Developmental and Comparative Immunology 113 (2020) 103807

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Short communication

Molecular characterization and expression analysis of the chicken-type and goose-type lysozymes from totoaba (*Totoaba macdonaldi*)

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ARTICLE INFO

Keywords:
Totoaba
c-type lysozyme
g-type lysozyme
Lysozyme superfamily
Innate immunity

ABSTRACT

Lysozymes play a key role in innate immune response to bacterial pathogens, catalyzing the hydrolysis of the peptidoglycan layer of bacterial cell walls. In this study, the genes encoding the c-type (TmLyzc) and g-type (TmLyzg) lysozymes from *Totoaba macdonaldi* were cloned and characterized. The cDNA sequences of TmLyzg and TmLyzc were 582 and 432 bp, encoding polypeptides of 193 and 143 amino acids, respectively. Amino acid sequences of these lysozymes shared high identity (60–90%) with their counterparts of other teleosts and showed conserved functional-structural signatures of the lysozyme superfamily. Phylogenetic analysis indicated a close relationship with their vertebrate homologues but distinct evolutionary paths for each lysozyme. Expression analysis by qRT-PCR revealed that TmLyzc was expressed in stomach and pyloric caeca, while TmLyzg was highly expressed in stomach and heart. These results suggest that both lysozymes play important roles in defense of totoaba against bacterial infections or as digestive enzyme.

1. Introduction

Totoaba (*Totoaba macdonaldi*) is a demersal teleost fish and one of the largest members of the *Sciaenidae* family, commonly known as croakers or drums. This species is endemic and only found in the central and northern Gulf of California, Mexico (Cisneros-Mata et al., 1997). Due to its extremely high value in Asian markets, totoaba was an important species for commercial fisheries until 1975, when its natural population was severely reduced due to unregulated fishing, by-catch and habitat loss. Since then, totoaba is declared to be a threatened species and is currently classified as critically endangered (Bobadilla et al., 2011). Aquaculture of totoaba represents a feasible strategy to recover the natural population (Mata-Sotres et al., 2015; True et al., 1997). Nevertheless, large-scale hatchery rearing of this species faces notable technical difficulties, including those related to disease outbreak control. For this reason, understanding the immune response of totoaba against pathogens is critical to prevent diseases, promote fish health and develop immunoprophylactic strategies to improve farming conditions. However, knowledge of this fish at genetic, biochemical and immunological levels is lacking and demands further investigation (González-Félix et al., 2018; Reyes-Becerril et al., 2016).

Amongst the vast innate immune arsenal, lysozyme is one of the most important molecules that participate in protection against bacterial pathogens (Ragland and Criss, 2017). Lysozyme (EC 3.2.1.17) catalyzes the hydrolysis of β-(1,4)-glycosidic bonds between *N*-acetyl-muramic acid and *N*-acetyl-glucosamine present in the peptidoglycan layer of bacterial cell walls (Kirby, 2001; Sukhithasri et al., 2013). This enzyme is ubiquitously distributed in diverse organisms, including animals,

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https://doi.org/10.1016/j.dci.2020.103807
Received 10 April 2020; Received in revised form 23 July 2020; Accepted 23 July 2020
Available online 29 July 2020

plants, fungi, bacteria and bacteriophages (Wohlkönig et al., 2010). The three types of lysozymes found in the animal kingdom, c-type (chicken), g-type (goose) or i-type (invertebrates), differ in their biochemical and immunological characteristics, catalytic mechanisms and genomic organization (Callewaert and Michiels, 2010; Peregrino-Uriarte et al., 2012). Nevertheless, they share very similar three-dimensional structures despite sharing a low overall amino acid sequence identity (Callewaert and Michiels, 2010; Leysen et al., 2013; Vargas-Requena et al., 2020). Particularly in teleost fish, the presence of at least one of each type (c- or g-type) of lysozyme genes and their functional products have been reported (Saurabh and Sahoo, 2008). In these organisms, lysozymes are highly expressed in lymphoid tissues and are distributed in tissues susceptible to bacterial invasion.

In this work, we cloned the cDNA sequences encoding *T. macdonaldi* g-type (TmLyzg) and c-type (TmLyzc) lysozymes and examined the expression profile of both lysozyme genes in different tissues of healthy totoaba individuals by qRT-PCR. The results derived from this study will contribute basic knowledge of the innate immune system of totoaba and will serve as a foundation to elucidate the molecular and biochemical characteristics that support the unique antibacterial activity of lysozymes in this fish.

2. Materials and methods

2.1. Experimental fish

Healthy totoaba juveniles (15 cm of average body size) were kindly provided by the Center of Reproduction of Marine Species of the State of Sonora, located in Kino Bay, Sonora, Mexico (28°52′29.8″N, 112°01′55.4″W). Fish were maintained in an indoor recirculating aquaculture system at 23 ± 1.0 °C. Tank conditions were kept at 35 ± 0.5% of salinity, oxygen concentration higher than 6 mg L^{-1} and a photoperiod cycle of 12 h light/12 h dark. The juveniles were euthanized and the spleen, kidney, pyloric caeca, stomach, heart and brain tissues were surgically removed. Sampled tissue was immediately soaked in RNAlater® (Invitrogen) and stored at −80 °C until RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA from sampled tissues was isolated using Quick-RNA™ Miniprep kit (Zymo Research), according to the manufacturer's specifications. Thereafter, first-strand cDNA was synthesized from spleen RNA using SuperScript® III first-strand synthesis system for RT-PCR (Invitrogen), according to the manufacturer's protocol. The resulting cDNA was stored at −20 °C and was used to obtain the full-length cDNA sequences of totoaba g-type and c-type lysozymes. RNA samples for the rest of tissues were immediately processed to perform one-step qRT-PCR analysis as described in section 2.5.

