Short communication

Molecular characterisation, evolution and expression analysis of g-type lysozymes in *Ciona intestinalis*

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A R T I C L E   I N F O

A B S T R A C T

Lysozyme is an important defense molecule of the innate immune system. Known for its bactericidal properties, lysozyme catalyzes the hydrolysis of b-(1,4)-glycosidic bonds between the N-acetyl glucosamine and N-acetyl muramic acid in the peptidoglycan layer of bacterial cell walls. In this study, the complete coding sequence of four g-type lysozymes were identified in *Ciona intestinalis*. Phylogenetic analysis and modelling supported the hypothesis of a close relationship with the vertebrate g-type lysozymes suggesting that the *C. intestinalis* g-type lysozyme genes (*Ci*Lys-g1, *Ci*lys-g2, *Ci*Lys-g3, *Ci*Lys-g4) share a common ancestor in the chordate lineage. Protein motif searches indicated that *C. intestinalis* g-type lysozymes contain a GEWL domain with a GXXQ signature, typical of goose lysozymes. Quantitative Real-Time PCR analysis results showed that transcripts are expressed in various tissues from *C. intestinalis*. In order to determine the involvement of *C. intestinalis* g-type lysozymes in immunity, their expression was analyzed in the pharynx, showing that transcripts were significantly up-regulated in response to a challenge with lipopolysaccharide (LPS). These data support the view that *Ci*Lys g-type are molecules with potential for immune defense system against bacterial infection.

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1. Introduction

Lysozyme is a ubiquitous bacteriolytic enzyme produced by diverse groups of organisms, ranging from bacteria and bacteriophages to fungi, plants and animals (Jollès and Jollès, 1984). It catalyzes the hydrolysis of 1, 4-beta-linkages between N-acetyl-*d*-glucosamine (NAG) and N-acetylmuramic acid (NAM) in peptidoglycan heteropolymers of prokaryotic cell walls, leading to the breakdown of bacterial cells (Smirnow and Wislowska, 2001; Nilsen et al., 1999). As a result, lysozyme acts directly on Gram-positive bacteria, causing the lysis of their outermost peptidoglycan layer. Gram-negative bacteria, however, are not directly damaged by lysozyme as their outer membrane is significantly coated with lipopolysaccharide (LPS) moieties. Instead, the outer membranes of Gram-negative bacteria must first be disrupted by cationic antimicrobial peptides that expose the inner peptidoglycan layer of bacteria to lysozyme (Banks et al., 1986; Hancock and Scott, 2000; Ibrahim et al., 2002).

Based on differences in structural, catalytic and immunological characteristics, lysozymes are generally classified into six main types: chicken (c-type) (Hultmark, 1996), goose (g-type) (Prager and Jollès, 1996), invertebrate (i-type) (Jollès and Jollès, 1975), T4 phage (phage-type) (Fastrez, 1996), bacterial (Holtje, 1996), and plant (Beintema and Terwissscha van Scheltinga, 1996). The g-type lysozyme was initially identified as an antibacterial enzyme in egg whites from the Embden goose (Canfield and McMurry, 1967), but it was later found to exist in the egg whites of several other bird species (Prager et al., 1974). More recently, the g-type lysozyme has been identified in vertebrate species, including mammals (Nakano and Graf, 1991), fish (Mohanty and Sahoo, 2010), urochordates (Nilsen et al., 2003) and molluscs (Zhao et al., 2007; Zhang et al., 2012).

Lysozyme is a well-known antibacterial protein which is active against Gram-positive bacteria such as *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus stearothermophilus* and *Clostridium tyrobutyricum* (Proctor and Cunningham, 1988). It is also known to act as an opsonin and as an activator of the complement system and circulating phagocytes (Jollès and Jollès, 1984; Grinde, 1989). In addition to these antibacterial functions, some lysozyme family members have been demonstrated to have antiviral (Ferrari et al., 1959; Lee-Huang et al., 1999), anti-inflammatory activities (Jollès and Jollès, 1984; Samarayanake et al., 1997; Ogundele, 1998;

Zhang et al., 2008), to be involved in immune modulatory (Valisena et al., 1996; Rymuszka et al., 2005) and antitumor activities (Sava et al., 1989), thus, it is possible that lysozymes function as multipurpose defense factors.

Ascidians (subphylum: Tunicata) occupy a key phylogenetic position in chordate evolution and are considered the sister group of vertebrates (Zeng and Swalla, 2005; Delsuc et al., 2006; Tsagkogeorga et al., 2009). They are proto-chordates which possess an innate immune system, including inflammatory humoral and cellular responses. An inflammatory response induced by LPS injection in the body wall of *C. intestinalis* is a well-established model for the analysis of regulator and effector inducible host defense molecules of the innate immune system (Bonura et al., 2009; Parrinello et al., 2008, 2010; Vizzini et al., 2012, 2013; 2015a, 2015b; 2016). In the present paper, we report on the identification, characterization and expression of *C. intestinalis* g-type lysozymes. Phylogenetic analysis was conducted to determine their evolutionary relationships. Real-Time PCR analysis revealed that *Ci*Lys-g(1-4) are expressed in several type of tissue, and their transcription is up-regulated by LPS inoculation.

2. Materials and methods

2.1. Tunicates and LPS inoculation

Ascidians were collected from Sciacca Harbour (Sicily, Italy), maintained in tanks with aerated sea water at 15 °C, and fed every second day with a marine invertebrate diet of coraliquid (Sera Heinsberg, Germany). LPS (Escherichia coli 055:B5, LPS, Sigma-Aldrich, Germany) solution was prepared in sterile sea water (12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 43 mM Tris HCl, 0.4 M NaCl, pH 8.0). LPS solution (100 µg LPS in 100 µl sea water per animal) was inoculated into the tunic matrix close to the pharynx wall at the median body region. Ascidians, both untreated (naive ascidians) and injected with MS (sham ascidians), were used as controls.

2.2. Total RNA extraction

Ascidian tissue fragments (200 mg) explanted at various times (from 1 to 72 h) were immediately soaked in RNAlater Tissue collection solution (Ambion, Austin, TX), and stored at −80 °C. Total RNA extraction was performed by using an RNAqueous™-Midi Kit purification system (Ambion, Austin, TX).

