



## Gene profiling and characterization of arginine kinase-1 (MrAK-1) from freshwater giant prawn (*Macrobrachium rosenbergii*)

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### ARTICLE INFO

#### Article history:

Received 11 January 2011

Received in revised form

30 March 2011

Accepted 11 April 2011

Available online 28 April 2011

#### Keywords:

Arginine kinase

*Macrobrachium rosenbergii*

IHHNV virus

Gene expression

Kinetic activity

### ABSTRACT

Arginine kinase-1 (MrAK-1) was sequenced from the freshwater prawn *Macrobrachium rosenbergii* using Illumina Solexa Genome Analyzer Technique. MrAK-1 consisted of 1068 bp nucleotide encoded 355 polypeptide with an estimated molecular mass of 40 kDa. MrAK-1 sequence contains a potential ATP:guanido phosphotransferases active domain site. The deduced amino acid sequence of MrAK-1 was compared with other 7 homologous arginine kinase (AK) and showed the highest identity (96%) with AK-1 from cherry shrimp *Neocaridina denticulate*. The qRT-PCR analysis revealed a broad expression of MrAK-1 with the highest expression in the muscle and the lowest in the eyestalk. The expression of MrAK-1 after challenge with the infectious hypodermal and hematopoietic necrosis virus (IHHNV) was tested in muscle. In addition, MrAK-1 was expressed in *Escherichia coli* by prokaryotic expression plasmid pMAL-c2x. The optimum temperature (30 °C) and pH (8.5) was determined for the enzyme activity assay. MrAK-1 showed significant ( $P < 0.05$ ) activity towards 10–50 mM ATP concentration. The enzyme activity was inhibited by  $\alpha$ -ketoglutarate, glucose and ATP at the concentration of 10, 50 and 100 mM respectively. Conclusively, the findings of this study indicated that MrAK-1 might play an important role in the coupling of energy production and utilization and the immune response in shrimps.

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### 1. Introduction

Arginine kinase (AK) plays a central role in both temporal and spatial ATP buffering in cells with high, fluctuating energy requirements (muscle, nerves, etc.). AK catalyzes the reversible phosphorylation of arginine by MgATP to form phosphoarginine and MgADP, thereby regenerating ATP during bursts of cellular activity [1]. AK is the most widely distributed among invertebrates. AK activity has been identified even in protozoa and its gene has been found in the genomes of *Paramecium* and *Tetrahymena*, indicating AK has an ancient origin. As a member of the phosphagen kinase family, it is mainly distributed in invertebrates, and plays a crucial role in the energy mechanism, which is analogous to the creatine kinase (CK) reaction in vertebrates [2]. AK is an ideal paradigm for the classical enzymology of bimolecular reactions [3].

According to Iwanami et al. [4] AK evolved independently at least three times. Firstly, at an early stage of phosphagen kinase evolution

(typical AK), second from the cytoplasmic creatine kinase (CK) gene later in metazoan evolution (*Stichopus* AK) and third from the mitochondrial CK gene in the course of annelid radiation (*Sabellastarte* AK). Iwanami et al. [4] also reported that three AKs (AK1, AK2 and AK3) from cherry shrimp *Neocaridina denticulate*. Among the three expressed AKs, AK2 is the typical AK, containing the conserved key residues established in *Limulus* AK for the substrate binding site and expressed among arthropods including crustaceans. AK1 and AK3 lacked some of these key residues, indicating a similar evolution to *Stichopus* AK and *Sabellastarte* AK. The size of amino acid of these three AKs ranged between 355 and 356 residues and all these AKs showed monomeric nature similar to typical AKs. Moreover, phylogenetic analysis of phosphagen kinases indicated that *N. denticulate* AK1 and AK3 diverged at the deepest branching point close to the root of the tree and formed a distinct cluster outside the typical AK cluster, which included *N. denticulate* AK2. Kinetic constants of *N. denticulate* AKs were similar to those of other AKs. However, activation energy for the transition state of AK1 and AK3 was about 1.5 fold larger than that of AK2.

Most of the AKs are monomers of 40 kDa, while the other phosphagen kinase, creatine kinase, glycoamine kinase and lombricine

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kinase are dimeric, or octameric in the case of mitochondrial CK [5,6]. In 1997 an unusual 80 kDa AK with a two-domain structure has been isolated from the primitive sea anemone *Anthopleura* sp by Suzuki et al. [7]. Interestingly, AK appears to have evolved at least twice during evolution of PKs: firstly at an early stage of phosphagen kinase evolution and secondly, from CK at later time in metazoan evolution [8]. Recently, a few researchers have also discovered AK with 2 domain structure from deep-sea clam *Calyptogena kaikoi* [9], clams *Solen strictus* and *Corbicula japonica* [10] have an molecular mass of 80 kDa AK. But their enzymatic properties were comparable to those of usual 40 kDa AKs.

Recently, a number of cDNA from cucumber *Stichopus japonicus* [13], oyster *Crassostrea* sp [5], kuruma prawn *Marsupenaeus japonicus* [6], *Callinectes sapidus* [11], deep-sea clam *Calyptogena kaikoi* [9] clams *Solen strictus* and *Corbicula japonica* [10] chiton *Liophura japonica* and turbanshell *Battilus cornutus* [12], *Cellana grata*, *Aplysia kurodai* [13], horseshoe crab *Limulus polyphemus* [14], sea Chinese shrimp *Fenneropenaeus chinensis* [15], greasy-back shrimp *Metapenaeus ensis* [16], *Litopenaeus vannamei* [17] and sea anemone *Anthopleura japonicus* [18] encoding AKs in major groups of invertebrate have been made available to investigate AK's structure, catalysis mechanism and evolutionary relationship.

A crystal structure for the transition state analog complex of the monomeric AK from horseshoe crab *Limulus polyphemus* has appeared which suggests that AKs with 2 domains have a unique substrate binding system [19]. These two arginine kinase forms show very similar kinetic properties, such as both require a divalent cation ( $Mg^{2+}$  or  $Mn^{2+}$ ) for activity. High concentrations of free  $Mg^{2+}$  will inhibit both enzymes. Although no catalytic basis for a functional difference between the two forms was found, it is possible that *in vitro* differences in stability may reflect stabilities *in vivo* and thus provide a basis for regulating arginine kinase levels in various tissues thereby giving a selective advantage to species having both forms.

In recent studies, it was demonstrated that the expression of AK correlated closely with hypoxic stress in *M. japonicus* [6], the immune response to laminarin stimulation in *F. chinensis* [15], *E. coli* LPS immune response to *L. vannamei* [17], salinity change in *C. sapidus* [20], the exposure to lead in yabby, *Cherax destructor* [21], acclimation to cadmium in crab, *Eriocheir sinensis* [22] and the defense against the infection of virus in shrimp, *Penaeus stylirostris* [23] and *F. chinensis* [24] suggesting that AK might play an important role in many biological events in crustaceans.

The Malaysian freshwater giant prawn *Macrobrachium rosenbergii* is an important commercial species. However, infectious diseases especially, infectious hypodermal and hematopoietic necrosis virus (IHHNV) have affected aquaculture of *M. rosenbergii* enormously. Thus, research into freshwater prawn defense mechanisms is important to develop disease control strategies [17,24]. Yao et al. [17] reported that AK levels changed in prawn after immune stimulant, which suggested that the prawn immune response maybe correlated with the change of energy metabolism that was regulated by AK [25] and some studies showed that AK changed significantly after prawn were infected with virus [23,24] and bacteria [17]. However, the detailed functions and characterization of AK in prawn are poorly understood. To gain insight into the characterization of AK and its role in prawn, a full length cDNA of AK-1 (designated as MrAK-1) was identified from the *M. rosenbergii* transcriptome unigenes obtained by Illumina's Solexa sequencing technology. The expression profiles of AK mRNA in the muscle of *M. rosenbergii* after IHHNV challenge were studied and the enzyme activity of recombinant AK protein was assayed.

## 2. Materials and methods

### 2.1. Identification of *M. rosenbergii* arginine kinase-1

A full length MrAK-1 gene was identified from the *M. rosenbergii* transcriptome unigenes obtained by Illumina's Solexa sequencing technology. Briefly, unigenes obtained from the assembly of the Illumina Solexa short reads from the RNA sequencing of the muscle, gill and hepatopancrease transcriptomes of *M. rosenbergii* were mined for sequences which had been identified as arginine kinase 1 gene through BLAST homology search against the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast>).