2.3. Isolation and cloning of TmLyzc and TmLyzg full-length cDNA sequences

Degenerate primers for totoaba g-type (TmLyzg) and c-type (TmLyzc) lysozymes were designed based on the available amino acid sequences deduced from *Larimichthys crocea* (GenBank ABR66917.1 and XP_019114159.1) and closely related teleost fish to amplify highly conserved regions in g-type and c-type lysozymes. The set of gene specific and degenerate primers used is listed in Table S1.

Partial cDNA sequences of TmLyzg and TmLyzc were cloned by RT-PCR using a GoTaq® Green Master Mix (Promega) and their respective degenerate primer set. The cDNA amplification reactions for each gene were performed in a DNA Engine® thermal cycler (Bio-Rad), with an initial denaturation of 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C (TmLyzg) or 52 °C (TmLyzc) for 1 min and extension at 72 °C for 45 s, and a final extension step of 72 °C for 5 min. PCR products were analyzed on a 1% agarose gel and purified using QIAEX II gel extraction kit (Qiagen). Thereafter, the purified PCR products were cloned into the pCR2.1® vector (Invitrogen) and used to transform *Escherichia coli* TOP10 competent cells for plasmid DNA isolation. Positive clones were sequenced using M13Rv and T7Fw universal primers at the USSDNA facilities (IBT-UNAM).

A new first-strand cDNA for 5′and 3′ RACE was synthesized from total spleen RNA using the SMARTer™ RACE cDNA amplification kit (Takara Bio), following the manufacturer's protocol. The gene specific primers TmLyzcFw3RACE, TmLyzcRv5RACE and UP (supplied by the kit) were used for the first PCR experiment. Amplification conditions were conducted at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 63 °C for 30 s and 72 °C for 3 min, and a final extension step of 72 °C for 5 min. A nested PCR was carried out using TmLyzcFw3NRACE and TmLyzcRv5NRACE, as gene specific primers, and UP-short (supplied by the kit) under identical amplification conditions. RACE for TmLyzg was carried out using gene specific primers TmLyzgFw3RACE, TmLyzgRv5RACE and UP for first round amplification run by 25 cycles of 94 °C for 30 s, 65 °C for 30 s (3′-RACE) or 63 °C for 45 s (5′-RACE) and 72 °C for 3 min. Nested 5′-RACE was conducted using TmLyzgRv5N-RACE and UP-short with the same amplification conditions. The resulting RACE products were analyzed, cloned and sequenced using M13Fw and M13Rv universal primers as described above.

2.4. Sequence analysis of TmLyzg and TmLyzc

Conserved domains in the TmLyzg and TmLyzc amino acid sequences were identified using the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/cdd) (Marchler-Bauer et al., 2017). Physicochemical parameters, such as molecular mass and theoretical isoelectric point, were computed using ProtParam tool on (https://web.expasy.org/protparam/) (Gasteiger et al., 2005). In addition, the presence of signal peptide and the subcellular location of the lysozymes were predicted using the SignalP v4.1 server (http://www.cbs.dtu.dk/services/SignalP/) and YLoc tools (http://abi.inf.uni-tuebingen.de/Services/YLoc/webloc.cgi), respectively (Briesemeister et al., 2010; Petersen et al., 2011). The evolutionary histories of TmLyzg and TmLyzc were inferred through phylogenetic analysis using MEGA X software, through the Maximum Likelihood method at 1000 bootstraps per analysis (Kumar et al., 2018). Finally, the tridimensional structures of TmLyzg and TmLyzc were predicted by homology modeling in MOE software v2018.01 (Chemical Computing Group), using as templates the crystal structures of the g-type lysozyme from *Gadus morhua* (PDB: 3GXK) (Helland et al., 2009) and the c-type lysozyme from *Oncorhynchus mykiss* (PDB: 1LMN) (Karlsen et al., 1995).

2.5. Tissue distribution of TmLyzg and TmLyzc mRNA

Expression profile of TmLyzg and TmLyzc genes in healthy tissues (spleen, kidney, intestine, stomach, pyloric caeca, heart and brain) from totoaba was evaluated by qPCR analysis using Brilliant III Ultra-Fast SYBR® Green qRT-PCR master mix (Agilent). The reactions were conducted on a StepOne™ Real-Time PCR System thermal cycler (Thermo Fisher Scientific) under the following amplification conditions: 50 °C for 10 min, 95 °C for 3 min and 35 cycles of 94 °C for 10 s and 55 °C for 5 s. Primers used to amplify amplicons from each lysozyme gene are shown in Table S1. Elongation Factor-1 α from totoaba was used to normalize gene expression in each sample using the C_T method ($2^{-\Delta Ct}$) (Livak and Schmittgen, 2001) based on previous reports of totoaba gene expression (Angulo et al., 2019; Reyes-Becerril et al., 2016). Three healthy totoaba individuals were used as biological replicates. All data were presented as relative mRNA expression (mean ± S.D.) with experimental triplicates (n = 3). Descriptive statistics were performed using Prism version 8.4.1 for MacOS (GraphPad Software).