2.3. Cloning and sequence analysis

A search conducted in the Ensembl genome browser identified the sequence: *Ci*Lys-g1 (ENSCING00000007365), *Ci*Lys-g2 (ENSCING00000024417), *Ci*Lys-g3 (ENSCING00000018223), *Ci*Lys-g4 (ENSCING00000007365). The sequence of the cDNA was obtained by using the GeneRacer™ kit (Invitrogen, USA). 5′-and 3′ RACE was conducted using the primers listed in Table 1. The overlapping RACE products were cloned into the pCR™IIvector (TA Cloning Kit, Invitrogen) and sequenced. They contained the complete coding regions.

2.4. Bioinformatic analysis

The full length *Ci*Lys-g(1-4) cDNA were analyzed using the ExPASy translation tool (http://web.expasy.org/translate/) to obtain their ORF region, leader and trailer sequences (UTR), and the nucleotide sequence was translated into a protein sequence. A BLAST (http://blast.ncbi.nlm.nih.gov/Blast) search was conducted to identify the known protein sequences that are homologous to

Table 1
Primers used for cloning and expression.

Gene	Primer sequence (5′-3′)	Application
*Ci*Lys-g1	5′-TAACCCATACCCATGCCCGT-3′	RACE5′
	5′-GCTTGCCCAATCTCGTCTTT-3′	NESTED5′
	5′-TGACGGGGATTCGAACAGGA-3′	RACE3′
	5′-ATCTGGAAAGTGTGGAGGGC-3′	NESTED3′
*Ci*Lys-g2	5′-CTCCCAAACCCTTGCTGTTC-3′	RACE5′
	5′-AATTAGATCGGGCGGCCTTA-3′	NESTED5′
	5′-GCAATGGCGACTCGTTTCAA-3′	RACE3′
	5′-GCTGAACGAGAATGGCTACG-3′	NESTED3′
*Ci*Lys-g3	5′-TGCGACGACTCTAACACTTG-3′	RACE5′
	5′-CGTACATTGCTGACCCCAAA-3′	NESTED5′
	5′-AAAAGATCTGCGTGCGATGG-3′	RACE3′
	5′-AGGTTGATAAGCGGCACCAT-3′	NESTED3′
*Ci*Lys-g4	5′-TCCTGCCACCATTTTACCAC-3′	RACE5′
	5′-AGCCACTACATCGCTAGAGT-3′	NESTED5′
	5′-TTGCTTTTCCTGTTCGTCGG-3′	RACE3′
	5′-GACGATCGTTACCACACCAT-3′	NESTED3′
*Ci*Lys-g1	5′-AACTTTGTATGGACGCTGCTG-3′	Real-time PCR
	5′-GCCCCTGCACGACTTTCA-3′	Real-time PCR
*Ci*Lys-g2	5′-CACGGTGCCGACACAAAGT-3′	Real-time PCR
	5′-GCGCCTTGTAAAATGTGATCTC-3′	Real-time PCR
*Ci*Lys-g3	5′-GCAAGCCGCGAAAGCA-3′	Real-time PCR
	5′-TCACCAAGCCCGTCTTTGTC-3′	Real-time PCR
*Ci*Lys-g4	5′-CGGCCGTAGCCATCGCTTA-3′	Real-time PCR
	5′-CGGTGGTGTCAGTGTTGTAGAT-3′	Real-time PCR
Actin	5′-TGATGTTGCCGCACTCGTA-3′	Real-time PCR
	5′-TCGACAATGGATCCGGT-3′	Real-time PCR

*Ci*Lys-g(1-4). The Exon-Intron Graphic Maker (http://wormweb.org/exonintron) was used to create an image of the genomic organization of *Ci*Lys-g genes and for comparison. 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 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/). The subcellular location of lysozyme proteins was predicted using the MultiLoc tool (http://abi.inf.uni-tuebingen.de/Services/MultiLoc/). Multiple sequence alignment was conducted using CLC (Version 7.0.0). A secondary structure was evaluated using Polyview (http://polyview.cchmc.org). 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) to obtain the best structure among those predicted. Finally, a phylogenetic tree using Neighbor-joining method was constructed using MEGA 6.0 after 1000 bootstrap iterations.

2.5. Real-Time PCR analysis

Tissue Differential expression of the *Ci*Lys-g(1-4) 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. Tissue 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 PCRMasterMix (Applied Biosystems).

The 50 cycles of the two-step PCR program consisted of 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.

The 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 *Ci*Lys-g(1-4) transcripts from different tissues was normalised to actin in order to compensate for variations in input RNA amounts. Relative *Ci*Lys-g(1-4) expression was determined by dividing the normalised value of the target gene in each tissue by the normalized value obtained from the untreated tissue.

2.6. Statistical methods

Multiple comparisons were performed using 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 g-type lysozymes in C. intestinalis

A search in the Ensembl genome browser identified only g-type lysozymes: *Ci*Lys-g1 (ENSCING00000007365), *Ci*Lys-g2 (ENSC-ING00000024417), *Ci*Lys-g3 (ENSCING00000018223), *Ci*Lys-g4 (ENSCING00000007365).

The full-length mRNA of g-type lysozymes was isolated using a 5′ and 3′ RACE strategy. The cDNA and amino acid sequence analysis showed that: *Ci*Lys-g1(GenBank Accession No. KX 761980) presents a 5′-UTR of 80 bp, an ORF of 906 bp and a 3′-UTR of 84 bp, encoding 301 amino acids with a predicted molecular size of 39.3 kDa and a pI of 6.93 (Fig. 1); *Ci*Lys-g2 (KX761981) presents a 5′-UTR of 41 bp, an ORF of 564 bp, and a 3′-UTR of 98, encoding 186 amino acids with a predicted molecular size of 26.4 kDa and a pI of 7.8 (Fig. 1); *Ci*Lys-g3 (KX761982) presents a 5′-UTR of 59 bp, an ORF of 456 bp and a 3′-UTR of 197 bp, encoding 151 amino acids with a predicted molecular size of 27.7 kDa and a pI of 9.79 (Fig. 1); *Ci*Lys-g4 (KX761983) presents a 5′-UTR of 58 bp, an ORF of 609 bp and a 3′-UTR of 91 bp, encoding 202 amino acids, predicted molecular size of 28.5 kDa and a pI of 7.76 (Fig. 1). In silico analysis, using Signal P 4.1 and the DELTA BLAST program to evaluate the presence of conserved domains, showed that: *Ci*Lys-g1 presents a predicted signal peptide of 16 amino acids and a Lytic transglycosylase-like (SLT) domain (51–162); *Ci*Lys-g2 presents a signal peptide of 20 amino acids and a Lysozyme-like domain (2–174); *Ci*Lys-g3 does not present a signal peptide and shows a Lytic transglycosylase-like (SLT) domain (19–134);*Ci*Lys-g4 presents a signal peptide of 16 amino acids and a Lytic transglycosylase-like 2 (SLT2) domain (26–101) (Fig. 1). The MultiLoc tool was used to predict the subcellular location of lysozyme proteins: *Ci*Lys-g1, *Ci*Lys-g3, *Ci*Lys-g4 showed extracellular lysozymes, while *Ci*Lys-g2 showed an intracellular lysozyme. The deduced amino acid sequences of *C. intestinalis* g-type lysozymes, examined in GeneBank through BLAST analysis, showed significant homologies with components of the g-type lysozymes family: *Ci*Lys-g1 has an identity of 56% and a positivity of 67% with the Lysozyme g-like 1 of *Danio rerio* (NP 001002706.1); an identity of 39% and a positivity of 57% with the Lysozyme g 2 precursor of *Homo sapiens* (NP_783862.2); *Ci*Lys-g2 has an identity of 31% and a positivity of 54% with the Lysozyme g 1 of *D. rerio* (NP 001002706.1); an identity of 26% and a positivity of 51% with the Lysozyme g 2 precursor of *H. sapiens* (NP_783862.2); *Ci*Lys-g3 has an identity of 51% and a positivity of 68% with the