### 2.2. Bioinformatics analysis

The Basic Local Alignment Tool (BLAST) program was used to search similar nucleotide and protein sequences to the MrAK-1 [26]. The open reading frame (ORF) and amino acid sequence of MrAK-1 was obtained by using DNAssit 2.2. Characteristic domains or motifs were identified using the PROSITE profile database [27]. Identity, similarity and gap percentages were calculated using FASTA program [28]. The N-terminal transmembrane sequence was determined by DAS transmembrane prediction program (<http://www.sbc.su.se/~miklos/DAS>). Signal peptide analysis was done using the SignalP worldwide P server (<http://www.cbs.dtu.dk>). Pair-wise and multiple sequence alignment were analyzed using the ClustalW version 2 program [29]. The phylogenetic relationship of the MrAK-1 was determined using the neighbor-joining (NJ) method and MEGA 4.1 program [30].

### 2.3. *M. rosenbergii* and IHHNV challenge

Specific pathogen free (SPF) prawns (average body weight 10 g) were obtained from the Bandar Sri Sendayan, Negeri Sembilan, Malaysia. Prawns were maintained in flat-bottomed glass tanks (300 L) with aerated and filtered freshwater at  $28 \pm 1$  °C in the laboratory. All prawns were acclimatized for 1 week before challenged to IHHNV. A maximum of 15 prawns per tank were maintained during the experiment.

For IHHNV induced mRNA expression analysis, the prawns were injected with IHHNV. Briefly, IHHNV infected prawn tail tissue, tested positive by nested PCR was homogenized in sterile 2% NaCl (1:10, w/v) solution and centrifuged in a tabletop centrifuge at 5000 rpm for 5 min at 4 °C. The supernatant was filtered through 0.45 µm filter and used for injecting (100 µl per 10 g prawn) the animals. Samples were collected before (0 h), and after injection (3, 6, 12, 24 and 48 h) and were immediately snap-frozen in liquid nitrogen and stored at  $-80$  °C until the total RNA was isolated. Using a sterilized syringe, the haemolymph (0.2–0.5 ml per prawn) was collected from the prawn heart and immediately centrifuged at  $3000 \times g$  for 10 min at 4 °C to allow haemocyte collection for total RNA extraction. All samples were analyzed in three duplications and the results are expressed as relative fold of one sample as mean  $\pm$  standard deviation.

### 2.4. RNA extraction and cDNA synthesis

Total RNA was isolated from the tissues of each animal using TRI Reagent following manufacturer's protocol (Guangzhou Dongsheng Biotech, China). Total RNA was treated with RNase free DNA set (5 Prime GmbH, Hamburg, Germany) to remove the contaminating DNA. The total RNA concentration was measured spectrophotometrically (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd, England). First-strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Promega, USA) following the

manufacturer's protocol with AOLP primer (5'GGCCACGCTCGAC-TAGTAC(T)<sub>16</sub>(A/C/G)3').

## 2.5. Quantitative real-time PCR analysis of MrAK-1 mRNA expression

The relative expression of MrAK-1 in the hemocytes, pleopods, walking legs, eye stalk, gill, hepatopancreas, stomach, intestine, brain and muscle were measured by qRT-PCR. qRT-PCR was carried out using a ABI 7500 Real-time Detection System (Applied Biosystems) in 20 µl reaction volume containing 4 µl of cDNA from each tissue, 10 µl of Fast SYBR<sup>®</sup> Green Master Mix, 0.5 µl of each primer (20 pmol/µl) and 5 µl dH<sub>2</sub>O. The qRT-PCR cycle profile was 1 cycle of 95 °C for 10s, followed by 35 cycles of 95 °C for 5s, 58 °C for 10s and 72 °C for 20s and finally 1 cycle of 95 °C for 15s, 60 °C for 30s and 95 °C. The same qRT-PCR cycle profile was used for the internal control gene, β-actin. The primers used in this study are presented in Table 1. β-actin primers were designed based on EST of 1357 bp (GenBank accession No. AY651918) from *M. rosenbergii*. After the PCR program, data were analyzed with ABI 7500 SDS software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative CT method ( $2^{-\Delta\Delta CT}$  method) was used to analyse the expression level of MrAK-1 [31]. All data were given in terms of relative mRNA expressed as means ± standard deviation. For comparison of relative MrAK-1 mRNA expression, statistical analysis was performed using one-way ANOVA and mean comparisons were performed by Tukey's Multiple Range Test using SPSS 11.5 at 5% significant level.

## 2.6. In vitro expression construction

All of the cloning experiments were carried out according to Sambrook et al. [32] with slight modifications [33]. The primer set of MrAK-1 F5, MrAK-1 R6 was designed with the corresponding restriction enzyme sites for *EcoRI* and *HindIII* at the N- and C-termini for MrAK-1, respectively (Table 1) in order to clone the coding sequence into the expression vector, pMAL-c2X (New England Biolabs UK Ltd, United Kingdom). Using plasmid DNA of MrAK-1 as a template and Taq DNA polymerase (Invitrogen Bio-Services India Pvt. Ltd, Bangalore, India), PCR was carried out to amplify the coding sequence. The PCR product was purified using the QIAquick Gel Extraction Kit (QIAGEN India Pvt. Ltd., New Delhi, India). Then, both insert and vector were digested with the respective restriction enzymes. The ligated product was transformed into XL1 blue cells and the correct recombinant product (as confirmed by restriction enzyme digestion and sequencing) was transformed into competent *E. coli* BL21 (DE3) cells for protein expression.

## 2.7. In vitro purification of recombinant protein MrAK-1

Transformed *E. coli* BL21 (DE3) cells were incubated in ampicillin (100 µg/mL) Luria broth (LB) overnight. This culture was then used to inoculate 100 mL of LB broth in 0.2% glucose-rich medium

with ampicillin at 37 °C until cell density reached 0.7 at OD<sub>600</sub>. *E. coli* BL21 (DE3) harboring pMAL-c2x-MrAK-1 was induced for over expression with 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and incubated at 15 °C for 4 h. Cells were harvested by centrifugation (4000 × g for 20 min at 4 °C). *E. coli* BL21 (DE3) uninduced culture was used as a negative control. Then the cells were resuspended in column buffer (Tris–HCl, pH 7.4, 200 mM NaCl) and frozen at –20 °C overnight. After thawing on ice, cells were disrupted by sonication. The crude MrAK-1 fusion protein fused with maltose binding protein (MBP) was purified using pMAL<sup>TM</sup> protein fusion and purification system protocol (New England Biolabs UK Ltd, United Kingdom). Further the fusion protein was digested with thrombin and purified as described by Yao et al. [17], then the purity of the expressed enzyme was verified by 12% SDS-PAGE and the molecular weight of target protein was evaluated using protein molecular weight standards. Proteins were visualized by staining with 0.05% Coomassie blue R-250. The purified enzyme was kept at –80 °C until determination of enzymatic activity.

## 2.8. Enzyme assay and protein determination

MrAK-1 activity was assayed using a modification of previous procedures [17, 34]. For the phosphate assay, the absorbance was measured using a UV-spectrophotometer. Appropriate controls consisted of assay samples lacking the prepared MrAK-1. Inorganic phosphate solutions were used to prepare a calibration curve. Briefly, the assay was performed using a stock solution prepared by combining 5.7 mM arginine, 6.6 mM magnesium acetate, complex acid-base indicator (consisting of 0.15% g/ml Thymol blue and 0.025% g/ml Cresol red), and 53.2 mg ATP, then adjusting the pH to 8.5 with 1 M NaOH. The reaction solution was pre-incubated for 10 min at 30 °C, prior to initiation of the enzymatic reaction upon the addition of 10 µl to 1 ml of reaction solution. The reaction progress was followed as a liner decrease in absorbance measured at 575 nm for 30 s using an analytic spectrophotometer Specord 200 UV VIS (Coslab, India). The slope was multiplied by the time to calculate the absorbance decrease. All measurements were carried out at 30 °C.

## 2.9. Measurement of optimum temperature, pH and MrAK-1 activity

For determination of the optimum temperature, aliquots of the purified recombinant MrAK-1 were incubated with standard buffer and assayed as described by Wang et al. [16] with the exception that the enzymatic activities were determined at different temperature gradients. For determination of optimum pH, the purified recombinant MrAK-1 preparation was added to various buffers with pH adjusted from 5.0 to 12.0, then incubated for 30 min at 30 °C prior to the recombinant MrAK-1 activity assay as described by Wang et al. [16].