3. Results and discussion

3.1. Characteristics of TmLyzg and TmLyzc cDNA sequences

The full-length cDNA sequences of TmLyzg (GenBank MT211628) and TmLyzc (GenBank MT211627) were obtained by RT-PCR and RACE techniques. For TmLyzg, we cloned a cDNA sequence of 711 bp in length, which includes an open reading frame (ORF) of 582 bp and a 3′-UTR of 129 bp with a typical polyadenylation signal (AATAAA) and a poly(A) tail (Fig. S1A). The deduced amino acid sequence for TmLyzg has 193 residues, with a calculated molecular mass of 21.5 kDa and a theoretical isoelectric point (*p*I) of 7.7. The subcellular location prediction indicated that TmLyzg might be an intracellular lysozyme. This is in accordance with the features exhibited by most g-type lysozymes from fish (Irwin, 2014). The highest identity of TmLyzg was 92% and 83% with members of the *Sciaenidae* family large yellow croaker (*L. crocea*) and big head croaker (*Collichthys lucidus*), respectively (Zheng et al., 2007). High identity values were also found with other bony fishes, such as 76% with European seabass (*Dicentrarchus labrax*) (Buonocore et al., 2014) and 64% with Atlantic cod (*Gadus morhua*) (Larsen et al., 2009).

On the other hand, the full-length cDNA of TmLyzc contains a 5′-UTR of 130 bp, a 3′-UTR of 280 bp with a 25 bp poly (A) tail and a consensus polyadenylation signal sequence AATAAA (Fig. S1B). The ORF of 432 bp for TmLyzc encodes a polypeptide of 143 amino acids with a calculated molecular mass of 16.12 kDa and a theoretical *p*I of 8.45. Moreover, this lysozyme was predicted to localize extracellularly, as a signal sequence of 15 residues was identified at the N-terminus of the deduced protein. This is consistent with the characteristics displayed by most of the c-type lysozymes from the vertebrate phylum, including fish, birds and mammals. The inferred amino acid sequence of TmLyzc showed high homology with c-type lysozymes from other fish species and even higher vertebrates. TmLyzc presented identity values of 92% with large yellow croaker (Ao et al., 2015), 91% with big head croaker, 75% with rainbow trout (*Oncorhynchus mykiss*) (Dautigny et al., 1991), and 55% with chicken (*Gallus gallus*) protein sequences.

3.2. Analysis of conserved domains, signature motifs and structural features in TmLyzg and TmLyzc

The amino acid sequence alignment of TmLyzg with homologous g-type lysozymes from teleost fish is shown in Fig. S2A. The analysis indicated that TmLyzg possesses a highly conserved goose egg white lysozyme (GEWL) domain (from Gln17 to Tyr193) (Monzingo et al., 1996), that contains two conserved catalytic residues (Glu71 and Asp101), eleven *N*-acetyl-D-glucosamine binding sites residues (Glu71, Gln99, Val100, Asp101, Pro104, His109, Ile127, Phe131, Tyr155, Asn156, Gly158), and a GXXQ signature motif (Gly96, Leu97, Met98 and Gln99). Altogether, these residues are fundamental for the three dimensional structure and the biological activity of g-type lysozymes (Callewaert and Michiels, 2010). Furthermore, TmLyzg contained only one cysteine residue and no signal peptide sequence was predicted. These are common features in most of the known fish g-type lysozymes, but differ from mammalian and avian counterparts whose g-type lysozymes are secreted proteins (Irwin, 2014). Nevertheless, the presence of intracellular lysozymes in fish species might accomplish an important role in controlling cytosolic growth of intracellular bacteria, possibly mediated by their muramidase activity, bacterial membrane disruption capacity and aggregation of bacterial cells (Seppola et al., 2016).

The amino acid sequence alignment of TmLyzc with homologous c-type lysozymes from vertebrate species, including fish, birds and mammals is shown in Fig. S2B. The analysis revealed that TmLyzc presents a highly conserved α-lactalbumin/c-type lysozyme signature motif called LYZ1 (from Lys16 to Gly142) (Nitta and Sugai, 1989; Qasba et al., 1997). This region includes the two conserved catalytic residues Glu50 and Asp67, the residues that constitute the active site cleft (Asn61, Asp67, Gln72, Ile73, Asn74, Trp77, Trp78, Glu115, Ser117, Ala121, Trp122 and Val123) and a highly conserved signature sequence from Gly64 to Ser75. We found eight highly conserved cysteines (Cys21, Cys45, Cys79, Cys90 Cys94, Cys108, Cys129 and Cys141) in this motif that could be able to form disulfide bridges as predicted by the molecular model (below). Taken as a whole, these residues are essential to preserve the structure and function of the c-type lysozymes. These characteristics are consistent with those reported for c-type lysozymes from all vertebrate species, ranging from fish to mammals (Callewaert and Michiels, 2010). The presence of extracellular c-type lysozymes in fish indicate that these proteins may play important roles in the host defense against extracellular bacterial pathogens. Furthermore, some reports also suggest the implication of secreted lysozymes in the modulation of the immune response (Ragland and Criss, 2017). Secondary to their ability to kill bacteria, extracellular lysozymes can release immunomodulatory bacterial ligands, including peptidoglycan fragments, that in certain scenarios can further activate pro-inflammatory responses or either resolve inflammation.

In general, the predicted secondary structures indicated that these proteins present structural features commonly found in the members of the lysozyme superfamily. More specifically, six distinct α-helixes (α1-α6) and an antiparallel three-stranded β-sheet (β1-β3) were identified in both TmLyzg and TmLyzc. The conserved α3-α5 helixes in TmLyzg and α2-α5 in TmLyzc, along with the three-stranded β-sheet, constitute the catalytic and substrate binding sites in these lysozymes. Altogether, these are considered the only constant elements of the secondary structure in all members of the lysozyme superfamily, including g-type and c-type lysozymes (Monzingo et al., 1996; Wohlkönig et al., 2010). In accordance with these characteristics, the predicted molecular models of TmLyzg (Fig. 1A) and TmLyzc (Fig. 1B) also showed a common lysozyme fold, which consists of a large α-helical domain separated from the small β-stranded domain by a wide substrate binding cleft (Helland et al., 2009; Karlsen et al., 1995; Wohlkönig et al., 2010). In TmLyzg, the α4 and α5 helices contain hydrophobic residues important for substrate binding, and an ϕp sequence signature (Tyr156 and Asn157) that is commonly found in g-type lysozymes at the end of α5-helix. Regarding TmLyzc, key residues for substrate binding are distributed mainly in α2-α4 helixes as well as in β1-β3-sheets.

