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Short communication

Molecular characterization of big-belly seahorse (*Hippocampus abdominalis*) arachidonate 5-lipoxygenase *(HaALOX5)*: First evidence of an immune defensive role by induced immunological stress in teleost

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ABSTRACT

Arachidonate 5-lipoxygenase (ALOX5) is an essential enzyme for the biosynthesis of leukotrienes, which are pro-inflammatory and anti-inflammatory mediators. In this study, the ALOX5 paralog of the big-belly seahorse (*Hippocampus abdominalis; HaALOX5*) was identified from our transcriptome database, and then molecularly and functionally characterized to determine its oxygenation capability and expression under pathogenic stress. The coding sequence of *HaALOX5* consisted of 2025 bp and encoded a protein of 674 amino acids in length. Sequence and phylogenetic tree analysis of HaALOX5 revealed a close relationship with its corresponding teleost HaALOX5 counterparts. Structure prediction detected an N-terminal regulatory C2-like domain and a C-terminal catalytic domain, which are the two main functional domains in ALOX5 enzymes. Quantitative PCR showed that *HaALOX5* was expressed in all the analyzed tissues at different magnitudes. The highest expression was detected in the intestine and stomach. In blood cells, the liver and the intestine, *HaALOX5* transcripts were significantly elevated at many post injection time points, when immune challenged with lipopolysaccharide, poly-inosinic:polycytidylic acid, and *Streptococcus iniae*, indicating its contribution to post immune defense mechanisms in the seahorse.

1. Introduction

The arachidonate 5-lipoxygenase (ALOX5) enzyme plays a vital role in the process of leukotriene (LT) biosynthesis from arachidonic acid (AA) [1]. Leukotrienes (LTs) are recognized as a family of important inflammatory mediators and are categorized into different types, including LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4 which produce the signs of inflammation. LTA_4 is an unstable intermediate and is converted to LTB_4, which mediates leukocyte migration from the blood to inflamed tissues and also results in aggregation, superoxide generation, degranulation, and mobilization of the membrane-associated Ca^{2+} of neutrophils [2–4]. The cysteinyl-containing LTs (C_4, D_4, and E_4) cause vasopermeability, mucus secretion, and smooth muscle contraction [4–6]. ALOX5 is primarily expressed in leukocytes, such as monocytes/macrophages, neutrophils, eosinophils, mast cells, dendritic cells, and B-lymphocytes [6].

ALOX5 has two main catalytic functions, one of which is incorporation of molecular oxygen into AA, converting it to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by its dioxygenase activity, and further converting it into 5-hydroxyeicosatetraenoic acid (5-HETE) or LTA_4. LTA_4 is formed from the LTA_4 synthase activity through epoxide formation [7,8]. In addition to LT biosynthesis, ALOX5 is involved in the formation of anti-inflammatory lipoxins from 5-HPETE, together with the enzymes 12-LOX and 15-LOX. In addition to AA, some polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA), eicosatrienoic acids, and fatty acids with 5,8 -cis double bonds serve as good substrates for ALOX5 [9,10].

ALOX5 protein is monomeric, but a homodimer can also be formed. The protein structure of ALOX5 consists of two main domains, which are β-sheets, containing a N-terminal regulatory C2-like domain (~120 amino acids) with ligand binding loops, and several α-helices, containing a C-terminal catalytic domain [11].

Functional characteristics of ALOX5 was substantially studied with regards to involvement of immune defense in human and mouse [6,12–15]. In fish, regulation of the transcriptional level of ALOX5 has been studied with dietary AA in Atlantic salmon (*Salmo salar*) [16] and

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tongue sole (*Cynoglossus semilaevis*) [17]. In terms of the inflammatory response, another expression analysis of ALOX5 was carried out in a macrophage-like cell line from Atlantic salmon [18] and in the liver of gilthead sea bream (*Sparus aurata*) [19]. ALOX5 gene expression was induced in preadipocytes of Atlantic salmon after exposure to LPS [20]. Characterization of ALOX5 and its leukotriene synthesis activity has been examined in zebrafish (*Danio rerio*) [21]. However, there is no information available regarding the modulation of expression of teleost ALOX5 in response to immune challenges.

The big-belly seahorse (*Hippocampus abdominalis*) is used in folk medicine in China, Japan and Korea. These seahorses have been identified as biologically, ecologically, and economically important species [22,23]. As a result of pathogenic infections, the mariculture industry of the big-belly seahorse has faced economic loss and their natural habitats are vulnerable, increasing the potential for extinction of this seahorse species [24–26].

In this study, ALOX5 of *Hippocampus abdominalis* was characterized and its mRNA transcriptional profiles were determined following challenge with the immunological stressors lipopolysaccharide (LPS), *Streptococcus iniae* (*S. iniae*), and polyinosinic:polycytidylic acid (poly (I:C)). Furthermore, the protein coding sequence (CDS) of ALOX5 was cloned to analyze the functional activity of the recombinant protein.

2. Materials and methods

2.1. Identification and characterization of the ALOX5 sequence

The National Center for Biotechnology Information (NCBI) BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the ALOX5 sequence from the previously constructed big-belly seahorse transcriptome library [27]. The seahorse ALOX5 homolog was designated as HaALOX5, and the full-length open reading frame was obtained with the entire amino acid sequence. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) and ExPASy prosite (http://prosite.expasy.org) were used to identify the signal peptide sequences and the domains of the amino acid sequence, respectively. EMBOOS needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) and Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) online software were used for multiple sequence alignment analysis and pairwise sequence alignment, respectively. Phylogenetic analysis was carried out using the neighbor-joining method in the MEGA 6.0 software with 5000 bootstrap replicates. The predicted three-dimensional structure was obtained using SWISS-MODEL (https://swissmodel.expasy.org/).