To investigate the effect of some energy metabolism products on MrAK-1 activity, experiments were carried out [17] by supplementation with glucose, ATP, α-ketoglutarate, citrulline, ornaline or glycerol

**Table 1**  
Details of the primers used in this study.

| Name         | Target                   | Sequence (5'–3' direction)                  |
|--------------|--------------------------|---------------------------------------------|
| MrAK-1 (F1)  | qRT-PCR amplification    | aagatcaagggaacatcaacgcc                     |
| MrAK-1 (R2)  | qRT-PCR amplification    | aggcgctcttcatgaggttctcaa                    |
| β-actin (F3) | qRT-PCR internal control | accaccgaaattgctccatctct                     |
| β-actin (R4) | qRT-PCR internal control | acggctactgttcacatcgccatt                    |
| MrAK-1 (F5)  | ORF amplification        | GAGAGAGAATTaccgcgagaagatgacacagatca (EcoRI) |
| MrAK-1 (R6)  | ORF amplification        | GAGAGAAAGCTTactctgctgtgctgccatct (HindIII)  |

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ATGGGCGAACCATTCCCTGATATCAAGAGCAAGCACTCCCTGGTGGCCAAGCACGTCACCAAGGAGCGCTGGGAGAACT 80
m--g--e--p--f--p--d--i--k--s--k--h--s--l--v--a--k--h--v--t--k--e--r--w--e--k--l-- 27
CTCCGGCCACAAGACGCCACCTCCGGCTTCAACCTCAAGCAGGCCATTGCCTGCGCCGTCGAGTTCGACAACCAAGCACT 160
--s--g--h--k--t--a--t--s--g--f--t--l--k--q--a--i--a--c--a--v--e--f--d--n--q--h--c 54
GCGGCATCTACGCCGTGACTGGGATTCCTACAAGGATTCAGGATGTGTTCGACCCCATCATCCAGGAATACCATGGC 240
--g--i--y--a--g--d--w--d--s--y--k--d--f--k--d--v--f--d--p--i--i--q--e--y--h--g-- 80
ATCTCCCCCGATGCCGTCCACACCTCCGACATGGAAGTCGAGAAGATCAAGGGCAACATCAACGCCAAGTTCCTCGTCCA 320
i--s--p--d--a--v--h--t--s--d--m--e--v--e--k--i--k--g--n--i--n--a--e--v--p--v--h-- 107
CTCCGTCAGGATCCGCGTCGGCCGTAGCATCGATGGCTTCGGTCTTCCCCAGGCATCACCAGGAACAGCGCATGGCG 400
--s--v--r--i--r--v--g--r--s--i--d--g--f--g--l--s--p--g--i--t--k--e--q--r--i--g--v 134
TTGAGAACCTCATGAAGAGCGCTTCGGCAAGCTCTCCGAGATCTCGCCGGCACTACTACCCCTGACTGGCATGGAC 480
--e--n--l--m--k--s--a--f--g--k--l--s--g--d--l--a--g--n--y--y--p--l--t--g--m--d-- 160
GAGAAGGTCGCCAGCAGCTCGTCGATGACCACTTCTCTTCATGTCTGGTGATCGCAACCTTCAGGTGCGAGGTATGGA 560
e--k--v--r--q--q--l--v--d--d--h--f--l--f--m--s--g--d--r--n--l--q--v--a--g--m--e-- 187
GCGCGACTGGCTGAAGGCCGCGGTATCTACCACAATGCCGAGAAGACCTTCTTGGTCTGGGTCAACGAGGAGGATCAGC 640
--r--d--w--p--e--g--r--g--i--y--h--n--a--e--k--t--f--l--v--w--v--n--e--e--d--q--l 214
TCAGGATCATCTCCATGCAGATGGGCGGTGATGTCAGGGGCGTCTTCGAGCGCCTGGCCCGGTATCAAGGCCGTCGGT 720
--r--i--i--s--m--q--m--g--g--d--v--r--g--v--f--e--r--l--a--r--g--i--k--a--v--g-- 240
GACTCCGTCAAGGCCGAGAGCGGCAAGGACTTCATGCTCGACCCCAAGTACGGCTTCGTCCACTCCTGCCCAACCACT 800
d--s--v--k--a--e--s--g--k--d--f--m--l--d--p--k--y--g--f--v--h--s--c--p--t--n--l 267
TGGCACCGGCATGAGGGCTTCGTCACGTTGACTTGCCCGGTGGACCAAGGAAGTCTTGACGCCCTGAAGAAGAGAT 880
g--t--g--m--r--a--s--v--h--v--d--l--p--g--w--t--k--e--g--l--d--a--l--k--k--r--c 294
GTGAAGAACTGAAGGTTACGCCCCGTGGTACCCGCGGTGAATCCGCGGCCAGACTGGTCACACTTATGACATCTCCAAC 960
--e--e--l--k--v--q--p--r--g--t--r--g--e--s--g--g--q--t--g--h--t--y--d--i--s--n-- 320
AAGCATCGCTGGGTACTCCGAGGTCAAGTCTCGATGATGACGGTGTCAATACCTGTACGCTGAGGACGT 1040
k--h--r--l--g--y--s--e--v--e--l--v--q--c--m--i--d--g--v--n--t--l--y--a--e--d--v-- 347
TGCCCTCCAGAAGAAGCAGGCATCTAA 1068
--a--l--q--k--k--h--g--i--* 355

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**Fig. 1.** Nucleotide and deduced amino acid sequences of *M. rosenbergii* arginine kinase-1 (MrAK-1). The nucleotide sequence is numbered from 5' end, and the single letter amino acid code is shown below the corresponding codon. The start codon (ATG) and the end codon (TAA) highlighted in fluorescent green color. Potential ATP:guanido phosphotransferases active site highlighted in blue color. Six protein kinase C phosphorylation sites are highlighted in grey color. The termination code is marked with an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the reaction buffer at a final concentration of 10, 50, 100 and 200 mM, respectively, in sealed glass vials containing 20 µl of enzyme in reaction buffer, pH 8.5. Each was with three replicates and the mixtures were withdrawn at appropriate times and immediately assayed for enzyme activity at 30 °C. Statistical analysis was performed using one-way ANOVA and mean comparisons were performed by Tukey's Multiple Range Test using SPSS 11.5.

### 3. Results

#### 3.1. MrAK-1 sequence analysis

MrAK-1 was identified from the *M. rosenbergii* transcriptome unigenes obtained by Illumina's Solexa sequencing technology. The MrAK-1 sequences was submitted to GenBank under the accession number HQ191218. The nucleotide and deduced amino acid sequences of the MrAK-1 from rock bream are shown in Fig. 1.

MrAK-1 full length nucleotide consisted of 1068 base pair (bp) with an open reading frame (ORF) of 1065 bp. The ORF is capable of encoding a polypeptide of 355 amino acid (aa) with an estimated molecular mass of 40 kDa and a predicted isoelectric point (pI) of

**Table 2**

Pair-wise ClustalW analysis and comparisons of the deduced amino acid sequence of MrAK-1 with other known species. The GenBank accession number, gene and species details are given in the legend of Fig. 2.

| Species                  | Amino acid length | Identity (%) | Similarity (%) | Gap (%) |
|--------------------------|-------------------|--------------|----------------|---------|
| <i>N. denticulata</i> I  | 355               | 96           | 98             | 0       |
| <i>N. denticulata</i> II | 355               | 85           | 93             | 0       |
| <i>L. salmonis</i>       | 354               | 82           | 90             | 0       |
| <i>C. rogercresseyi</i>  | 405               | 78           | 88             | 0       |
| <i>C. clemensi</i>       | 357               | 78           | 88             | 0       |
| <i>M. ovata</i>          | 389               | 52           | 64             | 1       |
| <i>C. gracilis</i>       | 382               | 51           | 66             | 2       |

6.1. MrAK-1 aa sequence neither have a signal peptide nor a trans-membrane regions. The ExPASy motif analysis of MrAK-1 aa sequence contains a potential ATP:guanido phosphotransferases active site (Cys<sup>263</sup>-Pro<sup>264</sup>-Thr<sup>265</sup>-Asn<sup>266</sup>-Leu<sup>267</sup>-Gly<sup>268</sup>-Thr<sup>269</sup>), 6 protein kinase C phosphorylation sites and 6 caesin kinase II phosphorylation sites (<sup>63</sup>s-y-k-d<sup>66</sup>; <sup>89</sup>s-d-m-e<sup>92</sup>; <sup>157</sup>t-g-m-d<sup>160</sup>; <sup>247</sup>s-g-k-d<sup>250</sup>; <sup>304</sup>t-r-g-e<sup>307</sup> and <sup>327</sup>s-e-v-e<sup>330</sup>). A long ADP binding site is available in the aa sequence between 108 and 318. Another long phosphagen binding site is available in the aa sequence between 51 and 262. Additionally a small substrate specificity loop is also available in the aa sequence between 300 and 310.

