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Short communication

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

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ABSTRACT

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.

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 Terwisscha 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;

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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 multi-purpose 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 RNAqueousTM-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 GeneRacerTM 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 *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.

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′-CGGCGTAGCCATCGCTTA-3′	Real-time PCR
	5′-CGGTGGTGTCAGTGTTGTAGAT-3′	Real-time PCR
Actin	5′-TGATGTTGCCGCACTCGTA-3′	Real-time PCR
	5′-TCGACAATGGATCCGGT-3′	Real-time PCR

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 (ENSCING00000024417), *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     AGAAAATGTGTTTATATATATCGAACGAAACAAACCGCACTTCGGTAAAGAAGATTCAC   60
61    TAGAGGAACTAACTGTTCAAGATGAAGCAATTGTTGGTTATCTGCCTTTTGATTGAAATA  120
                M  K  Q  L  L  V  I  C  L  L  I  E  I
121   TGTGTAGCAAGCGACTTAGCATGCACACGCAATGGTGGGACTTGTTTAGATTATCGTTAC  180
      C  V  A  S  D  L  A  C  T  R  N  G  G  T  C  L  D  Y  R  Y
181   TACTTATGCACTGCTGGTTATGAACAAGGATTATGTGACGGGGATTCGAACAGGAAGTGC  240
      Y  L  C  T  A  G  Y  E  Q  G  L  C  D  G  D  S  N  R  K  C
241   TGCCAGGAATGCGATAATACTTGCATCTCAAACGAGAATAGTTATGCTTCATGCTGTGAC  300
      C  Q  E  C  D  N  T  C  I  S  N  E  N  S  Y  A  S  C  C  D
301   TCACAATGCACTCAAAGTGGTGGGAAATGTCAAGATAATTCAAACTACTGTAGTGGTTCT  360
      S  Q  C  T  Q  S  G  G  K  C  Q  D  N  S  N  Y  C  S  G  S
361   TATTCATCTGGAAAGTGTGGAGGGCCTTCATCAAGGCAGTGTTGCAGTGGGGCAAGTTCA  420