Lysozyme g 1 of *D. rerio* (NP 001002706.1); an identity of 37% and a positivity of 60% with the Lysozyme g 2 precursor of *H. sapiens* (NP_783862.2); *Ci*Lys-g4 has an identity of 55% and a positivity of 68% with the Lysozyme g 1 of *D. rerio* (NP 001002706.1); an identity of 35% and a positivity of 53% with the Lysozyme g 2 precursor of *H. sapiens* (NP_783862.2).

The genomic organization of the *Ci*Lys-g genes was determined and compared with those of human and *D. rerio* lysozyme type-g genes (Supplementary data, Fig. S1). The *Ci*Lys-g1 and *Ci*Lys-g4 genes contained five exons and four introns like the *D. rerio* type g lysozyme gene, but unlike the *Ci*Lys-g2 and *Ci*Lys-g3 genes that contained 3 exons and 2 introns, whereas the human g-type lysozyme 1 gene contained eight exons and seven introns, and lysozyme g-type 2 contained six exons and five introns, with an increase in the number of exons in the human lysozyme genes compared to the lysozyme genes *of C. intestinalis*.

3.2. Alignment and structural analysis

BLAST analysis with DELTA-BLAST program showed that the *Ci*Lys-g proteins have a GEWL domain, which belongs to a lysozyme-like superfamily, and a GXXQ motif, which is a signature of goose family lysozyme. In Fig. S2, by using the CLC workbench 6.4 alignments, amino acids potentially important for lysozyme catalytic activity (Glu, Asp, Asp) and for binding to ligands (Gly) were found conserved, and a Lytic transglycosylase-like (SLT) domain was also identified.

Secondary structure analysis using the Polyview online tool showed an α helix region but no β-sheet region in the *Ci*Lys-g proteins. Five distinct α-helices were found in the secondary structure, which is a conserved feature among the g-type lysozymes. Tertiary structure analysis in PyMol revealed that the 3D structures of *Ci*Lys-g proteins, which was predicted using the I-Tasser online server, also showed five helix regions (Fig. 1). The active residues of the NAG binding site were distributed in both the coil and helix regions. The conserved GXXQ motif and the conserved catalytic residues (Glu) and (Asp) were found to be located nearer to the hydrophobic core region.

Fig. 1 A–B shows the *Ci*Lys-g1 molecular model resulting from the super-imposition of the 117–301 residue sequence, which corresponds to the mature peptide and shares 55.43% identity with the template. The homology modelling process was performed on the basis of the known crystal structure of Australian black swan egg white lysozyme (1gbs.1.A). The amino acids involved in binding to the substrate (Gly^{118}, Tyr^{263}, Gly^{266}) and the catalytic site (Glu^{188}, Asp^{213}) were found to be conserved.

Fig. 1 C–D shows the *Ci*Lys-g2 molecular model resulting from the super-imposition of the 17–173 residue sequence, which corresponds to the mature peptide and shares 32.9% identity with the template. The homology modelling process was performed on the basis of the known crystal structure of *Anser anser* goose lysozyme (153l.1.A). The amino acids involved in binding to the substrate (Tyr^{135}, Gly^{138}) and the catalytic site (Arg^{60},Gln^{82},Gln^{103}) were found to be conserved.

Fig. 1 E–F shows the *Ci*Lys-g3 molecular model resulting from the super-imposition of the 4–151 residue sequence, which corresponds to the mature peptide and shares 51.70% identity with the template. The homology modelling process was performed on the basis of the known crystal structure of *Struthio camelus* lysozyme g (3mgw.1.A). The amino acids involved in binding to the substrate (Tyr^{113}, Gly^{116}) and the catalytic site (Glu^{38}, Asp^{48}, Asp^{63}) were found to be conserved.

Fig. 1 G–H show the *Ci*Lys-g4 molecular model resulting from the super-imposition of the 18–202 residue sequence, which corresponds to the mature peptide and shares 54.35% identity with the

