotechnol. 22 (2002) 51–86.
[10] A.J. Gearing, P. Beckett, M. Christodoulou, M. Churchill, J. Clements, A.H. Davidson, A.H. Drummond, W.A. Galloway, R. Gilbert, J.L. Gordon, Processing of tumour necrosis factor-alpha precursor by metalloproteinases, Nature 370 (1994) 555–557.
[11] Q. Yu, I. Stamenkovic, Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis, Genes Dev. 14 (2000) 163–176.
[12] M. Egeblad, Z. Werb, New functions for the matrix metalloproteinases in cancer progression, Nat. Rev. Canc. 2 (2002) 161–174.
[13] A.M. Moura-da-Silva, R.D. Theakston, J.M. Crampton, Evolution of disintegrin cysteine-rich and mammalian matrix-degrading metalloproteinases: gene duplication and divergence of a common ancestor rather than convergent evolution, J. Mol. Evol. 43 (1996) 263–269.
[14] R. Brunetti, C. Gissi, R. Pennati, F. Caicci, F. Gasparini, L. Manni, Morphological evidence that the molecularly determined Ciona intestinalis type A and type B are different species: Ciona robusta and Ciona intestinalis, J. Zool. Syst. Evol. Res. 53 (2015) 186–193.
[15] R. Pennati, G.F. Ficetola, R. Brunetti, F. Caicci, F. Gasparini, F. Griggio, A. Sato, T. Stach, S. Kaul-Strehlow, C. Gissi, Morphological differences between larvae of the Ciona intestinalis species complex: hints for a valid taxonomic definition of distinct species, PloS One 10 (2015) e0122879.
[16] F. Delsuc, H. Brinkmann, D. Chourrout, H. Philippe, Tunicates and not cephalochordates are the closest living relatives of vertebrates, Nature 439 (2006) 965.
[17] G. Tsagkogeorga, X. Turon, R.R. Hopcroft, M.-K. Tilak, T. Feldstein, N. Shenkar, Y. Loya, D. Huchon, E.J.P. Douzery, F. Delsuc, An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models, BMC Evol. Biol. 9 (2009) 187.
[18] L. Zeng, B.J. Swalla, Molecular phylogeny of the protochordates: chordate evolution, Can. J. Zool. 83 (2005) 24–33.
[19] S. Iwanaga, B.-L. Lee, Recent advances in the innate immunity of invertebrate animals, BMB Rep. 38 (2005) 128–150.
[20] M.R. Pinto, C.M. Chinnici, Y. Kimura, D. Melillo, R. Marino, L.A. Spruce, R. De Santis, N. Parrinello, J.D. Lambris, CiC3-1a-mediated chemotaxis in the deuterostome invertebrate *Ciona intestinalis* (Urochordata), J. Immunol. 171 (2003) 5521–5528.
[21] I. Zucchetti, R. Marino, M.R. Pinto, J.D. Lambris, L. Du Pasquier, R. De Santis, ciCD94-1, an ascidian multipurpose C-type lectin-like receptor expressed in *Ciona intestinalis* hemocytes and larval neural structures, Differentiation 76 (2008) 267–282.
[22] N. Parrinello, A. Vizzini, V. Arizza, G. Salerno, D. Parrinello, M. Cammarata, F.T. Giaramita, M. Vazzana, Enhanced expression of a cloned and sequenced *Ciona intestinalis* TNFα-like (CiTNFα) gene during the LPS-induced inflammatory response, Cell Tissue Res. 334 (2008), https://doi.org/10.1007/s00441-008-0695-4.
[23] N. Parrinello, A. Vizzini, G. Salerno, M.A. Sanfratello, M. Cammarata, V. Arizza, M. Vazzana, D. Parrinello, Inflamed adult pharynx tissues and swimming larva of *Ciona intestinalis* share CiTNFalpha-producing cells, Cell Tissue Res. 341 (2010) 299–311.
[24] A. Vizzini, D. Parrinello, M.A. Sanfratello, G. Salerno, M. Cammarata, N. Parrinello, Inducible galectins are expressed in the inflamed pharynx of the ascidian *Ciona intestinalis*, Fish Shellfish Immunol. 32 (2012) 101–109.
[25] A. Vizzini, A. Bonura, D. Parrinello, M.A. Sanfratello, V. Longo, P. Colombo, LPS challenge regulates gene expression and tissue localization of a *Ciona intestinalis* gene through an alternative polyadenylation mechanism, PloS One 8 (2013) e63235.
