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Original Article

Gene Expression and Enzymatic Activity of Alkaline Phosphatase in Different Tissues of *Penaeus monodon*

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Abstract: Black tiger shrimp (*Penaeus monodon*) is an economically significant seafood product in Vietnam and globally, yet shrimp farming faces various dangerous diseases leading to substantial economic losses. Understanding the molecular immune mechanisms of the shrimp is crucial for developing disease prevention solutions. Alkaline phosphatase (ALP) is an essential enzyme that influences the black tiger shrimp's immunity. However, knowledge about the enzyme's distribution in different tissues of the shrimp and its specific spacial function remains limited. In this study, expression and enzymatic activity of ALP across six different tissues including body muscle, carapace, gill, head muscle, hemolymph, and hepatopancreas of *P. monodon* was investigated. Using reverse transcription quantitative polymerase chain reaction (RT-qPCR), the expression of ALP gene was found in the range: hepatopancreas > head muscle > carapace > gill, and not detectable in body muscle and hemolymph. The highest ALP gene expression occurs in the hepatopancreas, significantly differing from the other parts, 27.98, 28.87 and 75.79 times higher than head muscle, carapace, grill. Consistently, the enzymatic ALP activity was shown to be highest in the hepatopancreas (134.2 ± 16.05 mU/mg protein), followed by gill (8.6±3.5 mU/mg protein), carapace (3.3±2.2 mU/mg protein), head muscle (2.7±2.2 mU/mg protein, body muscle (1.4±0.02 mU/mg protein) and hemolymph (0.48 ± 0.022 mU/mg protein). Our results suggest that ALP expresses highest in hepatopancreas compared to other tissues in the black tiger shrimp.

Keywords: Penaeus monodon, ALP, gene expression, enzymatic activity, hepatopancreas.

1. Introduction

The black tiger shrimp is a valuable seafood product, significantly contributing to the industry. However, the shrimp farming industry faces several challenges, including the increasing incidence of diseases caused by bacteria and viruses, such as White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), and Acute Hepatopancreatic Necrosis Disease (EMS/AHPND) [1-3]. Understanding the mechanisms and pathways of shrimp immune responses is essential for developing solutions to combat the pandemics.

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Various enzymes responsible for immune responses in shrimp have been discovered. They manifest different immune responses when pathogens infect the shrimp. For example, phenoloxidase (PO) is a well-characterized enzyme involved in the melanization pathway, enabling the encapsulation of pathogens [4]. Shrimps also produce lysozymes to break down pathogens. Phosphatases, such as alkaline phosphatase (ALP) and acid phosphatase (ACP), are essential lysosomal enzymes that participate in the humoral immune system of shrimp. Many studies refer to them as indicators of shrimp immune function [5, 6].

Alkaline phosphatase (different abbreviations: AKP/ALP/Aps) are homodimeric metalloenzymes found in various organisms from bacteria to humans [7]. As phosphatase, it dephosphorylates its substrates under alkaline conditions [8, 9]. Some studies have shown that ALP is involved in reducing inflammation in different animals like zebrafish [10], mice [11], rats [12], by dephosphorylating bacterial lipopolysaccharide (LPS) and extracellular nucleotides [11, 13, 14]. Another study showed that lipopolysaccharide (LPS)_from Gram-negative bacteria upregulates intestinal ALP gene expression in zebrafish [15]. Additionally, ALP activity in the hemolymph of the red swamp crayfish (*Procambarus clarkii*) was observed to increase in response to the invasion of pathogenic bacteria [16]. All of the evidence above suggests the crucial role of ALP in immune responses across various body tissues such as hemolymph or intestine. It needs further investigation on its function from different body tissues.

In this study, we investigate the distribution of ALP in various organs of the black tiger shrimp using gene expression and enzymatic activity to gain insights into the tissue-specific functions of the enzyme in immunization.

2. Materials and Methods

2.1. Materials

Live healthy *P. monodon* black tiger shrimps (25- 35 g each) were purchased from Thanh Cong local market, Hanoi, Vietnam. The shrimp were transported to the laboratory in the original shrimp-raising water and processed immediately for the experiment. Healthy, disease-free shrimps are selected based on following criteria: i) Physical appearance: firm, intact shell with a smooth surface; black or brownish with dark stripes without unusual spots or discoloration around gills area or under the shells which can be indication of disease in shrimp; ii) Movement: alive and active, swim well and react actively to stimuli; iii) Internal organs: muscles when extracted are translucent and firm.