*Ci*Lys-g1

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1    AGAAAAATGTGTTTATATATATCGAACGAAACAAACCGCACTTCGGTAAAGAAGATTCAC   60
61   TAGAGGAACTAACTGTTCAAGATGAAGCAATTGTTGGTTATCTGCCTTTTGATTGAAATA   120
                MKQLLVICLLIET
121  TGTGTAGCAAGCGACTTAGCATGCACACGCAATGGTGGGACTTGTTTAGATTATCGTTAC   180
        CVASDLACTRNGGTCLDYRY
181  TACTTATGCACTGCTGGTTATGAACAAGGATTATGTGACGGGGATTCGAACAGGAAGTGC   240
        YLCTAGYEQGLCDGDSNRKC
241  TGGCAGGAATGGCGATAATACTTGCATCTCAAACGAAATAGTTATGCTTCATGCTGTGAC   300
        CQECDNTCISNENSYASCCD
301  TCACAATGCACTCAAAGTGGTGGGAAATGTCAAGATAATTCAAACTACTGTAGTGGTTCT   360
        SQCTQSGGKCQDNSNYCSGS
361  TATTCATCTGGAAAGTGTGGAGGGCCTTCATCAAGGCAGTGTTGCAGTGGGGCAAGTTCA   420
        YSSGKCGGPSSRQCCSGASS
421  GGTGGGGCCTTCGGTTGCTACAACATCTACAATACCGACACCACTGGTGCTTCATGC     480
        GGAFGCYGNIYNTDTTGASC
481  ACCACGTGCAGTCAAGATAACTTAGGATATTGCGGCATATCAGCGTCTCGTCAATTGGCC   540
        TTSSQDNLGYCGISASRQLA
541  GCTACAGATCTTAACAGGATGTCCCAATACAAAGACGAGATTGAACAAGCCGGTTACCAA   600
        ATDLNRMSQYKDETEQAGYQ
601  CTTTGTATGGACGCTGCTGTTATTGCAGGTATCATCAGTCGTGAAAGTCGTGCAGGGGCA   660
        LCMDAAVIAGIISRESRAGA
661  GCTCTTAATTCTAATGGCTACGGTAGTGACGGGCATGGGTATGGGTTAATGCAGGTCGAC   720
        ALNSNGYGSDGHGYGLMQVD
721  GATCGTTACCACACCATACAAGGCGACCCTACATCTCTTGACCATATATTACAAGGCACT   780
        DRYHTIQGDPTSLDHILQGT
781  GGCATCCTTATATCTAATATTGGACAAAATATCTTCGAAATTTCCACCTTGGGACCAAAAC   840
        GILISNIGQISKEFPWDQN
841  ATGGACTTAAAAGGGGCATATGCGTTATAATAGGTGGTTGGGGGCGTGTGGTCTTAT   900
        MDLKGGICAYNIGVGGVWSY
901  GATAACATGGACGTCGGTACAACTGGGGATGACTACTCTAGCGATGTAGTGGCTAGAGCC   960
        DNMDVGTTGDDYSSDVVARA
961  CAGTATTATAAAGACAACGGGTATTAGGGAAATGATTATAAGCTATGATATGTAATAGGTGG   1020
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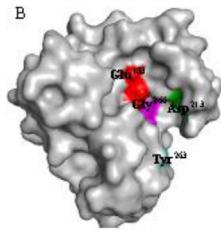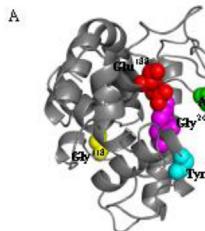
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        MAYEKDLRAMETYKEGIIQA
121  GCAAGAAATAACAATCTAGAACCTTCTCTGATTGCAGGTATTGCAAGCCGCGAAAGCAAT   180
        ARNNNLEPSLIAGIASRESN
181  GGCGGTAGAGCATTGGACAAAGACGGGCTTGGTGACCATGGAAACGGTTTCGGTATAATG   240
        GGRALDKDGLGDHGNGFGIM
241  CAGGTTGATAAGCGGCACCATCACCGATTGGCGGACCTTACTCCAACGAGCATATAAAC   300
        QVDKRHHTIPIGGPYSNEHIN
301  CAGGGTGCTACTATCCTGCGAAACTCAATCAATGCAGTGGAGAAAAAGTTTCCTACCTGG   360
        QGATILRNSINAVEKKFPTW
361  TCTCCTGACTTGCAACTGCGCGGAGGGGTGGCGGCGTACAACTTTGGGGTCAGCAATGTA   420
        SPDLQLRGGVAAYNFGVSNV
421  CGAACCCCTGATGGGATCGATAGAGGAACAACAGGCAATAACTATTCAGCTGATGTTATA   480
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481  AAACGAGCGGAGTATTTCAAACAGAACGGTTACTAAATTGATGAAAAATCGCCCATATAA   540
        KRAEYFKQNGY*
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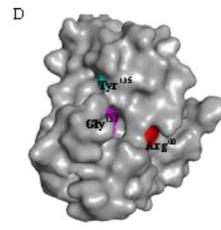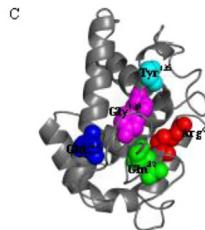
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121  AAAGACGATGTGGACGAGCTGTTAAAGTTAAGATCCAGTATTTGCTTACGCAGCTGAAAAA   180
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181  GCAGGTGTGCCGTCAGCGTTGCTTAGCTGGTATCATCAGTCGTGTAAGCGGGGCCGGTAGA   240
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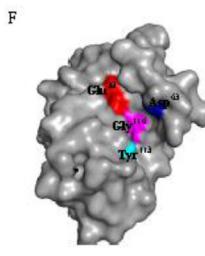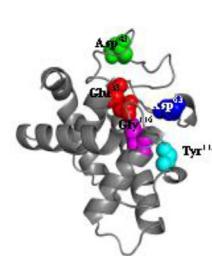
*Ci*Lys-g4