Although both lysozymes present a quite similar tridimensional structure, only the central α-helix (α4 in TmLyzg and α2 in TmLyzc) and the β-hairpin (β2-β3 strands in both lysozymes) are invariant structural motifs of the lysozyme superfamily, and constitute the core that shapes the protein's active site (Wohlkönig et al., 2010). Particularly, the central α-helix contains the catalytic residues Glu71 in TmLyzg or Glu50 in TmLyzc, while the β-hairpin has the second and third catalytic residues Asp101 and Asp84 in TmLyzg or only Asp67 in TmLyzc. These are fingerprints in g-type and c-type lysozymes and serve to differentiate between the catalytic mechanisms exhibited by each type. In both types of lysozymes, glutamate invariably acts as a general acid catalyst whereas aspartate acts as a general base. The c-type lysozymes exert their catalytic activity through a double displacement mechanism and are known as retaining glycosidases (Kirby, 2001; Zechel and Withers, 2000). In contrast, g-type lysozymes perform their catalytic activity by a single displacement mechanism and are known as inverting glycosidases (Zechel and Withers, 2000).

Moreover, the β-hairpin formed by β2 and β3 sheets has been acknowledged as the structural element with the highest conservation at the amino acid sequence level among the members of the lysozyme superfamily, and this motif displays a specific sequence for each type of lysozyme (Wohlkönig et al., 2010). In this sense, particular sequence motifs located in this region enable us to identify between families of lysozymes. The most distinctive signature motif is the GXXQ sequence, identified in TmLyzg as Gly96, Leu97, Met98 and Gln99, while in TmLyzc Gly69, Ile70, Phe71 and Gln72 (Pei and Grishin, 2005). These residues are part of the loop that links the β2 and β3 sheets and are known to participate in substrate binding, according to the findings in

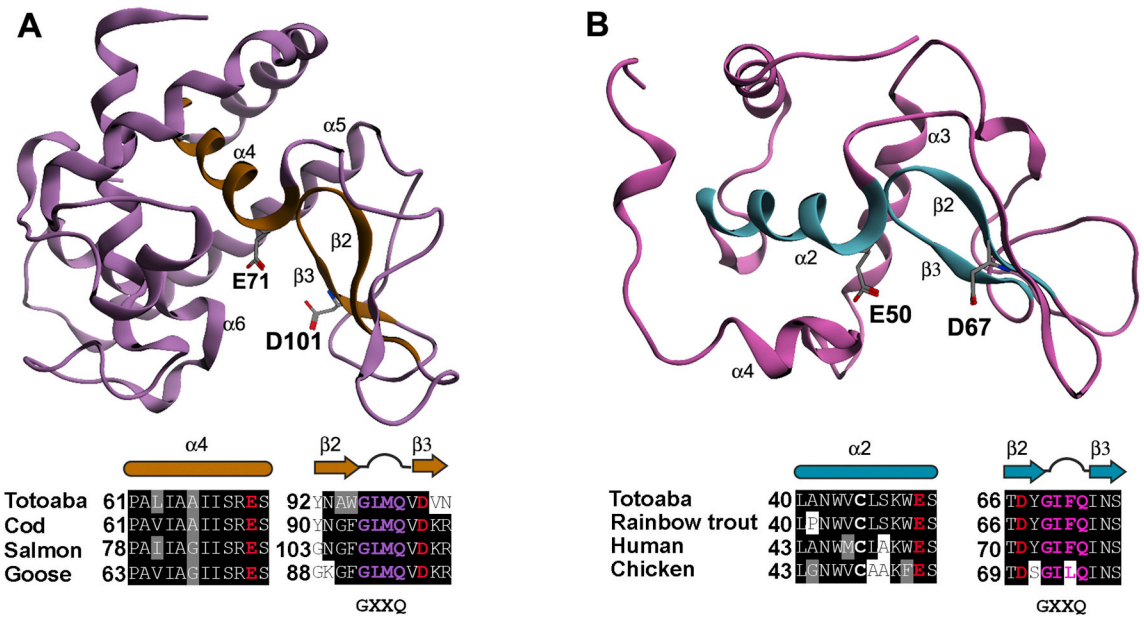

Fig. 1. Predicted three-dimensional structure of (A) TmLyzg and (B) TmLyzc, and localization of the active site and conserved secondary structure elements. GXXQ motif for TmLyzg and TmLyzc in alignments fragments is highlighted in purple and pink, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the crystal structures of *G. morhua*, *O. mykiss*, and their counterparts from avian and mammalian species (Helland et al., 2009; Karlsen et al., 1995). Amongst families of lysozymes, these signatures show low sequence similarity which reveals the high sequence plasticity in the β-hairpin region. For a given family, however, the high degree of conservation of a particular group of residues located in a specific structural region seems to be a requirement to adopt a specific structural conformation in order to accomplish a special functional role (Pils et al., 2005).