Chemicals used for the experiment included TOPreal qPCR 2X PreMix (SYBR Green with low ROX, UDG plus, RT500UM), M-MLV reverse transcriptase, and random hexamer primers, which were all purchased from Enzynomics (Daejeon, Korea). The Easy-spin Total RNA Extraction Kit was purchased from iNtRON (Gyeonggi-do, Korea). The substrate p-nitrophenyl phosphate (p-NPP) was purchased from Sigma Aldrich (St Louis, MO, USA). Oligonucleotide primers were purchased from Phusa (Cantho, Vietnam).

2.1. Methods

Total RNA isolation

100 mg of each body part of 3 shrimps including body muscle, carapace, gill, head muscle, and hepatopancreas, and hemolymph were collected in cold 50 mM Tris–HCl buffer, pH 8.0, 50 mM NaCl at a ratio of 1:1 (v/v). Total RNA from six different tissues including body muscle, carapace, gill, head muscle, hemolymph, and hepatopancreas was isolated using the Easy-spin Total RNA Extraction Kit (iNtRON, Gyeonggi-do, Korea) following the manufacturer's protocol. RNA concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA), with the qualified ratio of $A_{260/280}$ and $A_{260/230}$ within the range of 1.8-2.0 and 2.0-2.2, respectively.

cDNA synthesis

500 ng of total RNA was used for cDNA synthesis using random hexamers and M-MLV

reverse transcriptase (Enzynomics, Daejeon, Korea), according to the manufacturer's protocol. Thermal cycle conditions for PCR using Eppendorf Mastercycler ep Gradient S (Hamburg, Germany) including annealing at 25 °C (10 min), 42 °C (60 min) for cDNA synthesis, 95 °C (5 min) for the denaturation of M-MLV enzyme. The obtained cDNA was stored at -20 °C until use. The experiment includes three replications (n = 3). Each replication incorporates a mixture of three shrimps in a single purchase.

Gene expression evaluation using RT-qPCR

Primer sequences, product lengths, and GenBank accession numbers are shown in Table 1. RT-qPCR was carried out using freshly synthesized cDNA**.** The components of the RT-qPCR reaction (total volume of 20 µl) included 10 µl of 1× Enzynomics TOPreal qPCR 2× PreMIX (SYBR Green with low ROX, UDG plus), 0.5 µl of 10 µM Fw primer, 0.5 µl of 10 µM Rv primer, 2 µL pure cDNA, and 7 µl ddH_2O. For the negative control, cDNA was replaced with ddH_2O. SubF0 was used as the reference gene. Thermal cycle conditions for RT-qPCR included incubation with UDG at 50 °C (8 min) to remove any contaminated PCR product, an initial denaturation at 95 °C (15 min), followed by 40 cycles of denaturation at 95 °C (15 s), annealing at 62 °C (10 s), and final extension at 72 °C (30 s) on an Applied Biosystems™ 7500 Fast R. The expression of ALP was calculated using the formula $2^{-\Delta Ct} = 2^{-[Ct\ (targeted\ gene\ ALP)\ -Ct(reference\ gene\ subF0)]}$ [17].

Protein extraction

One gram of each tissue including body muscle, carapace, gill, head muscle, hepatopancreas, hemolymph was collected. They were then pulverized with a mortar and pestle in extraction buffer A (50 mM Tris-HCl, 50 mM NaCl, pH = 8), which was kept at 4 °C at a ratio of 1:5 (w/v). After centrifugation (10,000 ×g for 30 min at 4 °C), the clear supernatant was collected for the determination of enzyme activity. The supernatant was transferred into new tubes after centrifugation and centrifuged at the same condition again to collect the utilized clear supernatant. The protein concentration of the supernatant was initially determined using the Bradford Protein Assay [18].