2.2. Experimental fish

Healthy seahorses, with an average body size of 8 g (approximately 6 months old), were obtained from the Korean fish breeding center (Jeju, South Korea). Fish were adapted in tanks (300 L) at a temperature of 18 °C ± 2 °C and a salinity of 34 ± 0.6 g/L for one week before the experiments. Seahorses were fed with Mysis shrimps throughout the acclimatization period. Fish handling and challenge experiments were conducted according to the guidelines provided by Animal Care and Use Committee of Jeju National University.

2.3. Tissue dissection and immune challenge experiment

Tissue-specific transcript levels were examined by dissecting six healthy seahorses (three females and three males), with an approximate body weight of 8 g. Collected blood was subjected to immediate centrifugation at 3000 × *g* for 10 min at 4 °C to separate the peripheral blood cells (PBCs). The gill, brain, heart, liver, kidney, stomach, intestine, muscle, pouch, skin, testis, and ovary were dissected and snap-frozen in liquid nitrogen. Tissues were stored at −80 °C.

For the immune challenge experiment, seahorses (average body weight of 3 g) were randomly divided into four groups. In each group, 30 fish were intraperitoneally injected with poly (I:C) (1.5 μg/μL), LPS (1.25 μg/μL) or *S. iniae* (10^5 CFU/μL). These injections were prepared in PBS (final volume 100 μL), and the control group was only injected with 100 μL of PBS. As described above, the liver, PBCs, and intestine were sampled from five individual seahorses at 0, 3, 6, 12, 24, 48, and 72 h post-injection.

2.4. Total RNA extraction and cDNA synthesis

Equal amounts of the collected tissue samples were pooled from six healthy animal and five immune-challenged animal at each of the time points mentioned above. Total RNA was extracted using RNAiso plus (TaKaRa, Japan) reagent and then cleaned with RNeasy spin columns (Qiagen, USA). Quality of the RNA was assessed using 1.5% agarose gel electrophoresis, and the concentration was measured at 260 nm using a spectrophotometer with a μDrop plate (Thermo Scientific, USA). From each sample, 2.5 μg of RNA was used to prepare cDNA in a 20 μL reaction mixture using the PrimeScript™ cDNA Synthesis Kit (TaKaRa, Japan). Prepared cDNA was diluted to 800 μL in nuclease-free water (40-fold) and stored at −80 °C for further use.

2.5. Expression analysis of HaALOX5: quantitative real-time PCR (qPCR)

mRNA expression of HaALOX5 in the healthy and immune-challenged tissues was measured using qPCR. qPCR primers were designed according to the minimum information for publication of qPCR experiments (MIQE) guidelines (Table 1) and qPCR primer efficiencies were determined [28]. The internal control gene was the seahorse 40S ribosomal protein S7 (ShRP7; Accession number: KP780177). The qPCR reaction mixture consisted of 3 μL of the cDNA template from each tissue, 0.5 μL of each of the forward and reverse primers (10 pmoL/μL), 5 μL of 2 × EX Taq™ SYBR premix (TaKaRa, Japan), and 1 μL nuclease free dH_2O in a 10 μL reaction mixture. The thermal profile consisted of one cycle of 95 °C for 30 s, 45 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s, and one cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The qPCR reactions were carried out using a thermal cycler Dice™ system III TP950 (TaKaRa), and experiments were performed in triplicate. Relative expression of *HaALOX5* was analyzed according to the $2^{-\Delta\Delta Ct}$ method [29] and normalized compared to PBS-injected group at each time point.

2.6. Cloning of HaALOX5 into the pET28a(+) vector

Cloning primers were designed for the coding region of *HaALOX5*

Table 1
Sequences of primer used for experiments.

Primer name	Application	Sequence of primer (5′-3′)
HaALOX5_F	ORF	GAGAGAGAATTCATGCCGTCGTACACAGTGACCAT
HaALOX5_R	amplification	GAGAGAGTCGACTCAAACAGCAACACTGTTGGGTATT
HaALOX5_qF	qPCR	ACCAATTCCTGAACGGCTGCAATC
HaALOX5_qR	amplification	TGTAGATGTTACCCGCCTTCACTTCC
40S ribosomal protein S7_qF	qPCR internal	GCGGGAAGCATGTGGTCTTCATT
40S ribosomal protein S7_qR	reference	ACTCCTGGGTCGCTTCTGCTTATT