The MrAK-1 amino acid identity and similarity percentages were calculated using FASTA program. We analyzed the MrAK-1 sequence identity with other seven homologous arginine kinase including arginine kinase 1 and 3 from cherry shrimp *Neocaridina denticulata* and argininie kinase from *Lepeophtheirus salmonis*, *Caligus rogercresseyi*, and *C. clemensi* and from euglena *Monosiga ovata* and *Codonosiga gracilis* (Table 2). Their respective aa sequence identity with MrAK-1 is as follows: 96%, 85%, 82%, 78%, 78%, 52% and 51%. In addition, MrAK-1 was aligned with the same seven homologous sequences using ClustalW multiple analyses (Fig. 2). The results revealed that *C. rogercresseyi* has the longest amino acid

|                                     |                                                                   |
|-------------------------------------|-------------------------------------------------------------------|
| <i>Macrobrachium rosenbergii</i>    | -----MGEFFPDIKS 10                                                |
| <i>Neocaridina denticulata</i> I    | -----MGEFFPDIKS 10                                                |
| <i>Neocaridina denticulata</i> III  | -----MGEFFPDIKS 10                                                |
| <i>Caligus rogercresseyi</i>        | MAEQGGFSHYFNSTTIKGRANSKATLAVVGGAFAFYKFFKGNKTNLKAESAAPPFNIRS 60    |
| <i>Caligus clemensi</i>             | -----MSEVPFPPTIRS 12                                              |
| <i>Lepeophtheirus salmonis</i>      | -----MSDFPDIKS 9                                                  |
| <i>Monosiga ovata</i>               | -----MEDTYSLLLDIHERVKKIERNLGIKPGKKEAYG-AGFPSPFNE 42               |
| <i>Codonosiga gracilis</i>          | -----YAGIEARLSAIESSL---PGNGSPSVIDGYPRPFE 32                       |
|                                     | : * : .                                                           |
| <i>Macrobrachium rosenbergii</i>    | K-HSLVAKHVTKERWDLKLAHKATSGFTLKQAIACAVEFDNQHCIGIYAGDWSYKDFKD 69    |
| <i>Neocaridina denticulata</i> I    | K-HSLVAKHVTKERWDLKLAHKATSGFTLKQAIACAVEFDNQHCIGIYAGDWSYKDFKD 69    |
| <i>Neocaridina denticulata</i> III  | K-HSLVAKVYTKDLWDLKSGIKTATSGFTLGKAIACAVDFDNQHCIGIYAGDWSYKDFKD 69   |
| <i>Caligus rogercresseyi</i>        | G-HSLVAKHVTKERWDLKSGIKTATSGFTLAKAIACAVEFDNQHCIGIYAGDWSYADFAE 119  |
| <i>Caligus clemensi</i>             | S-HSLVAKHVTKERWDLKSGIKTATSGFTLAKAIACAVEFDNQHCIGIYAGDWSYADFAE 71   |
| <i>Lepeophtheirus salmonis</i>      | K-HSLVAKHVTKERWDLKSKIVTKTSGFTLAKAIACAVEFDNQHCIGIYAGDWSYKDFGE 68   |
| <i>Monosiga ovata</i>               | KGRSLKLYLTIDVVEQLKDKVTP-NGFTLERAISQGVNDQSGVGLYAGDADATYTFAP 101    |
| <i>Codonosiga gracilis</i>          | KCSLLKHLNSVEYLECAGRITP-SGFTLENVMQSGVDNFDGSGVGCYAGDESYTFAP 91      |
|                                     | ** : : : : : * .**** .. : . : . * **** : *                        |
| <i>Macrobrachium rosenbergii</i>    | VFDPIIQEYHG-ISPDAVHTSDMEVEKIKGNINA-EVPVHSVRIRVGRSIDGFLSPGIT 127   |
| <i>Neocaridina denticulata</i> I    | VFDPIIQEYHG-ISPDAVHTSDMEVEKIKGNINT-EVPVHSVRIRVGRSIDGFLSPGIT 127   |
| <i>Neocaridina denticulata</i> II I | VFDPIIQDYHG-IASDSKHTSDMDVGKLGKGNINP-ECVPVHSVRIRVGRNIDGFLSPGIT 127 |
| <i>Caligus rogercresseyi</i>        | VFIPLICEYHG-VPPSFHSHTSDMDSGKIKGNINP-DAPVHSTRIRVGRSIQGFGLSPGIT 177 |
| <i>Caligus clemensi</i>             | VFLPIICEYHG-VPPSFHSHTSDMDSVKINGNINP-DAPVHSTRIRVGRSIQGFGLSPGIT 129 |
| <i>Lepeophtheirus salmonis</i>      | VFLPIICEYHG-LPADFKHTSDMDSVKIKGNVNS-EVPVHSTRIRVGRSIEGFGLSPGIT 126  |
| <i>Monosiga ovata</i>               | LFDAVIDYHGGYKPTDKHVSMDVSKVGNPDPTGEDVSTRIRVGRNIRGLGLSPGIT 161      |
| <i>Codonosiga gracilis</i>          | LFDRVIDYHGGYKPTDKHVSMDASKLHGSVDP--DYVISTRIRVGRNIRGLGLSPGITS 149   |
|                                     | : * : * : . . * * : : : . . * * * : * : * : *                     |
| <i>Macrobrachium rosenbergii</i>    | KEQRIGVENLMKSAFGLKSGDLAGNYPLTGMDEKVRQQLVDDHFLFMSGDRNLQVAGME 187   |
| <i>Neocaridina denticulata</i> I    | KEQRIGVENLMKSAFGLKSGDLGNYPLTGMDEKVRQQLVDDHFLFMSGDRNLQVAGME 187    |
| <i>Neocaridina denticulata</i> III  | RQRLGIVENLMKHAQKLTGDLAGNYPLTGMDEKVRQQLVDDHFLFMSGDRNLQVAGME 187    |
| <i>Caligus rogercresseyi</i>        | KQRLDVESLMKTAFAKGLKSGSYPLTGMDEATRQQLVDDHFLFMSGDRNLKVGME 237       |
| <i>Caligus clemensi</i>             | QQRRLDVESLMKTAFAKGLKSGSYPLTGMDEATRQQLVDDHFLFMSGDRNLKVGME 189      |
| <i>Lepeophtheirus salmonis</i>      | QQRRLDVESLMKNAFKLPEGLNGNYPLTGMDEKVRQQLVDDHFLFMSGDRNLTVAGME 186    |
| <i>Monosiga ovata</i>               | RSQRREVERVVTGLSTFTGDLKGYYPPLGKMTAEARKQLVEDHFLFKKGRDFLESAGAN 221   |
| <i>Codonosiga gracilis</i>          | RAQRVRESILVDALSKVDGDLGKYYSLGNMSEEDRKQLVADHFLFKKGRDFLESAGAN 209    |
|                                     | : * * : : : . . . * * : . . * * * * * * * * * * * * * * *         |
| <i>Macrobrachium rosenbergii</i>    | RDWPEGRGIYHNAEKTFLVWVNEEDQLRIISMQGGDVGRVFERLARGIKAVGDSVKAES 247   |
| <i>Neocaridina denticulata</i> I    | RDWPEGRGIYHNAEKTFLVWVNEEDQLRIISMQGGDVGRVFERLARGIKAVGDSVKAES 247   |
| <i>Neocaridina denticulata</i> III  | REWPEGRGIYHNAEKTFLVWVNEEDQLRIISMQGGDVKAVFERLARGIKAVGDSVKAES 247   |
| <i>Caligus rogercresseyi</i>        | RDWPEGRGIYHNAEKTFLVWVNEEDQLRIISMQGGDVKGVFERLARGIKAVGDSVKAES 297   |
| <i>Caligus clemensi</i>             | RDWPEGRGIYHNAEKTFLVWVNEEDQLRIISMQGGDVKGVFERLARGIKAVGDSVKAES 249   |
| <i>Lepeophtheirus salmonis</i>      | RDWPEGRGIYHNAEKTFLVWVNEEDQLRIISMQGGDVKGVFERLARGIKAVGDSVKAES 246   |
| <i>Monosiga ovata</i>               | RDWPEGRGIYHNAEKTFLVWVNEEDQMRIISMQGGDVKGVFERLARGIKAVGDSVKAES 280   |
| <i>Codonosiga gracilis</i>          | RDWPEGRGIYHNAEKTFLVWVNEEDQARIISMQGGDVKGVFERLARGIKAVGDSVKAES 268   |
|                                     | * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *     |
| <i>Macrobrachium rosenbergii</i>    | GKDFMLDPKYGVFVHSCPTNLGTGMRASVHVDLPGWTKEGDLALKRCEELKVQPRGTRGE 307  |
| <i>Neocaridina denticulata</i> I    | GKDFMLDSKYGVFVHSCPTNLGTGMRASVHVDLPGWTKEGDLALKRCEELKVQPRGTRGE 307  |
| <i>Neocaridina denticulata</i> III  | GKEFAFDEKYGFHSCPTNLGTGMRASVHVDLPGWTKEGDLALKRCEELKVQPRGTRGE 307    |
| <i>Caligus rogercresseyi</i>        | GKEFLDPKLYLHSCPTNLGTGMRASVHVDLPGWTKEGDLALKRCEELKVQPRGTRGE 357     |
| <i>Caligus clemensi</i>             | GKEFLDPKLYLHSCPTNLGTGMRASVHVDLPGWTKEGDLALKRCEELKVQPRGTRGE 309     |
| <i>Lepeophtheirus salmonis</i>      | GKDFMLDPKLYLHSCPTNLGTGMRASVHVDLPGWTKEGDLALKRCEELKVQPRGTRGE 306    |
| <i>Monosiga ovata</i>               | GYESYNDHLGFIHSCPTNLGTGMRASVHVDLPVNSKH--ANFHAWCCKLRQLQPRGIHGE 338  |
| <i>Codonosiga gracilis</i>          | GYESYNDHLGFIHSCPTNLGTGMRASVHVDLPVNSKH--PDFKNWCEKLRQLQPRGIHGE 326  |
|                                     | * : : : : * : * : * : * : * : * : * : * : * : * : * : * : *       |
| <i>Macrobrachium rosenbergii</i>    | SGGQTGHTYDISNKHRLGYSEVELVQCMIDGVNTLYAEDVALQKKHGI----- 355         |
| <i>Neocaridina denticulata</i> I    | SGGQTGHTYDISNKHRLGYSEVELVQCMIDGVNTLYAEDVALQKKHGI----- 355         |
| <i>Neocaridina denticulata</i> III  | SGGQTGVTYDISNKHRLGYSEVELVQCMIDGVNLAHEEDIKLQKKHGL----- 355         |
| <i>Caligus rogercresseyi</i>        | SGGQTGVTYDISNKHRLGYSEVELVQTMIDGVNTLFKEDLELKKHSL----- 405          |
| <i>Caligus clemensi</i>             | SGGQTGVTYDISNKHRLGYSEVELVQTMIDGVNTLYKEDLELKKHGL----- 357          |
| <i>Lepeophtheirus salmonis</i>      | SGGQTGHTYDISNKHRLGYSEVELVQTMIDGVNTLYKEDLEFQKKHGM----- 354         |
| <i>Monosiga ovata</i>               | HSETEGGVYDISNKHRLGYSEVELVQTMIDGVNTLYNAEKALAAAG--KQPPAL-- 389      |
| <i>Codonosiga gracilis</i>          | HSESEGGVYDISNKHRLGYSEVELVQTMIDGVNTLYNAEKALAAAG--KQPPAL-- 382      |
|                                     | . * . * . * . * . * . * . * . * . * . * . * . * . * . * . *       |