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        MLKVFLLFLFVATAYTGCYG
121  TATGGCAACATCTACAACACTGACACCACCGGTTGTTCCAGTCAAACATCAAGTCAAGAT   180
        NIYNTDTTGCSSQDNLGYSG
181  AACTTGGGTTACAGCGGAATCTCCGCTTCGAGGCAAATGGCCGCAACAGATCTGTCCAGA   240
        ISASRQMAATDLSRMNQYKS
241  ATGAACCAATACAAAAGTAACATTGAGAATGCTGGACGTCAACTGTGTATGGATCCTTCT   300
        NIENAGRQLCMDPSVIAGII
301  GTCATTGCAGGCATCATTAGCAGGGAAACTCGTGCGGGGGCTACCCTTGGTTCAGACGGT   360
        SRETRAGATLGSDGYGADGH
361  TACGGCGCAGATGGACACGGTTACGGTTTGATGCAGGTCGACGATCGTTACCACACCATA   420
        GYGLMQVDDRYHTIQGGPTS
421  CAAGGTGGCCCTACATCTCTTGACCATATATTACAAGGCACTGGCATCCTTATATCAAAT   480
        LDHILQGTGILISNIGQISS
481  ATTGGACAAATATCTTCGAAATTTCCATCTTGGGACCAAAACATGGACTTAAAAGGCGGC   540
        KFPSWDQNMDLKGGICAYNI
541  ATATCGCGTTATAATAAGGTGTTGGGGGGCGTGTGGTCTTTATGATAGCATGGACGTCGGT   600
        GVGGVWSYDSMDVGTTGDDY
601  ACAACTGGGGATGACTACTCTAGCGATGTAGTGGCTAGAGCCCAGTATTATAAAGACAAC   660
        SSDVVARAQYYKDNGYYKDN
661  GGGTATTAGGGAAATGGGATTATAAGCTATGATGTAATAGGTGGTAAAATGGTGGCAGGA   720
        GT*
721  TATTGGCGATTCTGGTAATAAAATACAACCTATATAATTTAAAAAAAAAAAAAAAAAAAA   780
781  AAA
```
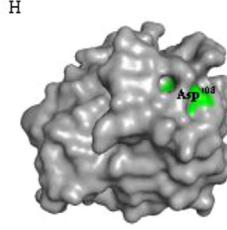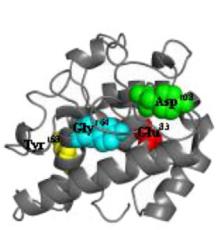
Fig. 1. *Ci*Lys-g1, *Ci*Lys-g2, *Ci*Lysg-3, *Ci*Lys-g4 nucleotide and amino acid sequence. Mid-grey shading indicates signal peptide, light grey shading indicates Lytic transglycosylase-like (SLT) domain; goose family lysozyme signature residues are indicated in bold and underlined. Three dimensional structure: A. *Ci*Lys-g1; C. *Ci*Lys-g2; E. *Ci*Lys-g3; G. *Ci*Lys-g4. Surface view: B. *Ci*Lys-g1; D. *Ci*Lys-g2; F. *Ci*Lys-g3; H. *Ci*Lys-g4. The active residues are highlighted as coloured spheres, with numbers representing the location.

template. The homology modelling process was performed on the basis of the known crystal structure of *Struthio camelus* lysozyme g (3mgw.1.A). The amino acids involved in binding to the substrate (Tyr[158], Gly[164]) and the catalytic site (Glu[83], Asp[108]) were found to be conserved.

3.3. Phylogenetic analysis

By using the MEGA 6 program, *Ci*Lys-g proteins were aligned with vertebrate and invertebrate members of lysozyme superfamily proteins and a phylogenetic tree was constructed using the Neighbor-joining method. The tree shows three main clusters (Fig. S3). The first cluster includes, vertebrate and invertebrate g-type lysozymes as well as *C. intestinalis* g-type lysozymes; the second cluster includes invertebrate i-type lysozymes; the third cluster includes vertebrate and invertebrate c-type lysozymes. This analysis suggests that g-type lysozymes share a common ancestor and that the i-type and c-type may be closely interrelated (Fig. S3).

3.4. Differential gene expression of g-type lysozymes in different tissues

The spatial expression pattern of g-type lysozyme mRNA in adult *C. intestinalis* was investigate by quantitative Real-Time PCR analysis of total RNA samples from different tissue (pharynx, ovary, stomach, intestine). Expression was detected in all tissues. As shown in Fig. 2 *Ci*Lys-g1 expression was higher in stomach and intestine, and lower in ovary and pharynx tissue; *Ci*Lys-g2 expression was higher in pharynx and intestine, and lower in ovary and stomach tissue; *Ci*Lys-g3 expression was higher in intestine, and lower in ovary, stomach and pharynx tissue; *Ci*Lys-g4 expression was higher in stomach and lower in intestine, ovary and pharynx tissue.

3.5. G-type lysozyme genes expression is upregulated by LPS

To study the involvement of the g-type lysozymes of *C. intestinalis* in inflammatory response induced after LPS challenge, the expression pattern of *Ci*Lys-g(1-4) mRNAs in naive, sham and LPS-challenged ascidians was examined by Real-Time PCR analysis.

RealTime PCR analysis of the inflamed ascidian pharynx showed enhanced g-type lysozymes mRNA levels as an effect of the LPS challenge (Fig. 2). To examine the temporal course of the response, four ascidians in three distinct experiments were examined at increasing post-inoculation time points (1, 2, 4, 8, 12, 24, 48, 72 h). At each time point, four sham ascidians were the controls (Fig. 2). *Ci*Lys-g1 and *Ci*Lys-g2 gene expression was significantly boosted at 24–72 h, *Ci*Lys-g3 gene expression was significantly boosted at 24 h and 72 h and *Ci*Lys-g4 gene expression was significantly boosted at 1 h and 2–4 h, and increased at 8–48 h. The response by sham ascidians indicates that the inoculation procedure did not significantly modulate mRNA expression (Fig. 2).

4. Discussion

The immune system is an important physiological mechanism that protects the organism against invading pathogens. Lysozyme has been characterised as an important protein of the innate immune response, and has been evolutionarily conserved throughout invertebrates and vertebrates. The archetypal lysozyme, which has served as a model for studies on enzyme structure and function is the c-type. Analysis of available genome sequences of *C. intestinalis* reveals only g-type homologue lysozyme genes. The goose egg lysozyme (Simpson and Morgan, 1983) was found to be a secreted protein with a signal peptide. G-type lysozyme genes in birds, with