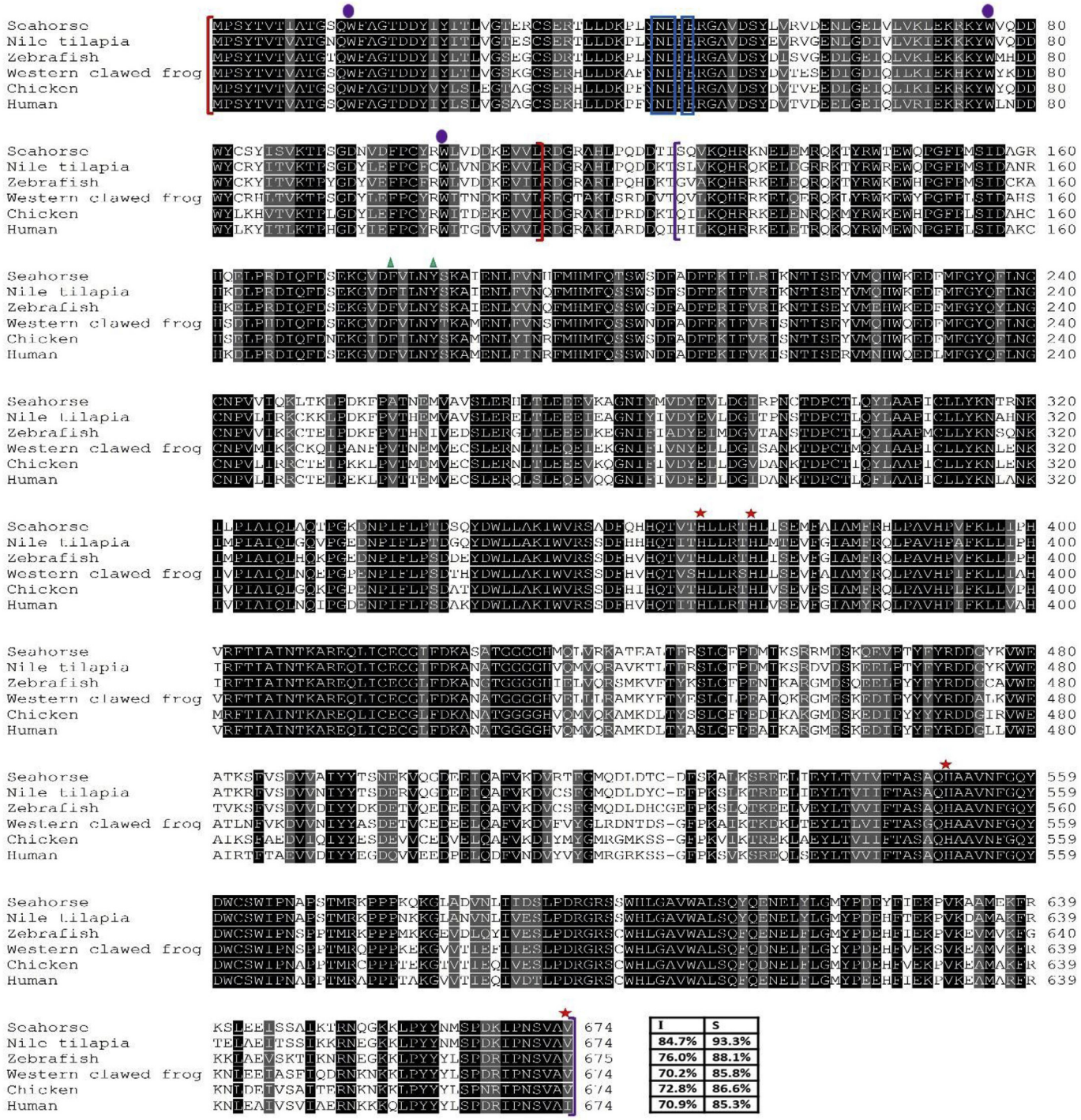

Fig. 1. Multiple sequence alignment of the amino acid sequences of HaALOX5 with ortholog sequences from different organisms. Fully conserved, strongly conserved, and weakly conserved amino acids are indicated in black, dark gray, and light gray, respectively. Colored shapes were used to indicate functionally important residues. Purple dots: membrane binding determinants. Blue boxes: Ca^{2+} binding residues. Green triangles: FY cork. Red Stars: iron binding residues. Red brackets and purple brackets enclose the N-terminal regulatory C2-like domain and the C-terminal catalytic domain, respectively. The similarities (S) and identities (I) for each of the amino acid sequences with HaALOX5 are shown as percentages (%) in the table. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with the relevant restriction sites (Table 1) for ligation into the pET28a (+) vector (Novagen, USA). The coding region was amplified using EXTaq™ DNA polymerase and a TaKaRa thermal cycler (TaKaRa, Japan). Amplified products and the expression vector were digested using the specific restriction enzymes. Ligation was carried out with 2 × Mighty mix in a 10 μL ligation mixture at 16 ˚C for 30 min, followed by a 12 h incubation at 4 ˚C. The ligation mixture was transformed into DH5α *Escherichia coli* competent cells and then sequences were confirmed (Macrogen, Korea).

2.7. Prokaryotic expression and purification of the recombinant HaALOX5 protein

The sequence confirmed gene, containing the recombinant pET28a

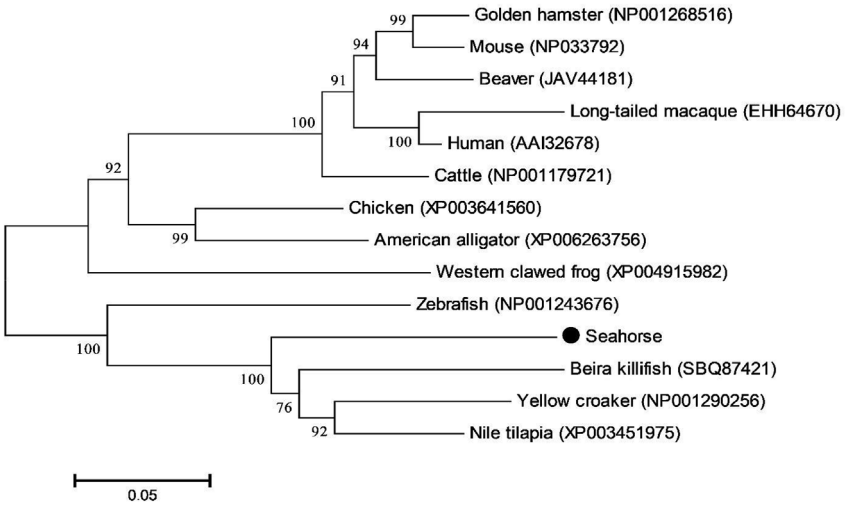

Fig. 2. A phylogenetic tree was built using the neighbor-joining method with different HaALOX5 counterparts. The corresponding boots-trap values are shown on the branches for each protein, and the common names are included with the NCBI accession numbers.