**Fig. 2.** Multiple sequence alignments of MrAK-1 with seven other homologous arginine kinase amino acid sequences. Argininie kinase 1 of cherry shrimp *N. denticulata* I (BAH56608), argininie kinase 3 of *N. denticulata* III (BAH56610), arginine kinase of sea louse *L. salmonis* (ACO11785), *C. rogercresseyi* (ACO11152), *C. clemensi* (ACO15761), euglena *M. ovata* (ABN49969) and *C. gracilis* (ABN49968) are shown. Asterisk marks indicates identical amino acids and numbers to the right indicate the aa position of arginine kinase in the corresponding species. Conserved substitutions are indicated by (:) and semi-conserved substitutions are indicated by (.) Deletions are indicated by dashes. GenBank accession numbers for the amino acid sequences of arginine kinase given in the parentheses.

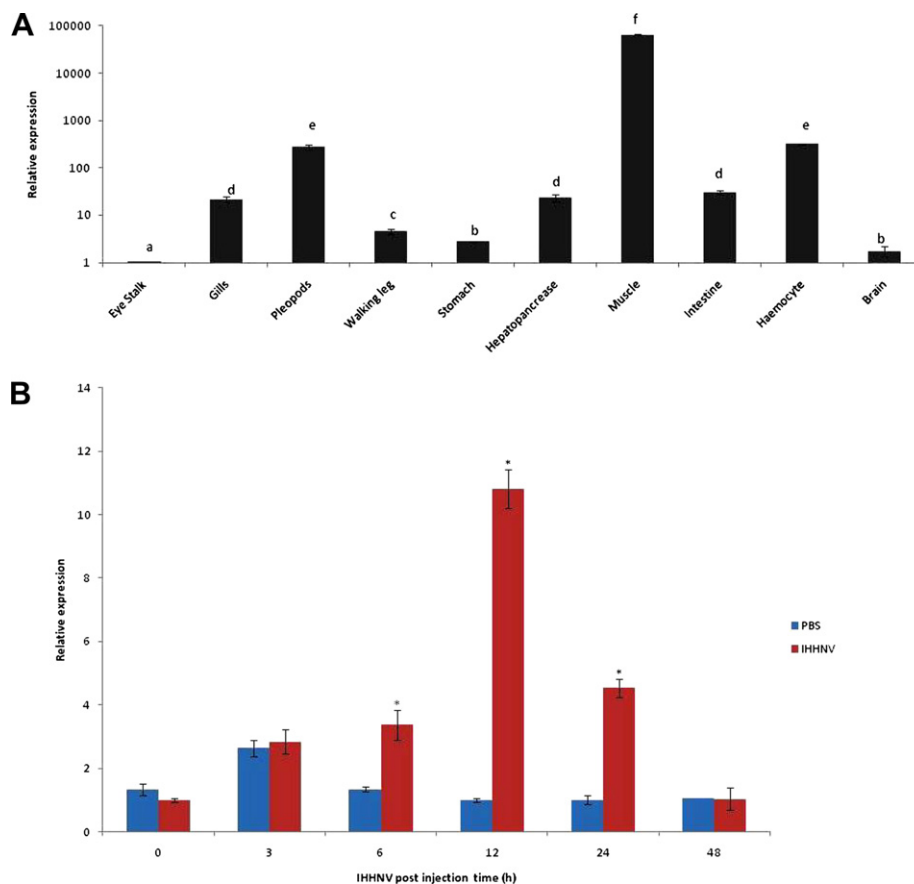


**Fig. 3.** Gene expression patterns of MrAK-1 by qRT-PCR. 3A: Tissue distribution of MrAK-1 in different tissues of *M. rosenbergii*. Data are expressed as a ratio to MrAK-1 mRNA expression in eye stalk. The different alphabets are statistically significant at  $P < 0.05$  level by one-way ANOVA and Tukey's Multiple Range Test. 3B: The time course of MrAK-1 mRNA expression in muscle at 0, 3, 6, 12, 24, and 48 h post-injection with IHNV. Data are expressed as a ratio to MrAK-1 mRNA in sample from unchallenged control group. The significant difference of MrAK-1 expression between the challenged and the control group were indicated with asterisks.

sequence (405 aa), even though conserved motifs were observed among the sequences, the length of the amino acids varied from species to species.

Phylogenetic analysis was performed using full length amino acid sequence of MrAK-1 and other 7 representatives [arginine kinase 1 of cherry shrimp *Neocardina denticulata* (BAH56608), arginine kinase 3 of *N. denticulata* (BAH56610), arginine kinase of sea louse *Lepeophtherirus salmonis* (ACO11785), *Caligus rogercrescey* (ACO11152), *Caligus clemensi* (ACO15761), euglena *Monosiga ovata* (ABN49969) and *Codonosiga gracilis* (ABN49968)]. The results of phylogenetic tree showed three distinct clusters of decapoda, copepoda and choanoflagellida (data not shown). MrAK-1 closely clustered with the homologous AK-1 from cherry shrimp *Neocardina denticulata* and formed a sister group with AK-3 from *N. denticulata* and finally clustered together with the AK from copepods. The results of the phylogenetic tree are agreeable with the traditional taxonomy.