[26] Vizzini, D. Parrinello, M.A. Sanfratello, V. Mangano, N. Parrinello, M. Cammarata, *Ciona intestinalis* peroxinectin is a novel component of the peroxidase–cyclooxygenase gene superfamily upregulated by LPS, Dev. Comp. Immunol. 41 (2013) 59–67.
[27] Vizzini, D. Parrinello, M.A. Sanfratello, M.R. Trapani, V. Mangano, N. Parrinello, M. Cammarata, Upregulated transcription of phenoloxidase genes in the pharynx and endostyle of *Ciona intestinalis* in response to LPS, J. Invertebr. Pathol. 126 (2015) 6–11, https://doi.org/10.1007/s00441-010-0993-5 share CiTNFα-producing cells, Cell Tissue Res. (2010) 1–13.
[28] Vizzini, F. Di Falco, D. Parrinello, M.A. Sanfratello, C. Mazzarella, N. Parrinello, M. Cammarata, *Ciona intestinalis* interleukin 17-like genes expression is upregulated by LPS challenge, Dev. Comp. Immunol. 48 (2015) 129–137.
[29] Vizzini, F. Di Falco, D. Parrinello, M.A. Sanfratello, M. Cammarata, Transforming growth factor b (CiTGF-b) gene expression is induced in the inflammatory reaction of *Ciona intestinalis*, Dev. Comp. Immunol. 55 (2016) 102–110.
[30] Vizzini, A. Bonura, V. Longo, M.A. Sanfratello, D. Parrinello, M. Cammarata, P. Colombo, LPS injection reprograms the expression and the 3′ UTR of a CAP gene by alternative polyadenylation and the formation of a GAIT element in *Ciona intestinalis*, Mol. Immunol. 77 (2016) 174–183.
[31] A. Vizzini, M.G. Parisi, L. Cardinale, L. Testasecca, M. Cammarata, Evolution of *Ciona intestinalis* Tumor necrosis factor alpha (CiTNFα): polymorphism, tissues expression, and 3D modeling, Dev. Comp. Immunol. 67 (2017) 107–116.
[32] S. Giacomelli, D. Melillo, J.D. Lambris, M.R. Pinto, Immune competence of the *Ciona intestinalis* pharynx: complement system-mediated activity, Fish Shellfish Immunol. 33 (2012) 946–952.
[33] A. Vizzini, M. Pergolizzi, M. Vazzana, G. Salerno, C. Di Sano, P. Macaluso, et al., FACIT collagen (1alpha-chain) is expressed by hemocytes and epidermis during the inflammatory response of the ascidian *Ciona intestinalis*, Dev. Comp. Immunol. 32 (2008) 682–692.
[34] G. Di Cara, G. Marengo, N.N. Albanese, M.R. Marabeti, R. Musso, P. Cancemi, I. Pucci-Minafra, Proteomic profiling of Trastuzumab (Herceptin(R))-sensitive and -resistant SKBR-3 breast cancer cells, Anticancer Res. 33 (2013) 489–503.
[35] R. Musso, G. Di Cara, N.N. Albanese, M.R. Marabeti, P. Cancemi, D. Martini, E. Orsini, C. Giordano, I. Pucci-Minafra, Differential proteomic and phenotypic behavior of papillary and anaplastic thyroid cell lines, J. Proteomics 90 (2013) 115–125.
[36] P. Cancemi, M. Buttacavoli, F. D'Anna, S. Feo, R.M. Fontana, R. Noto, A. Sutera, P. Vitale, G. Gallo, The effects of structural changes on the anti-microbial and anti-proliferative activities of diimidazolium salts, New J. Chem. 41 (2017) 3574–3585.
[37] M. Buttacavoli, N.N. Albanese, G. Di Cara, R. Alduina, C. Faleri, M. Gallo, G. Pizzolanti, G. Gallo, S. Feo, F. Baldi, P. Cancemi, Anticancer activity of biogenerated silver nanoparticles: an integrated proteomic investigation, Oncotarget 9

(2017) 9685–9705.
[38] P. Cancemi, N.N. Albanese, G. Di Cara, M.R. Marabeti, F. Costantini, S. Minafra, I. Pucci-Minafra, Multiple changes induced by fibroblasts on breast cancer cells, Connect. Tissue Res. 51 (2010) 88–104.
[39] P. Cancemi, G. Di Cara, N.N. Albanese, F. Costantini, M.R. Marabeti, R. Musso, I. Riili, C. Lupo, E. Roz, I. Pucci-Minafra, Differential occurrence of S100A7 in breast cancer tissues: a proteomic-based investigation, Proteonomics Clin. Appl. 6 (2012) 364–373.