the exception of the chicken lysozyme g2, and mammal contain predicted signal sequences for protein secretion (Nile et al., 2004). In contrast most fish g-type lysozymes do not have the signal peptide at their N-terminal sequence, suggesting that they are not secreted from cells (Irwin and Gong, 2003; Kyomuhendo et al., 2007).

In this study, we identified and characterised the mRNA of the four *C. intestinalis* *Ci*Lys-g proteinsthat encoded for tree extracellular lysozymes (*Ci*Lys-g1, *Ci*Lys-g3, *Ci*Lys-g4), and one intracellular lysozyme (*Ci*Lys-g2). G-type lysozymes are basic proteins and, in general, present a high isoelectric point (pI). We observed that the g-type lysozymes of *C. intestinalis* cover a wide range of pI values (from 6.93 to 9.79), suggesting a specialisation for specific tissue or for their intracellular or extracellular location.

Analysis of the alignment and structure of the *Ci*Lys-g proteins revealed that the mature proteins have a GEWL domain which is specific for the lysozyme g-type and a GXXQ signature which is specific for the Goose Lysozyme. In particular, in *Ci*Lys-g3 all three catalytic residues (Glu, Asp, Asp) responsible for the binding of the lysozyme to the bacterial cell wall turned out to be highly conserved (Fu et al., 2013) whereas in *Ci*Lys-g1 and *Ci*Lys-g4, only two catalytic residues (Glu, Asp) were found to be conserved. Structure and surface analysis revealed that the conserved GXXQ motif among the *Ci*lys-g lysozymes is located nearer to the hydrophobic core region. This suggests that the GXXQ motif is involved in the process of binding to the bacterial NAM-NAG and to the processing of pathogens. Multiple sequence alignments revealed that glycine residues remained conserved among the sequences, including fish, birds and humans and *C. intestinalis*. These findings indicate the importance of glycine residues in the structural conservation of g-type lysozymes. Thunnissen et al. (1995) confirmed, using a mutation study in which the mutant without glycine residue showed a declined activity, that the glycine residue is essential for lysozymal activity. Phylogenetic analysis of vertebrate and invertebrate g-type, i-type, c-type lysozymes supported the idea of their evolution from a common lysozyme ancestral gene and the conclusion that the i-type and c-type are more closely associated that the g-type. Genomic comparison among the g-type lysozymes of *D. rerio*, humans and *C. intestinalis* showed that the number of exons varies, ranging from only three for the *Ci*Lys-g2 and -g3 of ascidians to height in human lysozyme g1, and that the structural similarities of g-type lysozymes are not reflected by their genomic organization, since the exon-intron pattern of their genes is very different.

The spatial expression pattern of the mRNA of g-type lysozymes in adult *C. intestinalis* was investigated for different tissues (pharynx, ovary, stomach, intestine) and was detected in all tissues, but the level of mRNA was highest in the pharynx, stomach and intestine, which is consistent with suggestions that the g-type lysozyme gene is expressed predominantly in tissues of organs exposed to the external environment or in hematopoietic tissues (Hikima et al., 2001; Zheng et al., 2007). The expression pattern of g-type lysozyme genes has been investigated in various organisms. In chickens, a restricted expression pattern of the g-type lysozyme was found; however, it was only expressed in the bone marrow and lungs, and not in the oviduct, providing an explanation as to why its absence was observed in chicken egg white (Nakano and Graf, 1991). In contrast, g-type lysozyme was quite abundant in the egg white of many other birds, including geese, ostriches and swans (Irwin and Gong, 2003). Nile et al. (2004) identified a second chicken g-type lysozyme sequence (chicken g2) that was expressed in the liver, kidneys and intestines. In humans, two g-type lysozymes were identified, but neither of these genes was widely expressed in fetal and adult tissues (Irwin and Gong, 2003). These restricted distribution patterns in birds and mammals contrasted
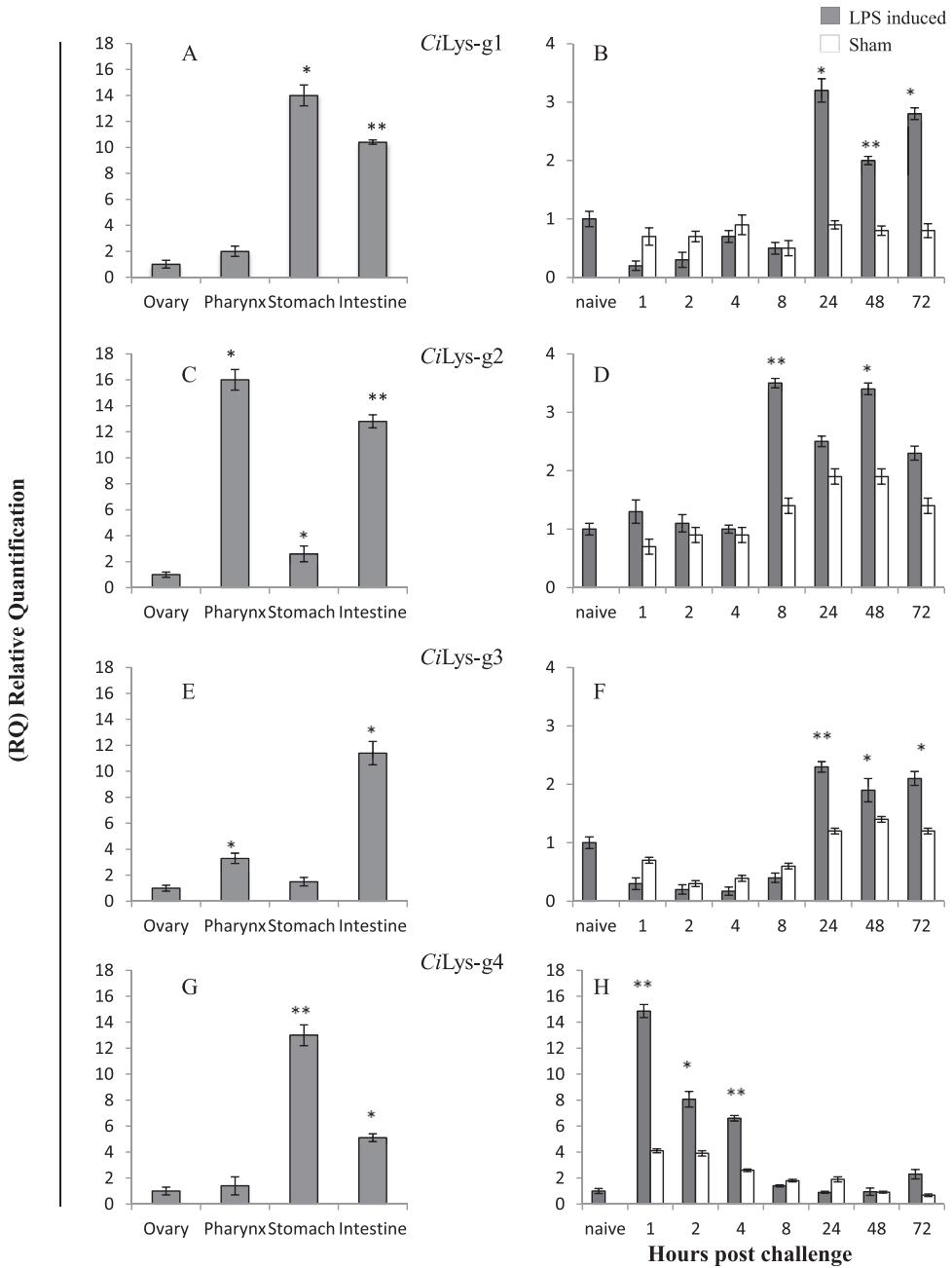
Fig. 2. Real-Time PCR analysis: (A, C, E, G) Tissue expression of g-type lysozymes of C. intestinalis. The mRNA expression level was calculated relative to actin expression and shown as mean ± SD (N = 4);. (B, D, F, H) Time-course of g-type lysozymes of C. intestinalis gene expression in the pharynx after inoculation into the body wall of 100 μg bacterial lipopolysaccharide (LPS) in 100 μl marine solution (MS) (in grey), 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; each assay was performed in triplicate. Asterisks indicate significant differences, at each time point, between LPS and MS inoculation (post hoc Tukey's t-test). *P < 0.05; **P < 0.01.