### 3.2. MrAK-1 mRNA expression in tissues

The mRNA transcripts of MrAK-1 could be detected by quantitative real-time RT-PCR mainly in muscle (Fig. 3A). The largest quantity of MrAK-1 was shown in muscle, then haemocyte, pleopods, intestine, hepatopancrease and gills, and the expression of MrAK-1 in walking leg, stomach, brain and eye stalk was very low. Further analysis results showed that MrAK-1 mRNA expression was significantly higher ( $P < 0.05$ ) in the muscle. Therefore, muscle tissue was selected to investigate the temporary expression of MrAK-1 gene after IHNV challenge.

### 3.3. MrAK-1 mRNA expression in muscle after IHNV challenge

To analyze the expression profile of *M. rosenbergii* MrAK-1 during infection, prawns were infected with IHNV and the muscle was analyzed by real-time PCR (Fig. 3B). The levels of MrAK-1 mRNA transcripts sharply increased until 12 h post-injection, a slight

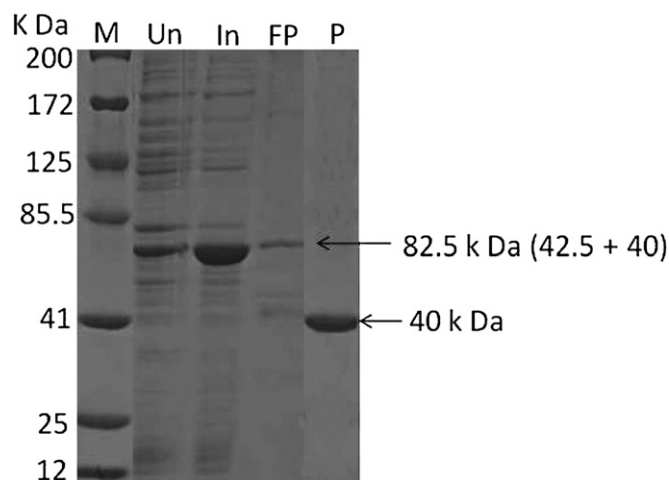


**Fig. 4.** Expression and purification of the recombinant MrAK-1 protein. Protein samples were separated by SDS-PAGE and stained with Coomassie brilliant blue. M, protein marker; Un, before induction with IPTG; In, after IPTG induction; FP, purified fusion protein and P, purified recombinant protein.

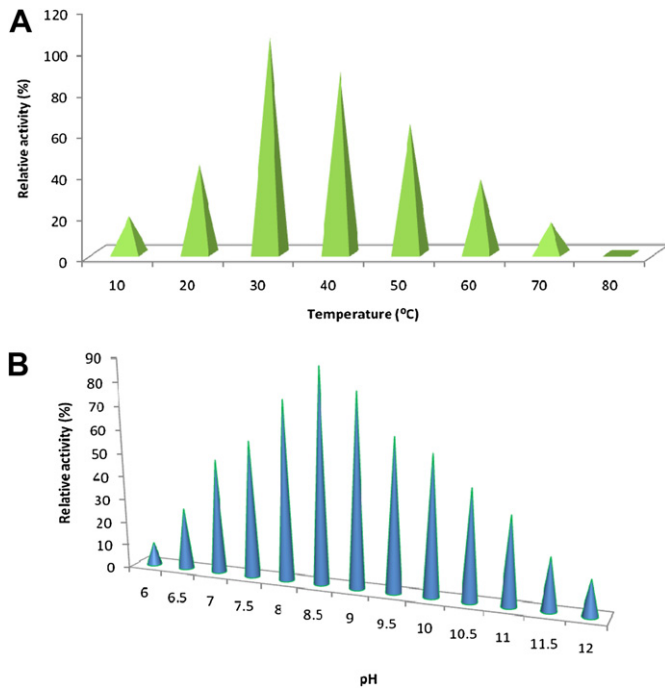


**Fig. 5.** The effect of temperature and pH on the activity of expressed MrAK-1. 5A: The assay systems were incubated for 30 min in the various temperatures, then added the enzyme to the system determining the enzyme activity. 5B: The assay systems were incubated at 30 °C for 5 min before the enzyme activity was determined. The pH 6.0–6.5 was sodium citrate–citrate buffer, pH 7.0–10.0 were Tris–HCl buffer and pH 10.0–12.0 were glycine–NaOH buffer.

decrease of MrAK-1 mRNA expression appeared at 24 h and at 48 h the expression was reached almost near to the basal level. Significant differences ( $P < 0.05$ ) in expression were found at 6, 12 and 24 h post-injection between the IHNV challenged and the PBS injected control group.

### 3.4. Expression and purification of recombinant MrAK-1 protein

The putative mature MrAK-1 molecule cDNA was expressed in *E. coli* cells after cloning the cDNA into the EcoRI and HindIII restriction sites of pMAL-c2x-MrAK-1 expression vector, IPTG driven expression of MrAK-1 was done in *E. coli* BL 21 (DE3) cells. The recombinant MrAK-1 was purified from the supernatant of induced cells. Fig. 4 (lane PF) shows the result of SDS-PAGE of the recombinant MrAK-1 along with fusion protein, the recombinant enzyme gave a major single band with molecular mass around 82.5 kDa (42.5 kDa for MBP and 40 kDa for MrAK-1 enzyme), suggesting that the enzyme is purified enough to determine its characterization. After digestion by thrombin, the recombinant MrAK-1 protein showed a single band with molecular weight about 40 kDa (Fig. 4).

### 3.5. Optimum temperature and pH for MrAK-1 activity

The optimum temperature was determined using the purified recombinant MrAK-1 protein along with the buffer. The assay results showed that the optimum temperature for MrAK-1 activity is 30 °C (Fig. 5A). And also we used the purified recombinant MrAK-1 protein along with various pH (5.0–12.0) buffers to determine the optimum pH. During the assay the recombinant MrAK-1 protein showed the highest activity in the synthesis direction of phosphoarginine and ADP at pH 8.5 (Fig. 5B), and this is taken into account as optimum pH for MrAK-1 activity.

### 3.6. MrAK-1 activity assay

The results of MrAK-1 enzyme activity and characterization assay are presented in Fig. 6. The purified recombinant MrAK-1 was used to determine its characterization. Following treatment with different energy metabolism products, MrAK-1 activity showed some changes (Fig. 6). MrAK-1 activity did not show significant variation after supplementation with 10 mM glucose. However, MrAK-1 activity decreased significantly when glucose concentration was higher than 50 mM ( $P < 0.05$ ) and almost all MrAK-1 activity was lost after treatment with 200 mM glucose ( $P < 0.05$ ).

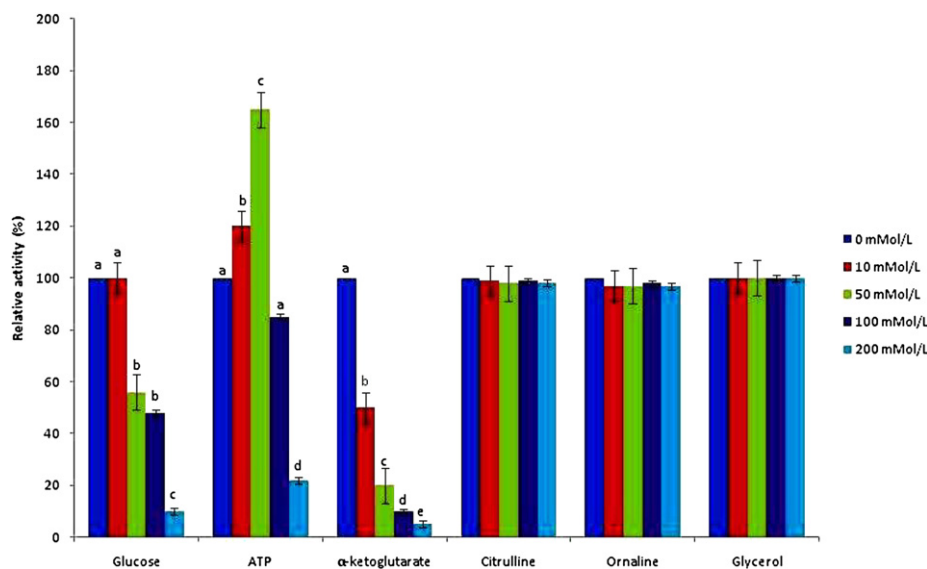


**Fig. 6.** Effect of different energy metabolism products, include 10, 50, 100 and 200 mM glucose, ATP, α-ketoglutarate, citrulline, ornithine and glycerol, on MrAK-1 activity. Each test was carried out in sealed glass vials containing 20 μl of enzyme in reaction buffer with different concentration of energy metabolism products at 30 °C. The data were analyzed by using SPSS 11.5. software. The significant difference of MrAK-1 activity between the treatment and the control group of each metabolic product were indicated with alphabets. The different alphabets are statistically significant at  $P < 0.05$  level by one-way ANOVA and Tukey's Multiple Range Test.