Protein quantification using Bradford Assay

Protein concentration of shrimp tissue extracts was determined using Bradford Assay [18]. The principle of the method is based on the reaction of proteins with Coomassie Brilliant Blue G-250 (CBB) reagent to produce a blue complex, which can absorb light at 595 nm (A_{595}). Color intensity is proportional to protein concentration. By using the standard protein (Bovine Serum Albumin - BSA) at different concentrations to build the standard curve, the sample protein concentration was calculated from the A_{595} value of the protein solution reacting with CBB reagent.

Determination of ALP activity

ALP activity was also determined according to the method described by González *et al.*, [19]. Briefly, 50 µL of the ALP sample was incubated at 37 °C in 250 µL of buffer B (100 mM Tris-HCl, 4 mM $MgCl_2$, pH = 9.1). Then 50 µL of 10 mM para-nitrophenyl phosphate (p-NPP) was added to start the reaction. The increase in absorbance at 405 nm (A_{405}) was recorded every minute for up to 5 minutes to monitor the production of p-nitrophenol. For the blank sample, the enzyme was replaced with buffer A, the other components remained the same. One unit of ALP activity was defined as the amount of enzyme needed to hydrolyze 1 µmole of p-NPP per minute at 37 °C.

The sample volume used can be adjusted depending on the kind of tissue due to the effect of the supernatant color on the reading number, such as hepatopancreas, to obtain a reading within the allowable range ($A_{405} < 1$). The enzyme volume could be compensated by the volume of ddH_2O to ensure the total reaction volume still reached 500 µL. The volume of the p-NPP substrate remained the same.

The formula used for the calculation of the enzyme activity.

$$Units/V_{enzyme} = \frac{\left(\frac{\Delta A405}{\min(test)} - \frac{\Delta A405}{\min(test)} 0.5.df\right)}{(18.5)V\text{enzyme used}}$$

Df: dilution factor; 18.5: Millimolar extinction coefficient of p-nitrophenol at 405 nm; 0.5 = Volume (in milliliters) of assay;$V_{enzyme\ used}$: Volume (in milliliters) of enzyme used

Enzyme specific activity (U/mg):

$$Units/mg\ protein = \frac{Units/ml\ enzyme}{mg\ protein/ml\ enzyme}$$

Statistical analysis

The statistical analysis was performed using one-way ANOVA. Statistical significance was set at $P < 0.05$. All statistical calculations were performed using the GraphPad Prism version 8.

Table 1. ALP and SubF0 primers used for RT-qPCR analysis in *P. monodon*

Genes	Genbank number	Primer sequence	Product size (bp)	Note
ALP	XM_037948113.1	Fw: 5'-GCCAGCAGTACAGGAAGTTCA	88	Designed using NCBI Primer Blast
		Rv: 5'-TCCTTCAGCCAGTATGCCTTG		
SubF0	NC_002184.1	Fw: 5'-TGTACCACAAGGAACGCCAG	94	[19]
		Rv: 5'-TCGAACAGCTAAGGTCCCTG		

3. Results and Discussion

3.1. ALP Gene Expression in Different Tissues of P. monodon

For the expression analysis of ALP gene, we used *SubF0* as a reference gene. The previous study [20] demonstrated that *SubF0* is one of the reference genes for different tissues of *P. monodon*. In this study, we examined the mRNA expression of ALP and in parallel with *SubF0* in six different shrimp tissues including body muscle, carapace, gill, head muscle, hemolymph, and hepatopancreas of.

As shown in Table 2, the expression of *SubF0* was with Ct values ranged from 25.4 ± 0.9303 in gill (lowest) to 28.18 ± 0.6983 in hepatopancreas (highest). The Ct values of ALP mRNA varied largely among the six tissues: 30.71 ± 0.9473 in hepatopancreas, 33.62 ± 0.7956 in gill, 35.18 ± 1.616 in carapace, 37.26 ± 3.344 in head muscle, and we could not detect ALP mRNA in body muscle and hemolymph samples, indicating that they had extremely low ALP expression levels (Table 2). Our results highlight that ALP mRNA expression was highest in the hepatopancreas, significantly differing from the remaining five tissues. Using $2^{-\Delta Ct}$ algorithm to determine the relative expression of ALP, it was shown in Figure 1 that ALP mRNA expression was highest in the hepatopancreas, significantly differing from the remaining five tissues, and the fold change of mRNA expression in hepatopancreas compared to that in the head muscle, the carapace, and the gill was 27.96, 28.8 and 75.8 times, respectively. In addition, the expression level of ALP was lower than that of *SubF0* ($2^{-\Delta Ct}$ values < 1) in all the tissues of *P. monodon*.