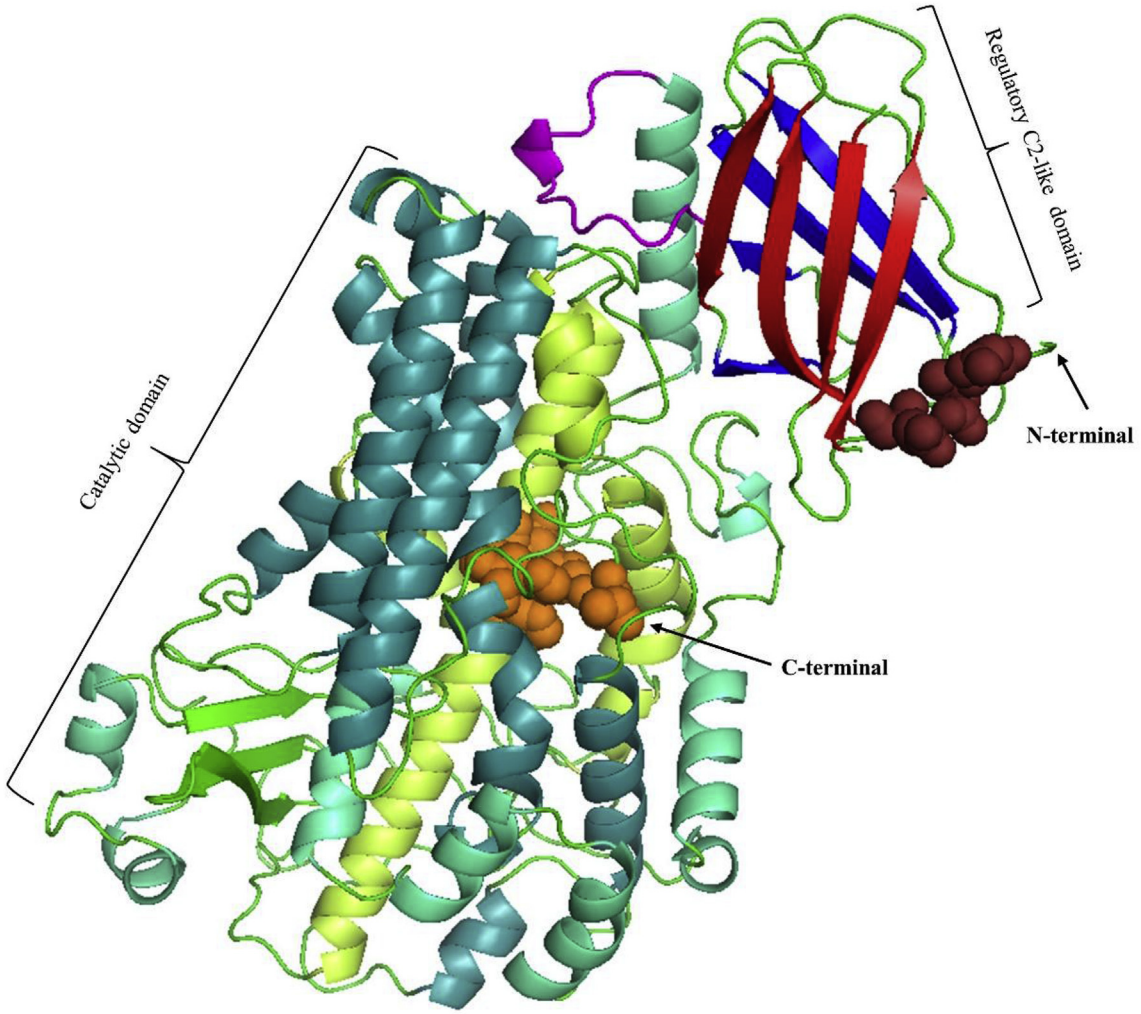

Fig. 3. Predicted tertiary structure of HaALOX5. The eight antiparallel *β*-sheets are indicated in red and blue in the N-terminal C2-like domain. Ca^{2+} binding residues and Fe^{2+} binding residues are indicated in ruby and orange, respectively. Domain linker residues are marked in magenta. C-terminal catalytic domain consisted of α-helices, which are shown in yellow, lime and teal. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

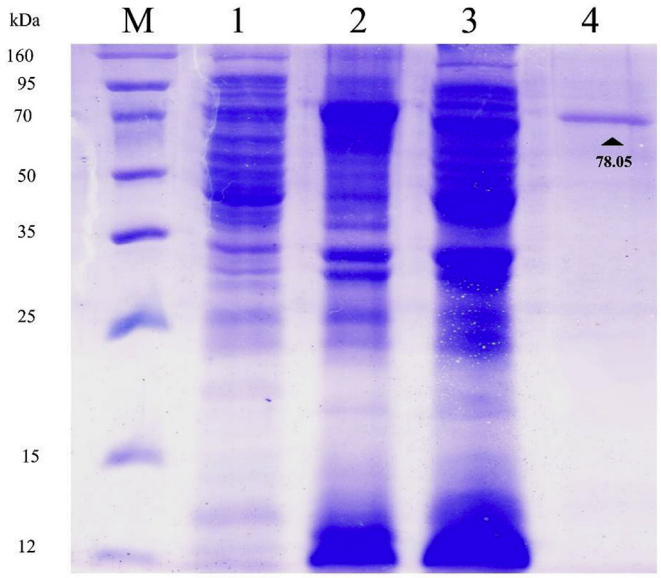

Fig. 4. SDS-PAGE of the purified ALOX5 protein on a polyacrylamide gel (12%). M: Protein marker (kDa). Lane 1: Sonicated lysate of *E. coli* ER2523 before IPTG induction. Lane 2: Soluble portion of the cell lysate after IPTG induction. Lane 3: Insoluble portion of the cell lysate proteins. Lane 4: Purified recombinant HaALOX5/His tag (rHaALOX5).