      Y  S  S  G  K  C  G  G  P  S  S  R  Q  C  C  S  G  A  S  S
421   GGTGGGGCCTTCGGTTGCTATGGCAACATCTACAATACCGACACCACTGGTGCTTCATGC  480
      G  G  A  F  G  C  Y  G  N  I  Y  N  T  D  T  T  G  A  S  C
481   ACCACGTCGAGTCAAGATAACTTAGGATATTGCGGCATATCAGCGTCTCGTCAATTGGCC  540
      T  T  S  S  Q  D  N  L  G  Y  C  G  I  S  A  S  R  Q  L  A
541   GCTACAGATCTTAACAGGATGTCCCAATACAAAGACGAGATTGAACAAGCCGGTTACCAA  600
      A  T  D  L  N  R  M  S  Q  Y  K  D  E  T  E  Q  A  G  Y  Q
601   CTTTGTATGGACGCTGCTGTTATTGCAGGTATCATCAGTCGTGAAAGTCGTGCAGGGGCA  660
      L  C  M  D  A  A  V  I  A  G  I  I  S  R  E  S  R  A  G  A
661   GCTCTTAATTCTAATGGCTACGGTAGTGACGGGCATGGGTATGGGTTAATGCAGGTCGAC  720
      A  L  N  S  N  G  Y  G  S  D  G  H  G  Y  G  L  M  Q  V  D
721   GATCGTTACCACACCATACAAGGCGACCCTACATCTCTTGACCATATATTACAAGGCACT  780
      D  R  Y  H  T  I  Q  G  D  P  T  S  L  D  H  I  L  Q  G  T
781   GGCATCCTTATATCTAATATTGGACAAATATCTTCGAAATTTCCACCTTGGGACCAAAAC  840
      G  I  L  I  S  N  I  G  Q  I  S  S  K  F  F  P  W  D  Q  N
841   ATGGACTTAAAAGGCGGCATATGCGCTTATAATATAGGTGTTGGGGGCGTGTGGTCTTAT  900
      M  D  L  K  G  G  I  C  A  Y  N  I  G  V  G  G  V  W  S  Y
901   GATAACATGGACGTCGGTACAACTGGGGATGACTACTCTAGCGATGTAGTGGCTAGAGCC  960
      D  N  M  D  V  G  T  T  G  D  D  Y  S  S  D  V  V  A  R  A
961   CAGTATTATAAAGACAACGGGTATTAGGGAAATGATTATAAGCTATGATGTAATAGGTGG 1020
      Q  Y  Y  K  D  N  G  Y  *
1021  TAAAATGGTGGCAGGATATTGGCGATTCTGGTAATAAATACAACCTATATAAAAAAAAAA 1080
1081  AAAAAAAAAAAA
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*Ci*Lys-g3

```
1     GGTGTACAGTCTAGTTAGGTTAGTACAGTGCGTAAGACTACATGAGCAGTTGTTTTAGT   60
                                                M  S  S  C  F  S
61    GTTTTAAGTTTACTTTACTAGTATTAGTTTTTTGCAATGGCGACTCGTTTCAAATTCCG  120
      V  I  S  L  L  L  V  L  V  F  C  N  G  D  S  F  Q  I  P
121   AAAGACGATGTGGACGAGCTGTTAAAGTTAAGATCCAGTATTTGCTACGCAGCTGAAAAA  180
      K  D  D  V  D  E  L  I  K  I  R  S  S  I  C  Y  A  A  F  K