Table 2. Ct values of ALP and SubF0 genes in six tissues of *P. monodon*

Tissues	***ALP*** **C_t ± S.D**	***SubF0*** **C_t ± S.D**
Body muscle	N/A	27.83 ± 0.686
Carapace	35.18 ± 1.616	25.85 ± 1.308
Gill	33.62 ± 0.795	25.4 ± 0.930
Head muscle	35.39 ± 1.174	28 ± 0.2722

Tissues	*ALP* C_t ± S.D	*SubF0* C_t ± S.D
Hemolymph	N/A	26.17 ± 1.669
Hepatopancreas	30.71 ± 0.947	28.18 ± 0.698

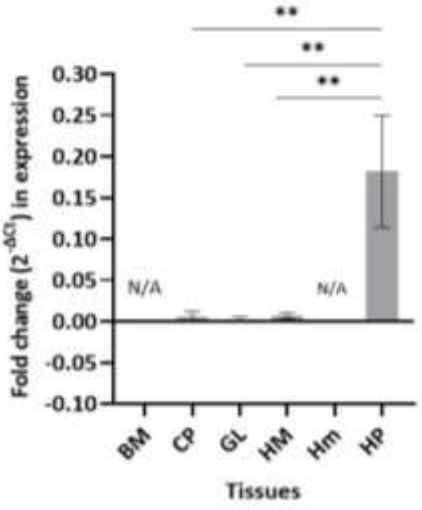

Figure 1. Relative expression of ALP gene in six different tissues of *P. monodon*.

Expression of ALP was normalized with the reference gene SubF0 to compare among different tissues using Livak analysis. Experiments were repeated three times using three different biological samples. One-way ANOVA statistical test was used. *ns: non significant (p-value>0.05); **: p- value*<0.01; BM: body muscle, CP: carapace, GL: gill, HM: head muscle, Hm: hemolymph, HP: hepatopancreas, N/A: non applicable.

3.2. Enzymatic Activity of in Different Tissues of P. monodon

ALP enzymatic activity was evaluated by monitoring the change in its p-nitrophenol substrate over time. As shown in Table 3, the ALP activity of hepatopancreas, gill, carapace, head muscles, body muscle, hemolymph was 134.2±16.05, 8.6±3.5, 3.3±2.2, 2.7±2.2, 1.4±0.02, and 0.48 ± 0.022, 0.48 ± 0.022 mU/mg protein, respectively. It is clear that the enzymatic activity data align with the gene expression data for ALP, with the highest ALP activity observed in the hepatopancreas. Statistical analysis indicated that the difference in ALP activity between the hepatopancreas and the gill (which had the highest activity in the hepatopancreas-excluded group) was significant, suggesting that this significance extends to comparisons with other tissues. Moreover, no significant differences were found among the other tissues.

Table 3. The enzymatic activity of ALP in different tissues of *P. monodon*

Tissues	*ALP activity (mU/mg protein)*	*P-value (others vs gill)*
Body muscle	1.931 ± 0.04003	ns
Carapace	3.296 ± 2.188	ns
Gill	8.591 ± 3.475	-
Head muscle	2.737 ± 2.224	ns
Hemolymph	0.4783 ± 0.02214	ns
Hepatopancreas	134.2 ± 16.05	****

ns: non significant (p-value> 0.05); ****: p- value<0.0001; One-way ANOVA was used.

Alkaline phosphatase from hepatopancreas of *P. monodon* has been purified and characterized [21]. However, to date, there is no information on the distribution of ALP in the shrimp, but in other Crustaceas. The highest expression level of *ALP* in the hepatopancreas was also observed among the 7 collected tissues: gill, hemocytes, hepatopancreas, intestine, muscle ovary and testis in river prawn *Macrobrachium nipponense* [22]. Similarly, ALP activity in hepatopancreas was 20-fold higher than that in lymphoid organs and gills in *Litopenaeus vannamei* compared to that in gill and muscle [23]. Here for the first time we have reported the gene expression and activity of alkaline phosphatase in six distinct tissues of *P. monodon.*

ALP transcripts were not detected in the body muscle and hemolymph; however, the active enzymes were present in these tissues. This suggests that the transcription of ALP might not be directly related to its protein location. One possible explanation is that ALP

is synthesized in the hepatopancreas and then translocated to the hemolymph and body muscle via unknown mechanism. This phenomenon has also been observed with other hepatopancreas-derived protein, such as hemocyanin [24].