3.3. Phylogenetic analysis of TmLyzg and TmLyzc

A phylogenetic tree was constructed using the maximum likelihood method to analyze the evolutionary relationship of TmLyzg and TmLyzc with other vertebrate g-type and c-type lysozymes (Fig. 2A). The results indicated that lysozymes were clustered into two distinct clades, separating the orthologous g-type lysozymes from the c-type lysozymes. In the case of g-type lysozymes, the clade was split into two distinct branches or nodes, setting aside the orthologs from teleost fish from those of mammals and birds. TmLyzg was positioned within the teleost

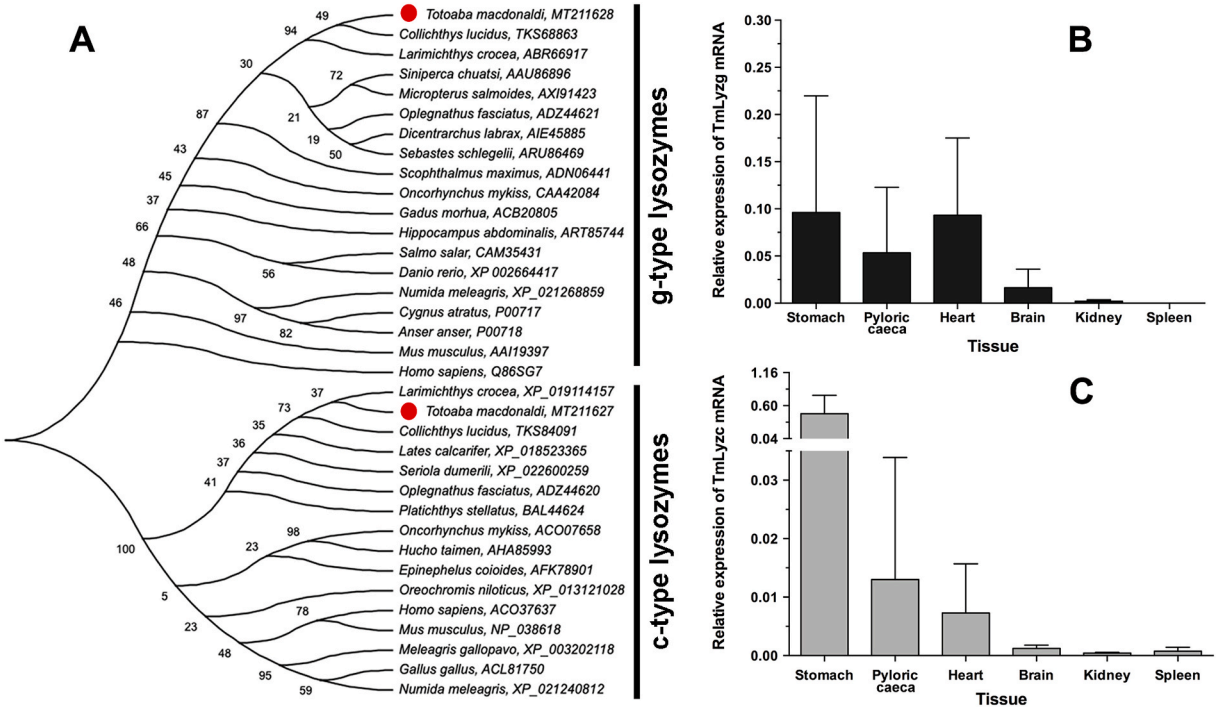

Fig. 2. (A) Phylogenetic analysis of TmLyzg and TmLyzc with known orthologs from vertebrate phylum. GenBank accession numbers of g-type and c-type lysozymes used for the analysis are listed behind scientific names. Relative expression level of g-type (B) and c-type (C) lysozymes mRNA in tissues of totoaba.

group and showed the closest relationship with the counterpart of large yellow croaker and big head croaker, consistent with a common recent ancestor between the three species in the *Sciaenidae* family. The diverse evolutionary paths of g-type lysozymes among vertebrates is in accordance with previous reports (Irwin, 2014; Ko et al., 2016). Particularly in fish species, the major contributors in the evolution of g-type lysozymes are the considerable insertions/deletions in their amino acid sequences, for example the absence of a signal peptide and the lack of disulfide bonds caused by replacement of cysteine residues (Irwin, 2014; Pooart et al., 2004).

Regarding c-type lysozymes, the clade was divided into two major nodes, classifying the orthologs from mammals apart from the fish and avian counterparts. Nevertheless, the evolutionary relationship between c-type lysozymes from distinct vertebrate species seem to be very close. In particular, TmLyzc was positioned within the teleost and avian group and showed the closest relationship with its counterparts from large yellow croaker and big head croaker. The close evolutionary path among c-type lysozymes from different species indicates that their structural characteristics and functional roles are well preserved during evolution (Nitta and Sugai, 1989). Fish species, including totoaba, clearly display the conservation of typical features of c-type lysozymes such as the presence of a signal peptide and cysteine residues that are able to form disulfide bonds.

It is generally accepted that proteins of the lysozyme superfamily have diverged from a common ancestor (Holm and Sander, 1994; Monzingo et al., 1996). This inference is based on the fact that although their amino acid sequences appear to be unrelated, the overall structure between families of lysozymes is strikingly similar. In the hypothesis of divergent evolution, this means that the ancestral fold has been conserved across species and during evolution, while the complete sequences have diverged (Grütter et al., 1983; Yoshikuni et al., 2006). This indicates the high sequence plasticity of these proteins in order to exert specific catalytic functions, but also their stunning ability to maintain an overall well-conserved fold to maintain physiological function.