181   GCAGGTGTGCCGTCAGCGTTGCTTGCAGGTATCATCAGTCGTGTAAGCGGGGCCGGTAGA  240
      A  G  V  P  S  A  L  L  A  G  I  I  S  R  V  S  G  A  G  R
241   ATGCTGAACGAGAATGGCTACGGGCAACATGGATATGGTGTGATGCAGATTTGCTGAAAAA  300
      M  L  N  E  N  G  Y  G  Q  H  G  Y  G  V  M  Q  I  A  E  K
301   CACGGTGCCGACACAAAGTTCGGGGCCTTTTTCAAGAGATCACATTTTACAAGGCGCACAA  360
      H  G  A  D  T  K  F  G  P  F  S  R  D  H  I  L  Q  G  A  Q
361   ATTCTCCACGACCTAATGCATTCCATACATGAAAAACACCCAGGAATCATGATGGACCAA  420
      I  L  H  D  L  M  H  S  I  H  E  K  H  P  G  I  M  M  D  Q
421   CAATTTCGAGGCGGAATATTGGCCTACCGAGTTGGTGTGGAAAATCTAAGGCCGCCCGAT  480
      Q  F  R  G  G  I  L  A  Y  R  V  G  V  E  N  L  R  P  P  D
481   CTAATTCATTCGGATATGCCCGAACAGCAATATGCGAATGACATTCTCAGGCGGACATCG  540
      L  I  H  S  D  M  P  E  Q  Q  Y  A  N  D  I  L  R  T  S
541   TTCTTTGAACAACAAGGGTTTGGGAGAAAAGAATGCACAAACATTCAGCATGAGGATCTT  600
      F  F  E  Q  Q  G  F  G  R  K  E  C  T  N  I  Q  H  E  D  L
601   TAAGTTAGAACAATTACACTTCTGGTGTGTAGTTTTCATGTTTGGTTTTGGTCTGATAAT  660
            *
661   GTTTGGTCGTAGTTTTCAATAAATTGGAGCAAATTAAATTAAAAAAAAAAAAAAAAAAAA  720
721   AAAAA                                                          780
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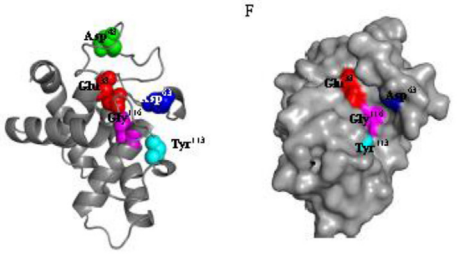

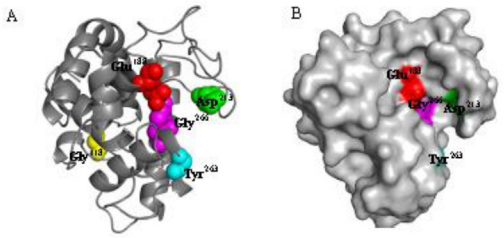

*Ci*Lys-g2

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1     GACAGGNCATTAATAACGGATAATTTGTCTGTGTATCAAAGTATAACATTGTAAAATAAC   60
61    ATGGCCTATGAAAAAGATCTGCGTGCGATGGAGACATATAAAGAGGGAATTATACAAGCT  120
      M  A  Y  E  K  D  L  R  A  M  E  T  Y  K  E  G  I  I  Q  A
121   GCAAGAAATAACAATCTAGAACCTTCTCTGATTGCAGGTATTGCAAGCCGCGAAAGCAAT  180
      A  R  N  N  N  L  E  P  S  L  I  A  G  I  A  S  R  E  S  N
181   GGCGGTAGAGCATTGGACAAAGACGGGCTTGGTGACCATGGAAACGGTTTCGGTATAATG  240
      G  G  R  A  L  D  K  D  G  L  G  D  H  G  N  G  F  G  I  M