(+)/HaALOX5 vector, was transformed into *E. coli* BL21 (DE3) competent cells (New England Biolabs, USA). A transformed colony was grown in kanamycin (100 μg/mL)-mixed LB broth at 37 °C overnight. Then, the culture was inoculated (1:100) in LB medium, containing glucose (2.0 g/L) and kanamycin (100 μg/mL). IPTG (0.5 mM) was added to the culture an OD at 600 nm of 0.5, and it was kept in a shaking incubator for 8 h at 20 °C. Cells were harvested by centrifugation at 10000 *g* for 10 min and then resuspended in 1× binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole) and stored at −20 °C overnight. Cells were thawed, sonicated, and centrifuged at 14000 *g* for 20 min at 4 °C. The supernatant was run through His-Bind resin (Novagen, USA), washed with 3 vol of 1× binding buffer and 3 vol of 1× wash buffer (0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole). The recombinant HaALOX5 (rHaALOX5) protein was eluted with 3 vol of elution buffer (0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazole), and the concentration of the eluted protein was determined by the Bradford method [30]. SDS-PAGE was carried out for the rHaALOX5 protein.

2.8. Arachidonic acid oxygenase activity assay

To quantify the arachidonic acid oxygenase activity, 3 μg of purified rHaALOX5 protein was diluted in PBS/EDTA (1 mM), ATP (1 mM) at pH 7.4. Reaction was initiated by the addition of 100 μM arachidonic acid and 1 mM $CaCl_2$ to a final volume of 1 mL. The reaction was carried out at 27 °C for 15 min, and ice-cold methanol was added to terminate the reaction [21]. The cloudy solution was centrifuged, and the clear supernatant was subjected to reversed-phase HPLC analysis.

2.9. HPLC analysis

HPLC analysis was conducted using an Atlantis™ dC18 column; (4.6 × 150 mm; 3 μm particle size) (USA). The solvent system consisted of acetonitrile/water/acetic acid (85/15/0.1, by volume), and the flow rate was set at 1 mL/min. The Absorbance detection range was 220–400 nm.

2.10. Statistical analysis

All the experiments were carried out in triplicate before analysis. The obtained results were expressed as the mean ± standard deviation. The level of significance between the groups was determined by the Student's t-test at $P < 0.05$.

3. Results and discussion

The *HaALOX5* cDNA sequence from the transcriptome library was identified by using BLAST with the highest sequence homology to its counterparts, and the sequence was deposited in the NCBI GenBank (accession number: **MG741005**). The open reading frame was 2025 bp long and encoded a protein of 674 amino acids in length, with a theoretical isoelectric point (pI) of 6.06 and predicted molecular weight of 78.05 kDa. A signal peptide was not detected within the HaALOX5 sequence, as determined by the SignalP server. The translated, primary protein structure of the cDNA sequence was aligned with the corresponding sequences of six known ALOX5 orthologs, and the functionally important, conserved amino acid residues were identified (Fig. 1.). The N-terminal regulatory C2-like domain was identified at $Met^{1}-Leu^{112}$ and marked by red brackets. Amino acid residues between Ser^{126} and Val^{674} build the C-terminal catalytic domain, which is marked by purple brackets. Membrane binding residues (purple dots), Ca^{2+} binding sites (blue boxes), and FY-cork (green triangle) are conserved in all of the aligned animals [31,32]. Iron-binding ligands (red stars) are conserved as His residues (His^{367}, His^{372}, His^{550}) and Val^{674}. Although, Ile^{674} appeared as a N-terminal iron-binding ligand in the human sequence [6], Val^{674} residues was conserved among the other aligned protein sequences. The highest similarity and the highest identity were obtained with Nile tilapia (93.3% and 84.7%, respectively), when HaALOX5 was matched pair-wise with six ALOX5 ortholog amino acid sequences (Fig. 1.). The neighbor-joining phylogenetic tree was generated to evaluate the evolutionary link between seahorse ALOX5 and other species (Fig. 2.). The phylogenetic tree was obtained with different ALOX5 members, aggregating to their corresponding evolutionary clusters and the seahorse clearly clustered with other fish. Yet, tree appeared in two distinct main clusters, one for fish species and another cluster for all other vertebrate species resulting common ancestral sequence origin. Moreover, extended seahorse branch distance represents higher genetic variations of HaALOX5 than that of zebrafish ALOX5.

The three-dimensional structure of HaALOX5 (Fig. 3.), obtained from SWISS-MODEL, was generated based on the human ALOX5 crystal structure (PDB ID: 3v98.1.A), which exhibited a 69.60% sequence identity with HaALOX5. The *β*-sheets mainly composed the N-terminal regulatory C2-like domain, which contained two sets of four-stranded, antiparallel *β*-sheets to form typical ligand binding loops. These binding loops bind to cellular membranes, Ca^{2+}, coactosin-like protein (CLP), and dicer [6,33]. The α-helices mainly appeared in the C-terminal catalytic domain, which helps to form the iron-binding site. In the process of peroxide formation, the bound, prosthetic iron can act as an electron acceptor, as well as an electron donor [6]. Collectively, HaALOX5 contains all the active sites comparable to human ALOX5 structure. It can be predicted that HaALOX5 could interact with its substrates performing similar functions to that of human ALOX5.

HaALOX5 was cloned into the pET28a (+) expression vector, which contains a His tag, and over-expressed in *E. coli* BL21 (DE3) cells. Purified recombinant protein was confirmed by SDS- PAGE analysis (Fig. 4.). Arachidonic acid oxygenase activity was examined for the rHaALOX5 protein, to determine the functional capability. The final product (5-HETE) of the reaction was detected by RP-HPLC (Fig. 5.). HPLC analysis revealed that the purified rHaALOX5 protein had the capability to convert the arachidonic acid to 5-HETE, as 5-HETE eluted as the major product in the supernatant of the completed oxygenation assay reaction (Fig. 5B.). Sample peaks were detected at 235 nm.