3.4. Expression profiles of TmLyzc and TmLyzg genes in different tissues

TmLyzc and TmLyzg were ubiquitously expressed in all examined tissues, with predominant expression in stomach, pyloric caeca and heart (Fig. 2B and C). The highest expression level of TmLyzc was detected in stomach and pyloric caeca, whereas TmLyzg was mainly expressed in stomach and heart. C-type and g-type lysozymes from other teleosts, such as orange-spotted grouper (Yin et al., 2003), large yellow croaker (Zheng et al., 2007), Atlantic cod (Larsen et al., 2009), Asian seabass (Fu et al., 2013), starry flounder (Kim and Nam, 2015) and Chinese black sleeper (Wei et al., 2020), were also expressed in the gastrointestinal tract (stomach and intestine) and in heart. Nevertheless, in contrast to our results, the highest expression level of these lysozymes was observed in spleen and kidney. Lysozyme genes in fish are not expressed in a tissue restricted fashion, as it is with their counterparts in birds and mammals (Irwin et al., 2011; Irwin and Gong, 2003; Nakano and Graf, 1991), but are expressed predominantly in hematopoietic organs including spleen, head kidney and liver, as well as tissues exposed to external environment that are sites of first interaction between host and bacteria such as gills, skin and gastrointestinal tract (Saurabh and Sahoo, 2008).

Farming conditions and diet of reared fish can influence the expression levels of the immune response genes in the gut-associated lymphoid tissue, as shown for Atlantic salmon (Løkka et al., 2014). In the gastrointestinal tract, lysozymes may prevent pathogen invasion through direct bactericidal effect as part of the humoral innate immunity in fish (Smith et al., 2019). In totoaba, a higher level of lysozyme activity in plasma was detected in fish fed with a basal diet in contrast with fish fed with a probiotic diet (González-Félix et al., 2018). However, there was no evidence of which type of lysozyme activity was affected in response to diet changes nor the expression profile in gastrointestinal tract. Since lysozyme activity is also increased in other farmed fish species as a possible marker of an inflammatory response to diet components, it will be interesting to explore the expression pattern of totoaba lysozymes with different challenges, such as immunostimulation or vaccination. In addition, high expression of lysozymes in the gastrointestinal tract suggest that besides their role on innate immunity, these enzymes may also be involved in digestion (Gao et al., 2012). All this suggests that g-type and c-type lysozymes in totoaba may play important roles in the defense against infectious diseases. However, temporal expression of lysozymes after immunostimulation and their functional characterization at the protein level in totoaba remains to be investigated.

4. Conclusions

In this work, the full-length cDNA sequences of *Totoaba macdonaldi* g-type lysozyme (TmLyzg) and c-type lysozyme (TmLyzc) were described. Both lysozymes were homologous to other g-type or c-type lysozymes from different fish and higher vertebrate species and presented conserved structural and functional signatures typical of the lysozyme superfamily as part of glycosyl-hydrolases. In addition, expression profiles of TmLyzg and TmLyzc mRNAs revealed that both genes are constitutively expressed in all tissues examined. However, the expression level of each lysozyme gene is higher in certain tissues and reflects its importance in innate immunity. These findings suggest that lysozymes play important roles in defense against bacterial infections in totoaba and also may be involved in digestion. In the long term, this knowledge will also contribute to the establishment of better immunoprophylactic strategies that improve the aquaculture of totoaba, including immunostimulation and vaccination.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

This work was supported by Texas A&M University and CONACyT [Collaborative Research Grant 2015-020]. E.N.M.C. acknowledges a graduate scholarship from CONACyT. M.A.I.O thanks a sabbatical fellowship from Universidad de Sonora. The authors thank to Instituto de Acuacultura del Estado de Sonora (IAES) and Centro Reproductor de Especies Marinas del Estado de Sonora (CREMES) for providing specimens and technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103807.

References

Angulo, C., Sanchez, V., Delgado, K., Reyes-Becerril, M., 2019. C-type lectin 17A and macrophage-expressed receptor genes are magnified by fungal β-glucan after Vibrio parahaemolyticus infection in Totoaba macdonaldi cells. Immunobiology 224, 102–109. https://doi.org/10.1016/j.imbio.2018.10.003.

Ao, J., Mu, Y., Xiang, L.X., Fan, D.D., Feng, M.J., Zhang, S., Shi, Q., Zhu, L.Y., Li, T., Ding, Y., Nie, L., Li, Q., Dong, W. ren, Jiang, L., Sun, B., Zhang, X.H., Li, M., Zhang, H.Q., Xie, S.B., Zhu, Y.B., Jiang, X.T., Wang, X., Mu, P., Chen, W., Yue, Z., Wang, Z., Wang, J., Shao, J.Z., Chen, X., 2015. Genome sequencing of the perciform fish larimichthys crocea provides insights into molecular and genetic mechanisms of stress adaptation. PLoS Genet. 11, 1–25. https://doi.org/10.1371/journal.pgen.1005118.

Bobadilla, M., Alvarez-Borrego, S., Avila-Foucat, S., Lara-Valencia, F., Espejel, I., 2011. Evolution of environmental policy instruments implemented for the protection of totoaba and the vaquita porpoise in the Upper Gulf of California. Environ. Sci. Pol. 14, 998–1007. https://doi.org/10.1016/j.envsci.2011.06.003.

Briesemeister, S., Rahnenführer, J., Kohlbacher, O., 2010. YLoc-an interpretable web server for predicting subcellular localization. Nucleic Acids Res. 38, 497–502. https://doi.org/10.1093/nar/gkq477.

Buonocore, F., Randelli, E., Trisolino, P., Facchiano, A., Pascale, D. de, Scapigliatia, G., 2014. Molecular characterization, gene structure and antibacterial activity of a g-type lysozyme from the European sea bass (Dicentrarchus labrax L.). Mol. Immunol. 62, 10–18. https://doi.org/10.1016/j.molimm.2014.05.009.

Callewaert, L., Michiels, C.W., 2010. Lysozymes in the animal kingdom. J. Biosci. 35, 127–160. https://doi.org/10.1007/s12038-010-0015-5.

Cisneros-Mata, M.A., Botsford, L.W., Quinn, J.F., 1997. Projecting viability of Totoaba macdonaldi, a population with unknown age-dependent variability. Ecol. Appl. 7, 968–980. https://doi.org/10.2307/2269447.