241   CAGGTTGATAAGCGGCACCATACACCGATTGGCGGACCTTACTCCAACGAGCATATAAAC  300
      Q  V  D  K  R  H  H  T  P  I  G  G  P  Y  S  N  E  H  I  N
301   CAGGGTGCTACTATCCTGCGAAACTCAATCAATGCAGTGGAGAAAAAGTTTCCTACCTGG  360
      Q  G  A  T  I  L  R  N  S  I  N  A  V  E  K  K  F  P  T  W
361   TCTCCTGACTTGCAACTGCGCGGAGGGGTGGCGGCGTACAACTTTGGGGTCAGCAATGTA  420
      S  F  D  L  Q  L  R  G  G  V  A  A  Y  N  F  G  Y  S  N  V
421   CGAACCCCTGATGGGATCGATAGAGGAACAACAGGCAATAACTATTCAGCTGATGTTATA  480
      R  T  P  D  G  I  D  R  G  T  T  G  N  N  Y  S  A  D  V  I
481   AAACGAGCGGAGTATTTCAAACAGAACGGTTACTAAATTGATGAAAAATGCCCATATAA  540
      K  R  A  E  Y  F  K  Q  N  G  Y  *
541   ACACGTATGCCTTTACGCAGCACAAAAAAGCGCTTAAGTCCATATATTCAGAAGTGTTTA  600
601   TTTAAAATATTATTAACTCATCATGACTAAATATCATTGCTTTCGTGTTCTCTAGCTTTT  660
661   AAATTGCAAGTGTTAGAGTCGTCGCATAAAATCAAATAAAACATGAGCTGTTCAAAAAAA  720
721   AAAAAAAAAAAAAAAAAAAAAAAAAA                                    780
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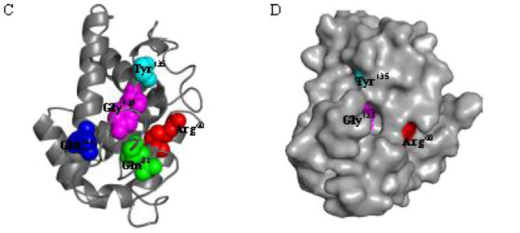

*Ci*Lys-g4

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1     AATCGCTGAAACCAAGACCGTTATCACAACTCGCGAGTTGAACGAATACGGTTTGAAA    60
61    ATGTTGAAGGTATTTTTGCTTTTCCTGTTCGTCGGCGTAGCCATCGCTTATACCGGTTGC  120
      M  L  K  V  F  L  L  F  L  F  V  A  I  A  Y  T  G  C  Y  G
121   TATGGCAACATCTACAACACTGACACCCACCGGTTGTTCCAGTCAAACATCAAGTCAAGAT  180
      N  I  Y  N  T  D  T  T  G  C  S  S  Q  D  N  L  G  Y  S  G
181   AACTTGGGTTACAGCGGAATCTCCGCTTCGAGGCAAATGGCCGCAACAGATCTGTCCAGA  240
      I  S  A  S  R  Q  M  A  A  T  D  L  S  R  M  N  Q  Y  K  S
241   ATGAACCAATACAAAAGTAACATTGAGAATGCTGGACGTCAACTGTGTATGGATCCTTCT  300
      N  I  E  N  A  G  R  Q  L  C  M  D  P  S  V  I  A  G  I  I
301   GTCATTGCAGGCATCATTAGCAGGGAAACTCGTGCGGGGGGCTACCCTTGGTTCAGACGGT  360
      S  R  E  T  R  A  G  A  T  L  G  S  D  G  Y  G  A  D  G  H
361   TACGGCGCAGATGGACACGGTTACGGTTTGATGCAGGTCGACGATCGTTACCACACCATA  420