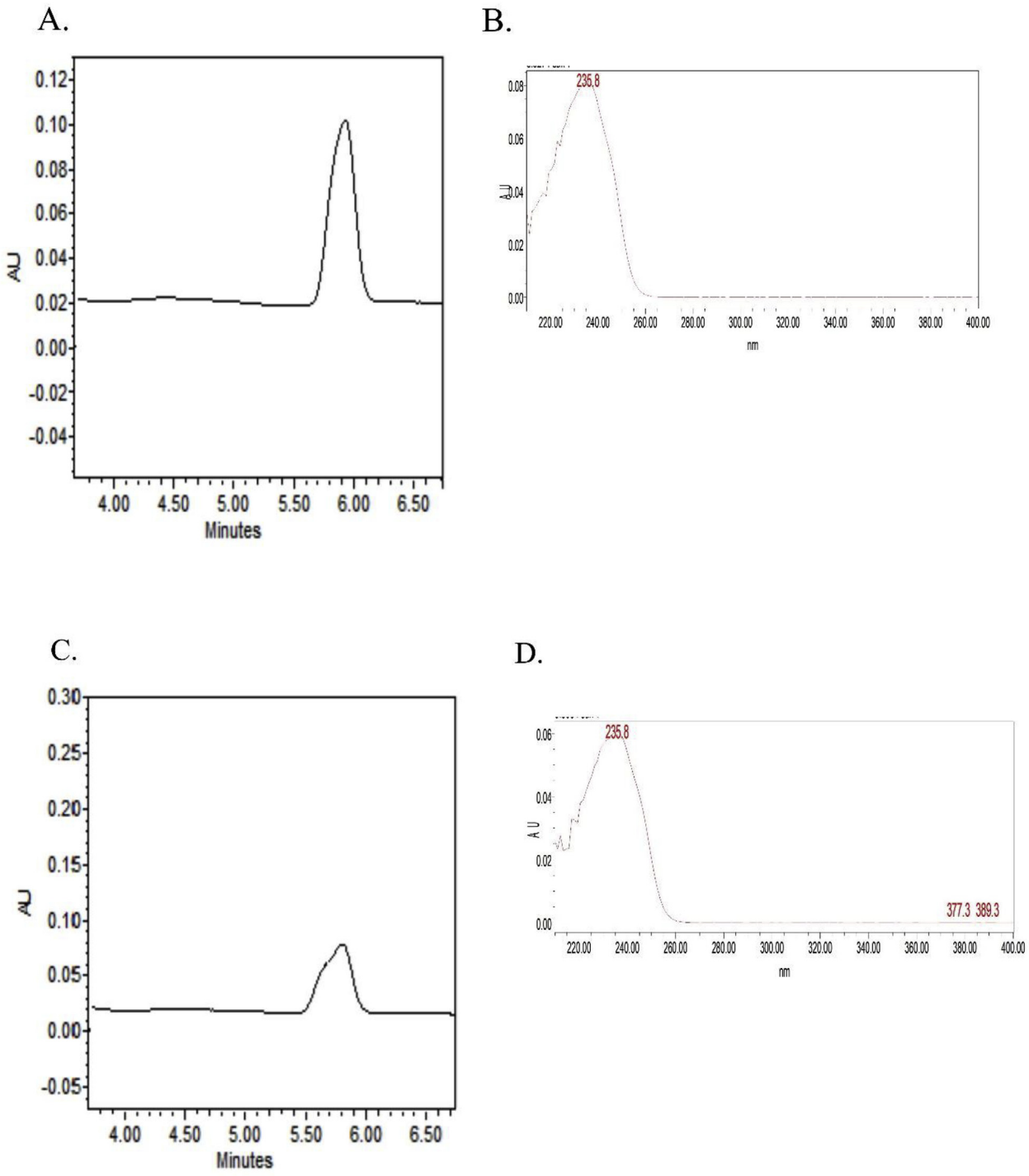

Fig. 5. HPLC chromatograms of 5-HETE, produced from the reaction of rHaALOX5 with the substrate arachidonic acid. A). 5-HETE standard. B). UV-vis spectrum of the standard peak. C). Final products of the AA oxygenation reaction of rHaALOX5. D). UV-vis spectrum of the peak of the oxygenation product. Absorbance of the elution products was measured at 235 nm.

Recombinant ALOX5 proteins of large yellow croaker *(Larimichthys crocea)* and zebrafish converted the AA to 5-HETE as the major oxygenation product [21,34]. Hence, according to activity obtained by rHaALOX5, the 5-lipoxygenase oxygenation functions related to leukotrienes synthesis pathway could be conserved in seahorse similarly to other fish ALOX5 orthologs.

Tissue-specific *HaALOX5* expression levels were quantified in 14 different tissues by qPCR. A single peak in the dissociation curves for *HaALOX5* and the 40S ribosomal protein S7 indicated specific amplification of individual genes and primer efficiencies were in the acceptable range [35]. The expression results revealed that *HaALOX5* transcripts were ubiquitously expressed in all the analyzed tissues (Fig. 6.). The highest expression levels were detected in the intestine with ~53-fold, the stomach with ~37-fold, and the kidney with ~32-fold higher values than the lowest level, which was in the ovary. In addition, a lower expression level was detected in the muscle tissue. In the large yellow croaker, the highest expression of *ALOX5* was detected in the kidney and intestine whereas the lowest was in the muscle [34]. *ALOX5* transcripts were detected in higher abundance during the initial stages of embryonic development of zebrafish. The kidney and intestine