Dautigny, A., Prager, E.M., Pham-Dinh, D., Jollès, J., Pakdel, F., Grinde, B., Jollès, P., 1991. cDNA and amino acid sequences of rainbow trout (Oncorhynchus mykiss) lysozymes and their implications for the evolution of lysozyme and lactalbumin. J. Mol. Evol. 32, 187–198. https://doi.org/10.1007/BF02515392.

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, 1–12. https://doi.org/10.1371/journal.pone.0079743.

Gao, F. ying, Qu, L., Yu, S. guo, Ye, X., Tian, Y. yuan, Zhang, L. li, Bai, J. jie, Lu, M., 2012. Identification and expression analysis of three c-type lysozymes in Oreochromis aureus. Fish Shellfish Immunol. 32, 779–788. https://doi.org/10.1016/j.fsi.2012.01.031.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., Bairoch, A., 2005. The proteomics protocols handbook. Proteomics Protoc. Handb. 571–608. https://doi.org/10.1385/1592598900.

González-Félix, M.L., Gatlin, D.M., Urquidez-Bejarano, Perla, de la Reé-Rodríguez, C., Duarte-Rodríguez, L., Sánchez, F., Casas-Reyes, A., Yamamoto, F.Y., Ochoa-Leyva, A., Perez-Velazquez, M., 2018. Effects of commercial dietary prebiotic and probiotic supplements on growth, innate immune responses, and intestinal microbiota and histology of Totoaba macdonaldi. Aquaculture 491. https://doi.org/10.1016/j.aquaculture.2018.03.031.

Grütter, M.G., Weaver, L.H., Matthews, B.W., 1983. Goose lysozyme structure: an evolutionary link between hen and bacteriophage lysozymes? Nature 303, 828–831.

Helland, R., Larsen, R.L., Finstad, S., Kyomuhendo, P., Larsen, A.N., 2009. Crystal structures of g-type lysozyme from Atlantic cod shed new light on substrate binding and the catalytic mechanism. Cell. Mol. Life Sci. 66, 2585–2598. https://doi.org/10.1007/s00018-009-0063-x.

Holm, L., Sander, C., 1994. Structural similarity of plant chitinase and lysozymes from animals and phage. An evolutionary connection. FEBS Lett. 340, 129–132. https://doi.org/10.1016/0014-5793(94)80187-8.

Irwin, D.M., 2014. Evolution of the vertebrate goose-type lysozyme gene family. BMC Evol. Biol. 14, 1–15. https://doi.org/10.1186/s12862-014-0188-x.

Irwin, D.M., Biegel, J.M., Stewart, C.B., 2011. Evolution of the mammalian lysozyme gene family. BMC Evol. Biol. 11 https://doi.org/10.1186/1471-2148-11-166.

Irwin, D.M., Gong, Z., 2003. Molecular evolution of vertebrate goose-type lysozyme genes. J. Mol. Evol. 56, 234–242. https://doi.org/10.1007/s00239-002-2396-z.

Karlsen, S., Eliassen, B.E., Hansen, L.K., Larsen, R.L., Riise, B.W., Smalas, A.O., Hough, E., Grinde, B., 1995. Refined crystal structure of lysozyme from the rainbow trout (Oncorhynchus mykiss). Acta Crystallogr. Sect. D Biol. Crystallogr. 51, 354–367. https://doi.org/10.1107/S0907444994010929.

Kim, Y.K., Nam, Y.K., 2015. Molecular characterization and expression pattern of c-type and g-type lysozyme isoforms in starry flounder Platichthys stellate infected with Streptococcus parauberis. Fish. Sci. 81, 353–363. https://doi.org/10.1007/s12562-015-0852-0.

Kirby, A.J., 2001. The lysozyme mechanism sorted — after 50 years. Nat. Struct. Biol. 8, 737–739.

Ko, J., Wan, Q., Bathige, S.D.N.K., Lee, J., 2016. Molecular characterization, transcriptional profiling, and antibacterial potential of G-type lysozyme from seahorse (Hippocampus abdominalis). Fish Shellfish Immunol. 58, 622–630. https://doi.org/10.1016/j.fsi.2016.10.014.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096.

Larsen, A.N., Solstad, T., Svineng, G., Seppola, M., Jørgensen, T., 2009. Molecular characterisation of a goose-type lysozyme gene in Atlantic cod (Gadus morhua L.). Fish Shellfish Immunol. 26, 122–132. https://doi.org/10.1016/j.fsi.2008.03.021.

Leysen, S., Vanderkelen, L., Weeks, S.D., Michiels, C.W., Strelkov, S.V., 2013. Structural basis of bacterial defense against g-type lysozyme-based innate immunity. Cell. Mol. Life Sci. 70, 1113–1122. https://doi.org/10.1007/s00018-012-1184-1.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Løkka, G., Austbø, L., Falk, K., Bromage, E., Fjelldal, P.G., Hansen, T., Hordvik, I., Koppang, E.O., 2014. Immune parameters in the intestine of wild and reared unvaccinated and vaccinated Atlantic salmon (Salmo salar L.). Dev. Comp. Immunol. 47, 6–16. https://doi.org/10.1016/j.dci.2014.06.009.

Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C.J., Lu, S., Chitsaz, F., Derbyshire, M.K., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Geer, L.Y., Bryant, S.H., 2017. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic Acids Res. 45, D200–D203. https://doi.org/10.1093/nar/gkw1129.

Mata-Sotres, J.A., Lazo, J.P., Baron-Sevilla, B., 2015. Effect of age on weaning success in totoaba (Totoaba macdonaldi) larval culture. Aquaculture 437, 292–296. https://doi.org/10.1016/j.aquaculture.2014.11.037.