MrAK-1 activity was significantly ( $P < 0.05$ ) higher than that of the control after treatment with 10 mM and 50 mM ATP, however, MrAK-1 activity dropped significantly following incubation with 200 mM ATP. The enzyme activity was inhibited by  $\alpha$ -ketoglutarate at the concentration between 10 and 200 mM, and the inhibition was in direct ratio to the  $\alpha$ -ketoglutarate concentration ( $P < 0.05$ ). MrAK-1 activity did not show significant variation after incubated with 10–200 mM citrulline, ornaline and glycerol.

#### 4. Discussion

AK plays a key role in the coupling of energy production and utilization in animals [35]. Several AK from shrimps such as *Mar-supenaeus japonicus* [6], *Fenneropenaeus chinensis* [15], *Metapenaeus ensis* [16], *Litopenaeus vannamei* [17,26], *Penaeus japonicus* [36], *P. aztecus* [37] and *P. monodon* [38] were purified, cloned or identified. In this study, a full-length cDNA of MrAK-1 was identified and sequenced from *M. rosenbergii*. An ORF of 1065 bp that coded for 355 aa was obtained (Fig. 1), which showed a high degree of similarity to known arginine kinase in shrimps and to other predicted proteins (Fig. 2 and Table 2). This finding supported a number of previous studies that provided evidence for an excellent conservation of both primary and secondary structures amongst phosphagen kinases [15,17,37,39]. Three conserved domains such as ATP:guanido phosphotransferases domain, ADP binding domain and phosphagen binding domain were predicted in the sequence, which corresponded to other AK [17,37].

A phylogenetic tree was constructed based on the amino acid sequence of MrAK-1 and other homologous sequences from decapoda, copepoda and choanoflagellida. The genetic distance among the species taken for phylogenetic analysis is 0.1. In the phylogenetic tree, MrAK-1 was clustered and had high similarity with AK-1 from *N. denticulata*, together with the structural features, indicating that MrAK-1 should be an isomer of arginine kinase. Although MrAK-1 and AK from the copepoda and choanoflagellida, their sequences and structural characteristic was disparate, and clustered separately in the phylogenetic tree, it may be due to their taxonomical differentiation as reported by Wang et al. [16].

In general, most research about AK has focused on the AK characterization and functions in muscle or gill in crustaceans [17,21,38–40]. In the present study, MrAK-1 transcripts were observed in most examined tissues in *M. rosenbergii* with the most predominant expression being in muscle (Fig. 3 A), which is similar with the AK in other shrimp species [15,17,38–41]. Previous research indicated that the variation of AK expression might be related to some external factors in invertebrates. It was reported that AK expression and activity change correlated closely with the salinity change in crabs [21,40]. AK concentration and specific activity showed significant variation after exposure to toxic and hypoxia stress in shrimps [6], shellfish lobster [21] and crabs [20]. Furthermore, AK also changed in shrimp after immune stimulation. For example, AK protein changed significantly in shrimp *F. chinensis* hemolymph after injection with laminarin [15]. Astrofsky et al. [23] demonstrated that AK transcript expression changed in shrimp *P. stylirostris* and *F. chinensis* [24] after white spot virus infection.

In this study, the mRNA expression of MrAK-1 was detected by qRT-PCR, which is the first report of it in shrimp muscle after IHNV challenge (Fig. 3B). At 12 h after IHNV challenge, the increased MrAK-1 expression in muscle may due to the up-regulation of gene expression, proliferation or recruitment of MrAK-1 expressing cells in the tissue. The pattern gene expression after IHNV challenge, showing that MrAK-1 might be involved in a transient systemic immune response to the stimulation inflicted by the immune challenge and the invasion of heterogenous substances as reported by Yao et al. [17].

This MrAK-1 sequence was validated by the pMAL-c2x-MrAK-1 expression vector and expressed in *E. coli* as fusion protein. Recombinant MrAK-1 was purified to homogeneity using pMAL<sup>TM</sup> protein fusion and purification system followed by digestion with thrombin. The molecular mass of protein was about 40 kDa on 12% SDS-PAGE gel (Fig. 4), similar to the earlier reported AK of Abe et al. [6], Holt and Kinsey [9], Yao et al. [15], Wang et al. [16], Yao et al. [17], Tada and Suzuki [18], Kinsey and Lee [20], Astrofsky et al. [23] and Wang et al. [24].

The optimum temperature and pH for MrAK-1 enzyme activity is 8.5 and 30 °C respectively (Fig. 5 A and B). This optimum levels are quite different from the optimum pH (7.5–8.0) reported by Qing-Yun et al. [41] for monomeric AK from scallop, but very similar to that of greasyback shrimp *M. ensis* [16], white shrimp *L. vannamei* [17] and American cockroach [42].

The results of enzyme activity showed that the MrAK-1 enzyme activity is enhanced when adding low concentration of ATP (10–50 mM), and the activity is reduced when increasing the concentration of ATP (100–200 mM). A similar result was demonstrated by Watts et al. [43] in *Trypanosoma cruzi*. MrAK-1 activity was inhibited significantly by 10 mM  $\alpha$ -ketoglutarate and almost all of the MrAK-1 activity was lost increasing concentrations, which may suggest it can combine with the active site of the enzyme. It has been indicated that MrAK-1 activity may be correlated with the tricarboxylic acid cycle and carbohydrate metabolism [17]. The presence of 10–200 mM citrulline, ornaline and glycerol did not decrease MrAK-1 activity significantly, suggesting that MrAK-1 activity was not affected significantly by the main products of arginine catabolism and the primarily products of triglyceride glycerol catabolism. However, further research is necessary on the detailed mechanism.

Conclusively, MrAK-1 was identified from the transcriptome database of *M. rosenbergii*. MrAK-1 belonged to the conserved ATP:guanido phosphotransferases family. MrAK-1 was predominantly expressed in most tissues in *M. rosenbergii* with the highest level in muscle and the lowest level in eyestalk. The expression of MrAK-1 in muscle changed rapidly and dynamically in response to IHNV challenge, which suggested that MrAK-1 expression correlated closely with the shrimp immune response. We successfully expressed the MrAK-1 gene from *M. rosenbergii* through an *E. coli* expression vector and acquired a highly purified functional enzyme. Some characterizations of the enzyme have been uncovered, however, most details of the active site and catalytic mechanism of the enzyme are still unclear, and the findings of this study may lead to further research.

#### Acknowledgements

The authors would like to thank the funding agency ABI (53-02-03-1030) for supporting this research. And also University of Malaya, Kuala Lumpur, Malaysia is gratefully acknowledged for providing the postdoctoral research fellowship grant to the first author J. Arockiaraj.

#### References

- [1] Guo SY, Guo Z, Guo Q, Chen BY, Wang XC. Expression, purification, and characterization of arginine kinase from the sea cucumber *Stichopus japonicus*. *Prot Expr Purif* 2003;29:230–4.
- [2] Shofer SL, Willis JA, Tjeerdema RS. Effects of Hypoxia and toxicant exposure on arginine kinase function as measures by <sup>31</sup>P-NMR magnetization transfer in living abalone. *Comp Biochem Physiol Part C* 1997;117:283–9.
- [3] Liu TT, Wang XC. Effects of N-terminal deletion mutation on arginine kinase from the sea cucumber *Stichopus japonicus*. *Int J Biol Macromol* 2008;42:68–74.
- [4] Iwanami K, Isono S, Uda K, Suzuki T. A novel arginine kinase from the shrimp *Neocaridina denticulata*: the fourth arginine kinase gene lineage. *Gene* 2009; 437:80–7.