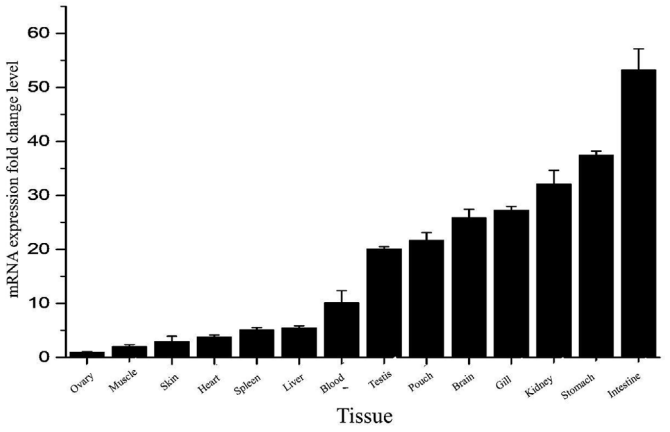

Fig. 6. Tissue-specific mRNA expression levels of ALOX5 in healthy *H. abdominalis*. Expression is shown relative to mRNA expression in the ovary. Data is shown as the mean ± SD of three samples (n = 3).

are known immune tissues [36]. Moreover, the ALOX5 metabolites of AA are involved in intestinal growth and differentiation [37]. LTC_4 and 5-HETE promote mucogenic and secretory functions in digestive tract [38,39]. Therefore, expression of *HaALOX5* may be detected at higher concentrations in intestine, and stomach.

As LT biosynthesis predominantly occurs in leucocytes [8], blood and liver tissue were selected with the highest specific expression tissue (the intestine), for the immune challenge experiment. *S. iniae* was used as a Gram-positive bacterial pathogen challenge. Poly (I:C) (a synthetic, double-stranded RNA) and LPS were used to mimic a viral challenge in fish [40] and a Gram-negative bacterial endotoxin [41], respectively. The selected tissues were examined for temporal mRNA expression of *HaALOX5*. In blood (Fig. 7A.), upon LPS challenge, *HaALOX5* was significantly overexpressed at 3 h post injection (p.i.) (6.25-fold); then, overexpression continued from 24 h to 72 h p.i. Following the poly (I:C) challenge, even though expression was downregulated at 6 h and 12 h p.i., *HaALOX5* expression gradually increased from 24 h (4-fold) to 72 h p.i. (73-fold). Upon *S. iniae* stimulation in blood, *HaALOX5* expression significantly increased at all the time points from 3 h p.i., with the highest at 72 h p.i. (18.8-fold). It clearly showed that the LPS and *S. iniae* challenges rapidly triggered the expression of *HaALOX5* in the blood after 3 h. In the intestine (Fig. 7B.), noticeable expression was detected at 3 h (1.4-fold), 12 h (1.8-fold) and 24 h p.i. (2.2-fold) with the LPS challenge. Except for the 6 h and 48 h time points, all the time points showed significant upregulation of *HaALOX5* transcripts with the highest at 12 h p.i. (2-fold), upon poly (I:C) challenge. Upon challenge with *S. iniae*, mRNA expression of *HaALOX5* was highly induced throughout the experiment and dramatically increased from 6 h (7.2-fold) to 72 h (33.5-fold) in the intestine. In the liver (Fig. 7C.), *HaALOX5* expression was downregulated at 3 h (0.4-fold) and a significantly higher expression was only observed at 24 h (1.6-fold) and 48 h (1.7-fold) p.i., upon LPS stimulation. The poly (I:C) challenge caused significantly elevated expression at 3 h (1.8-fold) and at 48 h p.i. (2.25-fold), with a significantly reduced level of *HaALOX5* expression at 12 h p.i. (0.65-fold). Upon *S. iniae* challenge, at 6 h, expression was significantly downregulated, then significant up-regulation, starting at 24 h (3-fold), decreased until 72 h (1.7-fold).

All the enzymes necessary for the biosynthesis of ALOX5 products, such as the phospholipase A2 (PLA2) enzymes, ALOX5, LTA_4H, LTC_4 synthases, and the 5-lipoxygenase-activating protein (FLAP), can be found in macrophages/monocytes, neutrophils, dendritic cells, B-lymphocytes, and mast cells. Transcellular LT biosynthesis can occur in some cell types (red blood cells, platelets, endothelial cells, and glial cells), as they contain only MGST2, LTA_4H and LTC_4 synthase [42]. Evidently, the transcellular biosynthetic process can occur in isolated cell-to-cell co-incubation, as well as in isolated complex organ systems,