with the broad expression patterns of g-type lysozymes in fish and invertebrate species. G-type lysozymes have been detected in the spleen, kidneys, gills, skin, heart, intestines and blood of Japanese flounder, orange-spotted grouper, large yellow croaker, Atlantic cod and grass carp (Hikima et al., 2001; Savan et al., 2003; Yin et al., 2003; Zheng et al., 2007), as well as in the gills, mantle, hepato-pancreas, hemocytes and muscles in *Mytilus galloprovincialis* (Wang et al., 2012). Real-Time analysis has shown that *C. intestinalis* g-type lysozymes are upregulated in the pharynx after LPS challenge, in particular, *Ci*Lys-g1, *Ci*Lys-g2 and *Ci*Lys-g3 gene expression were significantly boosted at 24–72 h, while *Ci*Lys-g4 gene expression was significantly boosted at 1 h and 2–4 h and

decreased at 8–48 h, supporting a defensive role for *Ci*Lys-g lyso-zymes. All of these findings suggest a functional role for *Ci*Lys-g(1-4) in innate immune defense and in the intracellular digestion of bacteria in *C. intestinalis*, a marine invertebrate that feeds by filtering seawater and which is often exposed to high concentra-tions of microorganisms.

thank M. Guarcello for collecting ascidians for the maintenance of the aquaria.

Appendix A. Supplementary data

References

Banks, J.G., Board, R.G., Sparks, N.H., 1986. Natural antimicrobial systems and their potential in food preservation of the future. Biotechnol. Appl. Biochem. 8, 103—147.

Beintema, J.J., Terwisscha van Scheltinga, A.C., 1996. Plant lysozymes. In: Jollès, P. (Ed.), Lysozymes: Model Enzymes in Biochemistry and Biology. Birkhäuser Verlag, Basel, pp. 75—86.

Bonura, A., Vizzini, A., Salerno, G., Parrinello, N., Longo, V., Colombo, P., 2009. 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.

Canfield, R.E., McMurry, S., 1967. Purification and characterization of a lysozyme from goose egg white. Biochem. Biophys. Res. Commun. 26, 38—42.

Delsuc, F., Brinkmann, H., Chourrout, D., Philippe, H., 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439, 965—968.

Fastrez, J., 1996. Phage lysozymes. In: Jollès, P. (Ed.), Lysozymes: Model Enzymes in Biochemistry and Biology. Birkhäuser Verlag, Basel, pp. 35—64.

Ferrari, R., Callerio, C., Podio, G., 1959. Antiviral activity of lysozyme. Nature 183, 548.

Fu, G.H., Bai, Z.Y., Xia, J.H., Liu, F., Liu, P., Yue, G.H., 2013. Analysis of two lysozyme genes and antimicrobial functions of their recombinant proteins in Asian seabass. PLoS One 8 (11), e79743.

Grinde, B., 1989. Lysozyme from rainbow trout, Salmo gairdneri Richardson, as an antibacterial agent against fish pathogens. J. Fish. Dis. 12, 95—104.

Hancock, R.E., Scott, M.G., 2000. The role of antimicrobial peptides in animal defenses. Proc. Natl. Acad. Sci. U. S. A. 97, 8856—8861.

Hikima, J., Minagawa, S., Hirono, I., Aoki, T., 2001. Molecular cloning, expression and evolution of the Japanese flounder goose-type lysozyme gene, and the lytic activity of its recombinant protein. Biochim. Biophys. Acta 30, 35—44.

Holtje, J.V., 1996. Bacterial lysozymes. In: Jollès, P. (Ed.), Lysozymes: Model Enzymes in Biochemistry and Biology. Birkhäuser Verlag, Basel, pp. 65—74.

Hultmark, D., 1996. Insect lysozymes. In: Jollès, P. (Ed.), Lysozymes: Model Enzymes in Biochemistry and Biology. Birkhäuser Verlag, Basel, pp. 87—102.

Ibrahim, H.R., Aoki, T., Pellegrini, A., 2002. Strategies for new antimicrobial proteins and peptides: lysozyme and aprotinin as model molecules. Curr. Pharm. Des. 8, 671—693.

Irwin, D.M., Gong, Z.Y., 2003. Molecular evolution of vertebrate goose-type lysozyme genes. J. Mol. Evol. 56, 234—242.

Jollès, J., Jollès, P., 1975. The lysozyme from Asterias rubens. Eur. J. Biochem. 54, 19—23.

Jollès, P., Jollès, J., 1984. What's new in lysozyme research? Always a model system, today as yesterday. Mol. Cell Biochem. 63, 165—189.

Kyomuhendo, P., Myrnes, B., Nilsen, I.W., 2007. A cold-active salmon goose-type lysozyme with high heat tolerance. Cell Mol. Life Sci. 64, 2841—2847.

Lee-Huang, S., Huang, P.L., Sun, Y., 1999. Lysozyme and RNases as anti-HIVcomponents in beta-core preparations of human chorionic gonadotropin. Proc. Natl. Acad. Sci. U. S. A. 96, 2678—2681.

Mohanty, B.R., Sahoo, P.K., 2010. Immune responses and expression profiles of some immune-related genes in Indian major carp, Labeo rohita to Edwardsiella tarda infection. Fish Shellfish Immunol. 28, 613—621.

Nakano, T., Graf, T., 1991. Goose-type lysozyme gene of the chicken: sequence, genomic organization and expression reveals major differences to chicken type lysozyme gene. Biochim. Biophys. Acta 1090, 273—276.

Nile, C.J., Townes, C.L., Michailidis, G., Hirst, B.H., Hall, J., 2004. Identification of chicken lysozyme g2 and its expression in the intestine. Cell Mol. Life Sci. 1, 2760—2766.

Nilsen, I.W., Myrnes, B., Edvardsen, R.B., Chourrout, D., 2003. Urochordates carry multiple genes for goose-type lysozyme and no genes for chicken- or invertebrate-type lysozymes. Cell. Mol. Life Sci. 60, 2210—2218.

Nilsen, I.W., Øverbø, K., Sandsdalen, E., Sandaker, E., Sletten, K., Myrnes, B., 1999. Protein purification and gene isolation of chlamysin, a cold-active lysozyme-like enzyme with antibacterial activity. FEBS Lett. 464, 153—158.