      G  Y  G  L  M  Q  V  D  D  R  Y  H  T  I  Q  G  G  P  T  S
421   CAAGGTGGCCCTACATCTCTTGACCATATATTACAAGGCACTGGCATCCTTATATCAAAT  480
      L  D  H  I  L  Q  G  T  G  I  L  I  S  N  I  G  Q  I  S  S
481   ATTGGACAAATATCTTCGAAATTTCCATCTTGGGACCAAAACATGGACTTAAAAGGCGGC  540
      K  F  P  S  W  D  Q  N  M  D  L  K  G  G  I  C  A  Y  N  I
541   ATATGCGCTTATAATATAGGTGTTGGGGGCGTGTGGTCTTATGATAGCATGGACGTCGGT  600
      G  V  G  G  V  W  S  Y  D  S  M  D  V  G  T  T  G  D  D  Y
601   ACAACTGGGGATGACTACTCTAGCGATGTAGTGGCTAGAGCCCAGTATTATAAAGACAAC  660
      S  S  D  V  V  A  R  A  Q  Y  Y  K  D  N  G  Y  Y  K  D  N
661   GGGTATTAGGGAAATGGGATTATAAGCTATGATGTAATAGGTGGTAAAATGGTGGCAGGA  720
      G  I  *
721   TATTGGCGATTCTGGTAATAAATACAACCTATATAATTTAAAAAAAAAAAAAAAAAAAAA  780
781   AAA
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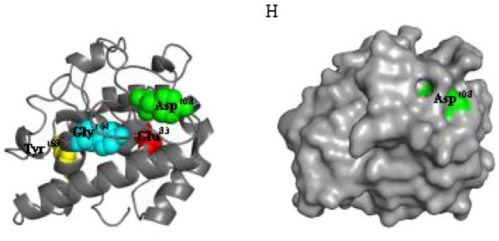

Fig. 1. *Ci*Lys-g1, *Ci*Lys-g2, *Ci*Lysg-3, *Ci*Lys-g4 nucleotide and amino acid sequence. Mid-grey shading indicates signal peptide, light grey shading indicates Lytic transglycosylase-like (SLT) domain; goose family lysozyme signature residues are indicated in bold and underlined. Three dimensional 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

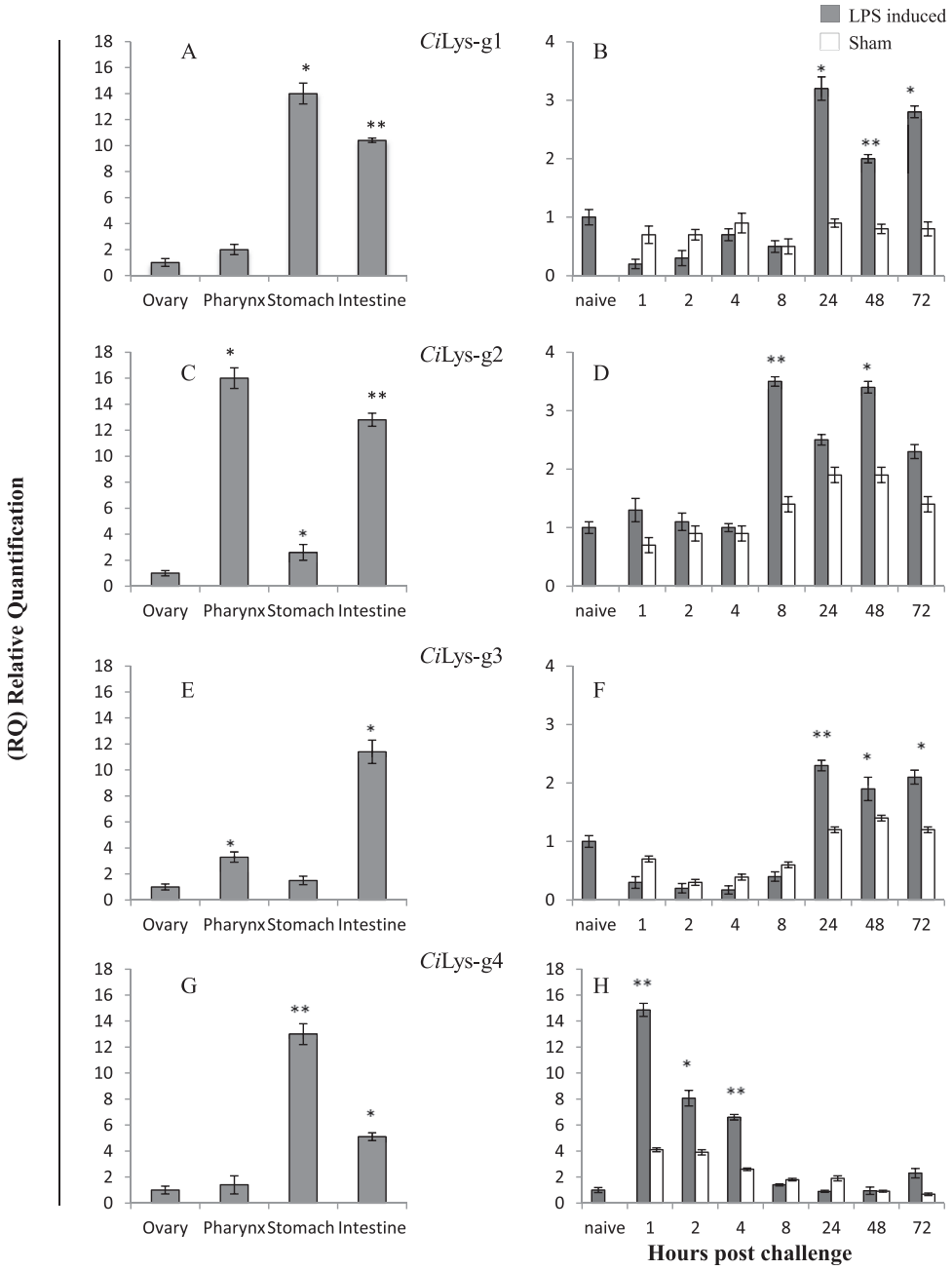

Fig. 2. Real-Time PCR analysis: (A, C, E, G) Tissue expression of g-type lysozymes of C. intestinalis. The mRNA expression level was calculated relative to actin expression and shown as mean ± SD (N = 4);. (B, D, F, H) Time-course of g-type lysozymes of C. intestinalis gene expression in the pharynx 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, hepatopancreas, 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 lysozymes. 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 concentrations of microorganisms.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.09.010.

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