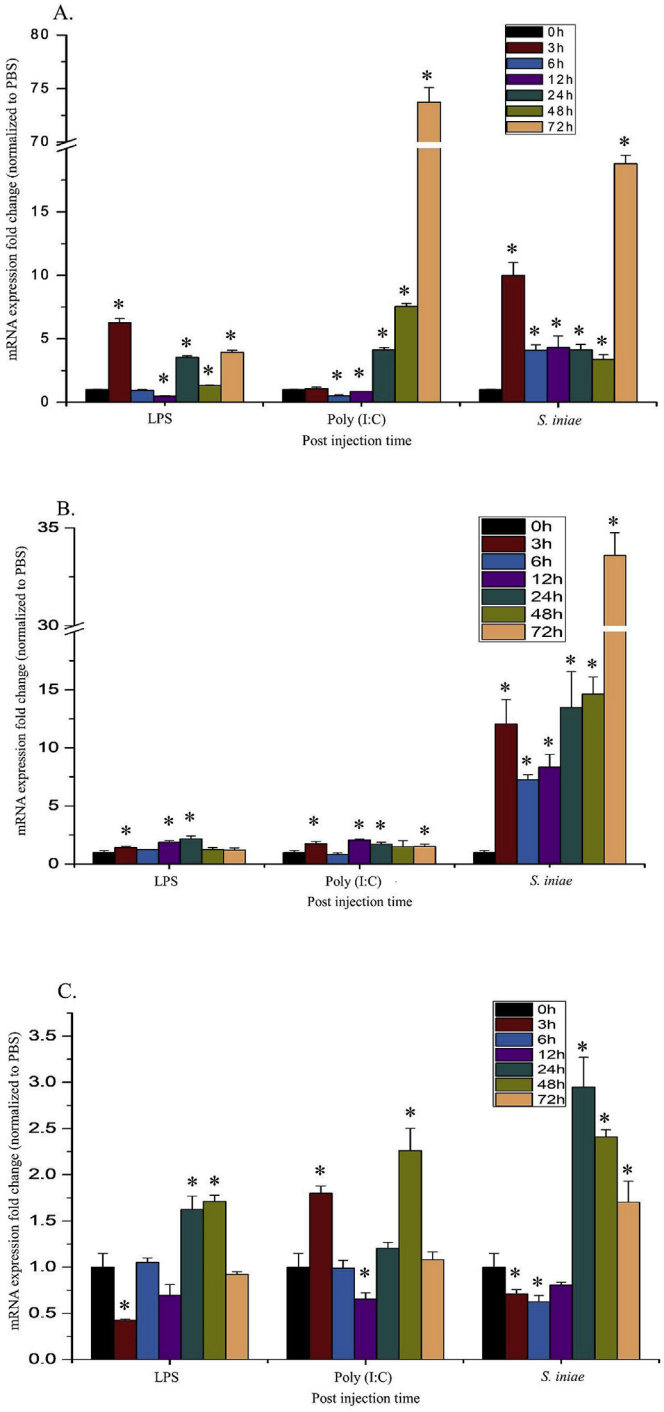

Fig. 7. mRNA expression fold change analysis after immune challenge with LPS, poly (I:C), and *S. iniae* by qPCR. HaALOX5 expression analysis in (A) peripheral blood cells (PBC), (B) intestine tissue, and (C) liver tissue. Data are shown as the mean ± SD of three samples (n = 3). The relative expressions of *HaALOX5* were calculated by the $2^{-\Delta\Delta Ct}$ method using an internal control gene and PBS-injected control. The asterisk (*) indicates significant expression difference between the un-injected control (0 h) ($P < 0.05$).

and during the production of LTs and lipoxins *in vivo* [43,44]. However, cells that include all of the essential enzymes or accessory cells for transcellular biosynthesis are needed for LT synthesis. These all essential enzymes included cell populations as well as accessory cells can be found in total blood cells [45]. During LT synthesis process, unstable LTA_4 is converted to LTB_4. When the LTB_4R1 receptor recognizes the produced LTB_4, regulation of ALOX5 is facilitated by a positive

feedback mechanism [46]. LTB_4 is predominantly synthesized in myeloid cells [47]. LTB_4 is involved in various physiological functions such as phagocytosis and cytokine secretion under pathogenic bacterial infections in neutrophils, monocytes and eosinophils [47–49]. Moreover, synthesis of LTB_4 can be increased in macrophages as results of *in vivo* immune stimulations such as zymosan (fungi-mimic) and calcium ionophore stimuli [50,51]. According to these evidences, LTB_4 synthesis could be increased in blood cells of *H. abdominalis* responding to LPS, *S. iniae* and poly (I:C) challenges. Hence, produced LTB_4 could lead to the higher expression of *HaALOX5* in the blood cells through the positive feedback mechanism comparable to the other analyzed seahorse tissues upon stimulations with LPS, *S. iniae* and poly (I:C).

In the cell, the synthesis of ALOX5 products is a firmly regulated process, with several modulating influences. LT synthesis is triggered by Ca^{2+}-mobilizing substances, such as thapsigargin or ionophores [8]. However, 'priming' substances, such as LPS, tumor necrosis factor-α, macrophage colony-stimulating factor, phorbol esters, or the Epstein Barr virus, strongly enhance LT synthesis. Similarly, phagocytic particles or opsonized zymosan can stimulate LT synthesis [6]. Independent of Ca^{2+} mobilization, phosphorylation of ALOX5 and subsequent LT formation can occur under cell stress conditions such as osmatic shock, heat shock, and oxidative or genotoxic agents [4]. The *S. iniae* immune challenges resulted in comparatively elevated expression of *HaALOX5* in all three of the challenged tissues. Unlike LPS or poly (I:C), *S. iniae* propagated in the fish, and its various pathogen associated molecular patterns (PAMPs) could be detected in all the tissues. In the process of phagocytosis of bacteria, fish neutrophils and macrophages produce reactive oxygen species (ROS), which cause oxidative stress during respiratory burst [52,53]. However, the phagocytic particles stimulate the LT synthesis pathway. Hence, elevated expression levels of *HaALOX5* in tissues may be noticed upon *S. iniae* challenge. In addition, ALOX5 completely preserves specific antibody production by regulating primary naive B-cells, memory B-cell phenotypes, and Tfh cell generation [54]. It has been reported that the balance of type I and II helper T cells (T_h cells) can be affected by a deficiency of ALOX5 [55], and LT receptors can be found on $CD4^+$ T cells that instructively facilitate mobility toward inflammatory locations [56,57]. These experimental evidences suggested the direct involvement of ALOX5 expression in immune regulation of the host. Further experimental studies are needed to find particular contributions of ALOX5 in terms of the teleost immune system.

In conclusion, the complete cDNA of the *HaALOX5* protein was identified and inserted into the pET28a (+) expression vector. The recombinant HaALOX5 protein was purified, and its oxygenation activity was determined using AA as a substrate for the production 5-HETE. Sequence characterization revealed that the functional domains were conserved. Expression of ALOX5 was ubiquitously distributed in healthy seahorse tissues. However, transcripts of *HaALOX5* increased significantly in PBCs, the liver, and the intestine upon LPS, poly (I:C), and *S. iniae* challenges. According to our results, HaALOX5 is modulated under immune stimuli and could be involved in the regulation of the seahorse immune defense system.

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