Ogundele, M.O., 1998. A novel anti-inflammatory activity of lysozyme: modulation of serum complement activation. Mediat. Inflamm. 7, 363—365.

Parrinello, N., Vizzini, A., Arizza, V., Salerno, G., Parrinello, D., Cammarata, M., Giaramita, F.T., Vazzana, M., 2008. 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.

Parrinello, N., Vizzini, A., Salerno, G., Sanfratello, M.A., Cammarata, M., Arizza, V., Vazzana, M., Parrinello, D., 2010. Inflamed adult pharynx tissues and swimming larva of Ciona intestinalis share CiTNFAlpha-producing cells. Cell Tissue Res. 341, 299—311.

Prager, E.M., Jollès, P., 1996. Animal lysozymes c and g: an overview. In: Jollès, P. (Ed.), Lysozymes: Model Enzymes in Biochemistry and Biology. Birkhäuser Verlag, Basel, pp. 9—31.

Prager, E.M., Wilson, A.C., Arnheim, N., 1974. Widespread distribution of lysozyme g in egg white of birds. J. Biol. Chem. 249, 7295—7297.

Proctor, V.A., Cunningham, F.E., 1988. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. Crit. Rev. Food Sci. Nutr. 26, 359—395.

Rymuszka, A., Studnicka, M., Siwicki, A.K., Sierosławska, A., Bownik, A., 2005. The immunomodulatory effects of the dimer of lysozyme (KLP-602) in carp (Cyprinus carpio L)—in vivo study. Ecotoxicol. Environ. Saf. 61, 121—127.

Samarayanake, Y.H., Samaranayake, L.P., Wu, P.C., So, M., 1997. The antifungal effect of lactoferrin and lysozyme on Candida krusei and Candida albicans. APMIS 105, 875—883.

Sava, G., Benetti, A., Ceschia, V., Pacor, S., 1989. Lysozyme and cancer: role of exogenous lysozyme as anticancer agent (review). Anticancer Res. 9, 583—591.

Savan, R., Aman, A., Sakai, M., 2003. Molecular cloning of G-type lysozyme cDNA in common carp (Cyprinus carpio L). Fish Shellfish Immunol. 15, 263—268.

Simpson, R.J., Morgan, F.J., 1983. Isolation and complete amino acid sequence of a basic low molecular weight protein from black swan egg white. Int. J. Pept. Protein Res. 22, 476—481.

Smirnow, J., Wislowska, M., 2001. Lysozyme and its biological value in rheumatoidarthritis (RA). Arthritis Res. 3 (Suppl. A), P014.

Thunnissen, A.M., Rozeboom, H.J., Kalk, K.H., Dijkstra, B.W., 1995. Structure of the 70-kDa soluble lytic transglycosylase complexed with bulgecin A. Implications for the enzymatic mechanism. Biochemistry 34, 12729—12737.

Tsagkogeorga, G., Turon, X., Hopcroft, R.R., Tilak, M.K., Feldstein, T., Shenkar, N., Loya, Y., Huchon, D., Douzery, E.J., Delsuc, F., 2009. An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. BMC Evol. Biol. 5, 9—187.

Valisena, S., Varaldo, P.E., Ingianni, A., Fontana, R., 1996. Modulatory effects of henegg-white lysozyme on immune response in mice. New Microbiol. 19, 15—24.

Vizzini, A., Di Falco, F., Parrinello, D., Sanfratello, M.A., Cammarata, M., 2016. Transforming growth factor β (CiTGF-β) gene expression is induced in the inflammatory reaction of Ciona intestinalis. Dev. Comp. Immunol. 55, 102—110.

Vizzini, A., Falco, F.D., Parrinello, D., Sanfratello, M.A., Mazzarella, C., Parrinello, N., Cammarata, M., 2015a. Ciona intestinalis interleukin 17-like genes expression is upregulated by LPS challenge. Dev. Comp. Immunol. 48, 129—137.

Vizzini, A., Parrinello, D., Sanfratello, M.A., Mangano, V., Parrinello, N., Cammarata, M., 2013. Ciona intestinalis peroxinectin is a novel component of the peroxidase-cyclooxygenase gene superfamily upregulated by LPS. Dev. Comp. Immunol. 41, 59—67.

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

Vizzini, A., Parrinello, D., Sanfratello, M.A., Trapani, M.R., Mangano, V., Parrinello, N., Cammarata, M., 2015b. Upregulated transcription of phenoloxidase genes in the pharynx and endostyle of Ciona intestinalis in response to LPS. J. Invertebr. Pathol. 126C, 6—11.

Wang, Q., Zhang, L., Zhao, J., You, L., Wu, H., 2012. Two goose-type lysozymes in Mytilus galloprovincialis: possible function diversification and adaptive evolution. PLoS One 7 (9), e45148.

Yin, Z.X., He, J.G., Deng, W.X., Chan, S.M., 2003. Molecular cloning, expression of orange spotted grouper goose-type lysozyme cDNA, and lytic activity of its recombinant protein. Dis. Aquat. Org. 55, 117—123.

Zeng, L., Swalla, B.J., 2005. Molecular phylogeny of the protochordates: chordate evolution. Can. J. Zool. 83, 24—33.

Zhang, S.H., Zhu, D.D., Chang, M.X., Zhao, Q.P., Jiao, R., Huang, B., Fu, J.P., Liu, Z.X., Nie, P., 2012. Three goose-type lysozymes in the gastropod Oncomelania hupensis: cDNA sequences and lytic activity of recombinant proteins. Dev. Comp. Immunol. 36, 241—246.

Zhang, X., Wu, F.X., Sun, M., Sang, Q.Y., Wang, Y.J., Wang, X.K., 2008. Study on antimicrobial and antiviral activities of lysozyme from marine strain S-12-86 in vitro. Agric. Sci. China 7, 112—116.

Zhao, J.M., Song, L.S., Li, C.H., Zou, H.B., Ni, D.J., Wang, W., et al., 2007. Molecular cloning of an invertebrate goose-type lysozyme gene from Chlamys farreri, and lytic activity of the recombinant protein. Mol. Immunol. 44, 1198—1208.

Zheng, W., Tian, C., Chen, X., 2007. Molecular characterization of goose-type lysozyme homologue of large yellow croaker and its involvement in immune response induced by trivalent bacterial vaccine as an acute-phase protein. Immunol. Lett. 113, 107—116.