In this study, the fresh shrimps were collected from the same seller at Thanh Cong market at different times. Our selection of healthy shrimp was primarily based on appearance criteria at the static state. A limitation is that the shrimps may have subjected to varying enviromental stress and other factors which could atribute to the variability in the data. Nevertheless, our stastically analyzed data clearly showed that the expression and enzymatic activity of ALP were distinctly different in various body parts of healthy *P. monodon.* This finding is consistent with the data on ALP expression and enzymactic activity of *Macrobrachium nipponense* shrimps [22] and to some extent with the data on the ALP enzymatic activity of *Fenneropenaeus chinensis* shrimp [25], and *Metapenaeus monoceros* shrimp [26]. To minize the variations in shrimp sampling, one ideal solution would be collecting shrimps from the same source and in laboratory settings. Healthy shrimps for experiment should be cultivated in seawater with consistent salinity, temperature and pH, and their body weight should be monitored for an increase over several weeks before sampling [22].

The physiological functions as well as the signalling pathway of ALP are still not clearly understood. Our study is one of the first to examine the expression levels of ALP at both mRNA and protein-enzyme levels in *P. monodon*, contributing to a better understanding of the enzyme distribution in the shrimp. ALP in the hepatopancreas organ has been shown in various species to play important roles in digestion and detoxification. ALP was concerned with several stages in mud crab *Scylla serrata* and in freshwater crab *Paratelphusa masoniana* [27, 28]. Moreover, ALP was reported to have a detoxification function, the fed diet containing aflatoxin B1 showed a correlation of elevated alkaline phosphatase activity in the hepatopancreas and aflatoxin concentration [29, 30], suggesting the potential role of ALP in the hepatopancreas in the elimination of toxins. However, further studies are needed to elucidate the physiological roles of ALP in *P. monodon.*

Our findings have shown the profile of gene expression and enzymatic activity of ALP in different tissues of *P. monodon*. Any changes in the ALP profile across different tissues might indicate pathogenic invasion, warranting further investigation. Indeed, previous research also demonstrated that various immune enzymes including ALP are generally selected as an indicator to evaluate the immune status and disease resistance of shrimp [31, 32]. Many research demonstrated an increase in ALP activity or mRNA expression when examined under a stress environment or challenged with pathogens such as WSSV and *Vibrio parahaemolyticus* in *Fenneropenaeus chinensis* [25, 33], *Macrobrachium nipponense* [22], in green tiger shrimp *Penaeus semisulcatus* [34]. These results indicate ALP prospective participation in the emergency response of the immune systems to pathological invasion.

To control the disease, immune-stimulants have been used to enhance ALP activity. Immune-stimulants, as an alternative of antibiotics, are used to enhance the immune system of shrimps in an eco-friendly manner [35]. Indeed, different immunostimulants were used to feed shrimps to enhance ALP activity such as β-1,3-glucan diet, AviPlus or marine yeast Candida aquaetextoris S527 or GSP [23, 36-38]. The above results elucidate that immunostimulant enhancement of ALP activity may result in increased immunity of the shrimp.

4. Conclusion

In this study, we for the first time reported the gene expression and activity of alkaline phosphatase in six different tissues of *P. monodon.* The ALP gene was detected in various tissues of the shrimp, with the highest

expression observed in the hepatopancreas, followed by the head muscle, the carapace, the gill and not detected in the body muscle and the hemolymph. The enzymatic activity of ALP was found to be most pronounced in the hepatopancreas (134.2 ± 16.05 mU/mg protein), followed by the gill (8.6±3.5 mU/mg protein), carapace (3.3±2.2 mU/mg protein), head muscle (2.7±2.2 mU/mg protein), body muscle (1.4±0.02 mU/mg protein), and hemolymph (0.48 ± 0.022 mU/mg protein). Consequently, the shrimp's hepatopancreas exhibited the highest level of both ALP gene expression and enzymatic activity.

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