- [5] Fujimoto N, Tanaka K, Suzuki T. Amino acid residues 62 and 193 play the key role in regulating the synergism of substrate binding in oyster arginine kinase. *FEBS Lett* 2005;579:1688–92.
- [6] Abe H, Hirai S, Okada S. Metabolic responses and arginine kinase expression under hypoxic stress of the kuruma prawn *Marsupenaeus japonicas*. *Comp Biochem Physiol Part A* 2007;146:40–6.
- [7] Suzuki T, Ban T, Furukohri T. Evolution of phosphagen kinase V. cDNA-derived amino acid sequences of two molluscan arginine kinases from the chiton *Liolophura japonica* and the turbanshell *Battilus cornutus*. *Biochim Biophys Acta* 1997;1340:1–6.
- [8] Suzuki T, Kamidochi M, Inoue N, Kawamichi H, Yazawa Y, Furukohri T, et al. Arginine kinase evolved twice: evidence that echinoderm arginine kinase originated from creatine kinase. *Biochem J* 1999;340:671–5.
- [9] Uda K, Yamamoto K, Iwasaki N, Iwai M, Fujikura K, Ellington WR, et al. Two-domain arginine kinase from the deep-sea clam *Calyptogena kaikoi*: evidence of two active domains. *Comp Biochem Physiol Part B* 2008;151:176–82.
- [10] Suzuki T, Sugimura N, Taniguchi T, Unemi Y, Murata T, Hayashida M, et al. Two-domain arginine kinases from the clams *Solen strictus* and *Corbicula japonica*: exceptional amino acid replacement of the functionally important D62 by G. *Int J Biochem Cell Biol* 2002;34:1221–9.
- [11] Holt SM, Kinsey ST. Osmotic effects on arginine kinase function in living muscle of the blue crab *Callinectes sapidus*. *J Exp Biol* 2002;205:1775–85.
- [12] Suzuki T, Kawasaki Y, Furukohri T. Evolution of phosphagen kinase, Isolation. *Biochem J* 1997;328:301–6.
- [13] Suzuki T, Inoue N, Higashi T, Mizobuchi R, Sugimura N, Yokouchi K, et al. Gastropod arginine kinases from *Cellana grata* and *Aplysia kurodai*: isolation and cDNA-derived amino acid sequences. *Comp Biochem Physiol Part B* 2000;127:505–12.
- [14] Strong SJ, Ellington WR. Expression of horseshoe crab arginine kinase in *Escherichia coli* and site-directed mutations of the reactive cysteine peptide. *Comp Biochem Physiol Part B* 1996;113:809–16.
- [15] Yao CL, Wu CG, Xiang JH, Dong B. Molecular cloning and response to laminarin stimulation of arginine kinase in haemolymph in Chinese Shrimp, *Fenneropenaeus chinensis*. *Fish Shellfish Immunol* 2005;19:317–29.
- [16] Wang JS, Zheng ZL, Lei J, Pan JC, Zou GL. Cloning, expression, characterization and phylogenetic analysis of arginine kinase from greasyback shrimp (*Metapenaeus ensis*). *Comp Biochem Physiol Part B* 2009;153:268–74.
- [17] Yao CL, Ji PF, Kong P, Wang ZY, Xiang JH. Arginine kinase from *Litopenaeus vannamei*: cloning, expression and catalytic properties. *Fish Shellfish Immunol* 2009;26:553–8.
- [18] Tada H, Suzuki T. Cooperativity in the two-domain arginine kinase from the sea anemone *Anthopleura japonicus*. II. Evidence from site-directed mutagenesis studies. *Int J Biol Macromol* 2010;47:250–4.
- [19] Blethen SL. Kinetic properties of the arginine kinase isoenzyme of *Limulus polyphemus*. *Arch Biochem Biophys* 1972;149:244–51.
- [20] Kinsey ST, Lee BC. The effects of rapid salinity change on in vivo arginine kinase flux in the juvenile blue crab *Callinectes sapidus*. *Comp Biochem Physiol Part B* 2003;135:521–31.
- [21] Morris S, van Aardt WJ, Ahern MD. The effect of lead on the metabolic and energetic status of the Yabby, *Cherax destructor*, during environmental hypoxia. *Aquat Toxicol* 2005;75:16–31.
- [22] Silvestre F, Dierick JF, Dumont C, Dieu M, Raes M, Devos P. Differential protein expression profiles in anterior gills of *Eriocheir sinensis* during acclimation to cadmium. *Aquat Toxicol* 2006;76:46–58.
- [23] Astrofsky KM, Roux MM, Klimpel KR, Fox JG, Dhar AK. Isolation of differentially expressed genes from white spot virus (WSV) infected Pacific blue shrimp (*Penaeus stylirostris*). *Arch Virol* 2002;47:1799–812.
- [24] Wang B, Li F, Dong B, Zhang X, Zhang C, Xiang J. Discovery of the genes in response to white spot syndrome virus (WSSV) infection in *Fenneropenaeus chinensis* through cDNA Microarray. *Mar Biotech* 2006;8:491–500.
- [25] Garcia-Orozco KD, Aispuro-Hernandez E, Yepiz-Plascencia G, Caldero n-de-la-Barca AM, Sotelo-Mundo RR. Molecular characterization of arginine kinase, an allergen from the shrimp *Litopenaeus vannamei*. *Int Arch Allergy Immunol* 2007;144:23–8.
- [26] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic alignment search tool. *J Mol Biol* 1990;215:403–10.
- [27] Bairoch A, Bucher P, Hofmann K. The PROSITE data base, its status in 1997. *Nucleic Acid Res* 1997;25:217–21.
- [28] Pearson WR. Rapid and sensitive sequence comparison with FASTP and FASTA. *Meth Enzymol* 1990;183:63–98.
- [29] Thompson JD, Higgins DG, Gibson TJ. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Res* 1994;22:4673–80.
- [30] Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 2004;5:150–63.
- [31] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402–8.
- [32] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press; 1989.
- [33] Nikapitiya C, De Zoysa M, Oh C, Lee Y, Ekanayake PM, Whang I, et al. Disk abalone (*Haliotis discus discus*) expresses a novel antistatin-like serine protease inhibitor: molecular cloning and immune response against bacterial infection. *Fish Shellfish Immunol* 2010;28:661–71.
- [34] Chen BY, Guo Q, Guo Z, Wang XC. Improved activity assay method for arginine kinase based on a Ternary Heteropolyacid System. *Tsinghua Sci Technol* 2003;8:422–7.
- [35] Ellington WR. Evolution and physiological roles of phosphagen systems. *Annu Rev Physiol* 2001;63:289–325.
- [36] Furukohri T, Okamoto S, Suzuki T. Evolution of phosphagen kinase (III). Amino acid sequence of arginine kinase from the shrimp *Penaeus japonicus*. *Zool Sci* 1994;11:229–34.
- [37] France RM, Sellers DS, Grossman SH. Purification, characterization, and hydrodynamic properties of arginine kinase from gulf shrimp (*Penaeus aztecus*). *Arch Biochem Biophys* 1997;345:73–8.
- [38] Yu CJ, Lin YF, Chiang BL, Chou LP. Proteomics and immunological analysis of a novel shrimp allergen. *J Immunol* 2003;170:445–53.
- [39] Wolf FK, Schnyder T, Wallimann T, Kabsch W. Structure of mitochondrial creatine kinase. *Nature* 1996;381:341–5.
- [40] Yao CL, Wang ZY, Zhang RY, Han XZ, Zhang ZJ, Kong P, et al. Purification of partial characterization of arginine kinase from muscle tissue of shrimp, *Litopenaeus vannamei*. *J Fish China* 2008;32:864–9.
- [41] Qing-Yun W, Feng L, Wen-Jing Z, Xiao-Yun W. Cloning, expression, purification, and characterization of arginine kinase from *Locusta migratoria manilensis*. *Comp Biochem Physiol Part B* 2007;148:355–62.
- [42] Brown AE, France RM, Grossman SH. Purification and characterization of arginine kinase from the American cockroach (*Periplaneta americana*). *Arch Insect Biochem Physiol* 2004;56:51–60.
- [43] Watts DC, Anosike EO, Moreland B, Pollitt RJ, Lee CR. The use of arginine analogues for investigating the functional organization of the arginine-binding site in lobster muscle arginine kinase. Role of the 'essential' thiol group. *J Biochem* 1980;185:593–9.