[40] G. La Rocca, I. Pucci-Minafra, A. Marrazzo, P. Taormina, S. Minafra, Zymographic detection and clinical correlations of MMP-2 and MMP-9 in breast cancer sera, Br. J. Canc. 5 (2004) 1414–1421.
[41] I. Pucci-Minafra, S. Minafra, G. La Rocca, M. Barranca, S. Fontana, G. Alaimo, Y. Okada, Zymographic analysis of circulating and tissue forms of colon carcinoma gelatinase A (MMP-2) and B (MMP-9) separated by mono- and two-dimensional electrophoresis, Matrix Biol. 20 (2001) 419–427.
[42] M. Fanjul-Fernández, A.R. Folgueras, S. Cabrera, C. López-Otín, Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models, Biochim. Biophys. Acta 1803 (2010) 3–19.
[43] A. Page-McCaw, A.J. Ewald, Z. Werb, Matrix metalloproteinases and the regulation of tissue remodeling, Nat. Rev. Mol. Cell Biol. 8 (2007) 221–233.
[44] R.G. Rowe, S.J. Weiss, Breaching the basement membrane: who, when and how? Trends Cell Biol. 18 (2008) 560–574.
[45] H.H. Ho, T. Antoniv, J.-D. Ji, L.B. Ivashkiv, LPS-induced expression of MMPs in human monocytes is suppressed by IFN-γ via superinduction of ATF-3 and suppression of AP-1 proteins, J. Immunol. 181 (2008) 5089–5097.
[46] J.S. Cho, J.H. Kang, J.Y. Um, I.H. Han, I.H. Park, H.M. Lee, Lipopolysaccharide induces pro-inflammatory cytokines and MMP production via TLR4 in nasal polyp-derived fibroblast and organ culture, PloS One 12 (2014) e90683.
[47] J. Vandooren, P.E. Van den Steen, G. Opdenakker, Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): the next decade, Crit. Rev. Biochem. Mol. Biol. 48 (2013) 222–272.
[48] Y. Liu, Y. Zhao, C. Lu, M. Fu, T. Dou, X. Tan, Signatures of positive selection at hemopexin (PEX) domain of matrix metalloproteinase-9 (MMP-9) gene, J. Biosci. 40 (2015) 885–890.
[49] A. Vizzini, Gene expression and regulation of molecules involved in pharynx inflammatory response induced by LPS in Ciona intestinalis, ISJ 14 (2017) 119–128.
[50] X. Wang, Y.Y. Yu, S. Lieu, F. Yang, J. Lang, C. Lu, Z. Werb, D. Hu, T. Miclau, R. Marcucio, C. Colnot, MMP9 regulates the cellular response to inflammation after skeletal injury, Bone 52 (2013) 111–119.
[51] B.A. Moore, C.L. Manthey, D.L. Johnson, A.J. Bauer, Matrix metalloproteinase-9 inhibition reduces inflammation and improves motility in murine models of post-operative ileus, Gastroenterology 141 (2011) 1283–1292.
[52] D. Singh, S.K. Srivastava, T.K. Chaudhuri, G. Upadhyay, Multifaceted role of matrix metalloproteinases (MMPs), Front Mol. Biosci. 13 (2) (2015) 19.
[53] H. Haro, H.C. Crawford, B. Fingleton, K. Shinomiya, D.M. Spengler, L.M. Matrisian, Matrix metalloproteinase-7-dependent release of tumor necrosis factor-α in a model of herniated disc resorption, J. Clin. Invest. 105 (2000) 143–150.
[54] L.J. McCawley, L.M. Matrisian, Matrix metalloproteinases: they're not just for matrix anymore!, Curr. Opin. Cell Biol. 13 (2001) 534–540.
[55] M.M. Shull, et al., Targeted disruption of the mouse transforming growth factor-β 1 gene results in multifocal inflammatory disease, Nature 359 (1992) 693–699.
[56] A.B. Kulkarni, S. Karlsson, Transforming growth factor-β 1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease, Am. J. Pathol. 143 (1993) 3–9.
[57] J.S. Munger, et al., The integrin αvβ6 binds and activates latent TGFβ1: a mechanism for regulating pulmonary inflammation and fibrosis, Cell 96 (1999) 319–328.
[58] Q. Yu, I. Stamenkovic, Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis, Genes Dev. 14 (2000) 163–176.
[59] M.D. Sternlicht, Z. Werb, How matrix metalloproteinases regulate cell behavior, Annu. Rev. Cell Dev. Biol. 17 (2001) 463–516.
[60] T. Dalmay, D.R. Edwards, MicroRNAs and the hallmarks of cancer, Oncogene 25 (2006) 6170–6175.