Monzingo, A.F., Marcotte, E.M., Hart, P.J., Robertas, J.D., 1996. Chitinases, chitosanases, and lysozymes can be divided into procaryotic and eucaryotic families sharing a conserved core. Nat. Struct. Biol. 3, 133–140.

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 Gene Struct. Expr. 1090, 273–276. https://doi.org/10.1016/0167-4781(91)90118-6.

Nitta, K., Sugai, S., 1989. The evolution of lysozyme and α-lactalbumin. Eur. J. Biochem. 182, 111–118. https://doi.org/10.1111/j.1432-1033.1989.tb14806.x.

Pei, J., Grishin, N.V., 2005. The P5 protein from bacteriophage phi-6 is a distant homolog of lytic transglycosylases. Protein Sci. 14, 1370–1374. https://doi.org/10.1110/ps.041250005.

Peregrino-Uriarte, A.B., Muhlia-Almazan, A.T., Arvizu-Flores, A.A., Gomez-Anduro, G., Gollas-Galvan, T., Yepiz-Plascencia, G., Sotelo-Mundo, R.R., 2012. Shrimp invertebrate lysozyme i-lyz: gene structure, molecular model and response of c and i lysozymes to lipopolysaccharide (LPS). Fish Shellfish Immunol. 32, 230–236. https://doi.org/10.1016/j.fsi.2011.10.026.

Petersen, T.N., Brunak, S., Heijne, G. von, Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.

Pils, B., Copley, R.R., Schultz, J., 2005. Variation in structural location and amino acid conservation of functional sites in protein domain families. BMC Bioinf. 6, 1–10. https://doi.org/10.1186/1471-2105-6-210.

Pooart, J., Torikata, T., Araki, T., 2004. The primary structure of a novel goose-type lysozyme from rhea egg white. Biosci. Biotechnol. Biochem. 68 https://doi.org/10.1271/bbb.68.159.

Qasba, P.K., Brew, D.K., Kumar, S., 1997. Molecular divergence of lysozymes and α-lactalbumin. Crit. Rev. Biochem. Mol. Biol. 32, 255–306. https://doi.org/10.3109/10409239709082574.

Ragland, S.A., Criss, A.K., 2017. From bacterial killing to immune modulation: recent insights into the functions of lysozyme. PLoS Pathog. 13, 1–22. https://doi.org/10.1371/journal.ppat.1006512.

Reyes-Becerril, M., Alamillo, E., Sánchez-Torres, L., Ascencio-Valle, F., Perez-Urbiola, J. C., Angulo, C., 2016. Leukocyte susceptibility and immune response against Vibrio parahaemolyticus in Totoaba macdonaldi. Dev. Comp. Immunol. 65, 258–267. https://doi.org/10.1016/j.dci.2016.07.016.

Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defence molecule of fish innate immune system. Aquacult. Res. 39, 223–239. https://doi.org/10.1111/j.1365-2109.2007.01883.x.

Seppola, M., Bakkemo, K.R., Mikkelsen, H., Myrnes, B., Helland, R., Irwin, D.M., Nilsen, I.W., 2016. Multiple specialised goose-Type lysozymes potentially compensate for an exceptional lack of chicken-Type lysozymes in Atlantic cod. Sci. Rep. 6, 1–14. https://doi.org/10.1038/srep28318.

Smith, N.C., Rise, M.L., Christian, S.L., 2019. A comparison of the innate and adaptive immune systems in cartilaginous fish, ray-finned fish, and lobe-finned fish. Front. Immunol. 10 https://doi.org/10.3389/fimmu.2019.02292.

Sukhithasri, V., Nisha, N., Biswas, L., Anil Kumar, V., Biswas, R., 2013. Innate immune recognition of microbial cell wall components and microbial strategies to evade such recognitions. Microbiol. Res. 168, 396–406. https://doi.org/10.1016/j.micres.2013.02.005.

True, C.D., Silva-Loera, A., Castro-Castro, N., 1997. Acquisition of broodstock of totoaba macdonaldi: field handling, decompression, and prophylaxis of an endangered species. Progress. Fish Cult. 246–248.

Vargas-Requena, C.L., Rodríguez-Romero, A., García-Ramírez, B., Sotelo-Mundo, R.R., Hernández-Santoyo, A., 2020. Crystal structure of a C-type lysozyme from Litopenaeus vanamei exhibiting a high binding constant to its chitotriose inhibitor. Fish Shellfish Immunol. 100, 246–255. https://doi.org/10.1016/j.fsi.2020.03.010.

Wei, K., Ding, Y., Yin, X., Zhang, J., Shen, B., 2020. Molecular cloning, expression analyses and functional characterization of a goose-type lysozyme gene from Bostrychus sinensis (family: eleotridae). Fish Shellfish Immunol. 96, 41–52. https://doi.org/10.1016/j.fsi.2019.11.067.

Wohlkönig, A., Huet, J., Looze, Y., Wintjens, R., 2010. Structural relationships in the lysozyme superfamily: significant evidence for glycoside hydrolase signature motifs. PloS One 5, 1–10. https://doi.org/10.1371/journal.pone.0015388.

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. https://doi.org/10.3354/dao055117.

Yoshikuni, Y., Ferrin, T.E., Keasling, J.D., 2006. Designed divergent evolution of enzyme function. Nature 440, 1078–1082.

Zechel, D.L., Withers, S.G., 2000. Glycosidase mechanisms: anatomy of a finely tuned catalyst. Acc. Chem. Res. 33, 11–18. https://doi.org/10.1021/ar970172+.

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. https://doi.org/10.1016/j.imlet.2007.08.001.