

Molecular cloning and response to laminarin stimulation of arginine kinase in haemolymph in Chinese shrimp, *Fenneropenaeus chinensis*

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Abstract

Arginine kinase (AK) was previously reported as a phosphagen-ATP phosphotransferase found in invertebrates. In this study, an 1184 bp cDNA was cloned and sequenced. It contained an open reading frame of 1068 bp that coded for 356 deduced amino acids of AK in *Fenneropenaeus chinensis*. The calculated molecular mass of AK is 40129.73 Da and pI is 5.92. The predicted protein showed a high level of identity to known AK in invertebrates and creatine kinase from vertebrates, which belong to a conserved family of ATP:guanidino phospho-transferases. In addition, AK protein in plasma of *F. chinensis* was identified using two-dimensional electrophoresis (2DE) and electrospray ionization mass spectrometry (ESI-MS) according to the calculated molecular mass and pI. AK was significantly decreased in the plasma of *F. chinensis* at 45 min and recovered at 3 h after laminarin injection as confirmed by 2DE and ESI-MS. The results showed that AK was one of the most significantly changed proteins on two-dimensional gel in the plasma proteins of *F. chinensis* at 45 min and 3 h after simulation.

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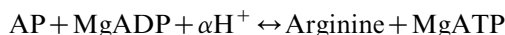
Keywords: Arginine kinase; Molecular cloning; *Fenneropenaeus chinensis*; Laminarin; 2-D electrophoresis; Electrospray ionization mass spectrometry

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1. Introduction

The enzyme arginine kinase (AK, EC 2.7.3.3) is a phosphagen kinase that is the key to energy metabolism in invertebrates. AK catalyses the reversible transfer of high-energy phosphate from arginine phosphate (AP) to ADP to form ATP, thereby regenerating ATP during bursts of cellular activity [1]. AK catalyses the reversible reaction as follows:



where α represents a partial proton. Some cDNA or amino acid sequences of arginine kinase were obtained from a variety of crustaceans in recent years, including crabs, *Carcinus maenas* and *Callinectes sapidus* [2,3], lobster, *Homarus gammarus* [4], shrimp, *Marsupenaeus japonicus* [5] and *Penaeus monodon* [6]. Analysis of the evolutionary relationship between arginine kinase and creatine kinase (CK) revealed that shrimp AK showed high identity with other invertebrate AK and significant homology with vertebrate CK [5]. Activity and structural changes of AK from shrimp *Fenneropenaeus chinensis* were found in trifluoroethanol solutions and oxidized dithiothreitol solutions [7,8]. France et al. [9] suggest that the overall conformation of arginine kinase from gulf shrimp (*Penaeus aztecus*) may differ from the prolate ellipsoidal subunits of the functionally analogous CK. Holt and Kinsey [10] reported that AK flux in isolated muscle was sensitive to prevailing osmotic conditions. In shrimp, AK cDNA was upregulated in WSSV infected *P. stylirostris* by EST analysis [11]. It was demonstrated that shrimp AK was an allergen that caused allergic reactions in hypersensitive individuals [6,12].

The activity of denatured arginine kinase was improved by glycerol, sucrose, and sorbitol [13]. As an immunostimulant, β -1,3-glucan enhances resistance of mammals [14], fish [15] and shrimp [16–19] against bacterial or viral infections. Some of the immune mechanisms by which β -1,3-glucan enhances resistance have been elucidated [20,21]. Dietary administration of β -1,3-glucan enhanced haemocyte phagocytic activity, cell adhesion, and superoxide anion production of shrimp after vibrio and white spot syndrome virus (WSSV) infection [22,23].

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) have been widely used in identification of specific protein variation. In this study, 2D-PAGE and electrospray ionization mass spectrometry (ESI-MS) was used to identify arginine kinase variation in plasma of Chinese shrimp, *F. chinensis*, after laminarin (a type of soluble branched β -1,3-glucan, primarily poly β -Glc- [1–3] with some β - [1–6]- interstrand linkages and branch points) injection. The cDNA of arginine kinase from Chinese shrimp haemocytes was also cloned.

2. Materials and methods

2.1. Experimental shrimps

Healthy cultured Chinese shrimp, *Fenneropenaeus chinensis* were obtained from Qingdao, China, and were placed in aerated 32‰ seawater at 21 °C for at least 2 days prior to each experiment. No sexual distinction and only intermolt shrimp were used in this study. Shrimp density was two individuals per litre. Artificial diet was given two times per day. The weight of the shrimp was 10.8 ± 1.7 g and the length was 10.1 ± 0.7 cm.

2.2. RNA extraction and cDNA cloning of arginine kinase

Total RNA was isolated and pooled from haemocytes of ten shrimps. After removing the plasma by centrifugation for 5 min at 800g at 4 °C, the haemocytes were homogenised in Unizol Reagent (Biostar

Genechip Inc.), and the RNA was extracted according to the manufacturer's protocol. This total RNA was used as a template for RT-PCR. The reverse transcription step was performed with M-MLV Reverse Transcriptase (Promega) according to the manufacturer's protocol using AOLP primer (5'-GGCC ACGCGTCGACTAGTACTTTTTTTTTTTTTTTT(A/C/G)(A/G/T/C)-3') to prime cDNA synthesis. The amplification of cDNA was performed with two specific primers, AK1F, 5'-GCC AGG AAT CTT CAA GCA ACA A-3' (forward) and AK1R, 5'-TCC TCC TAC ACC GCC ACA GTT A-3' (reverse), which were synthesised based on the nucleotide sequence of arginine kinase from EST sequence data for *F. chinensis* (the EST sequence database had been constructed in the Institute of Oceanology, Chinese Academy of Sciences) and used for PCR amplification (PCR cycles were: 94 °C, 2 min, followed by 35 cycles of 95 °C for 60 s, 61 °C for 60 s, 72 °C for 60 s, and a final cycle of 72 °C for 10 min). The amplified product was subsequently ligated into the pMD18-T vector (Takara), and transformed into *Escherichia coli* TOPO10F' competent cells (Invitrogen) and the DNA was subsequently sequenced.

2.3. Phylogenetic analysis

Fourteen kinds of AK from arthropods (shrimp, crab, insect) and four CK from vertebrates (fish, mouse and human) were used in this study. Phylogenetic analysis was carried out using the neighbour-joining (NJ) method provided by Phylip software version 3.5c [24] based on amino acid sequences in this study, *Homo sapiens* CK was chosen as the outgroup species. The sequences were aligned with the CLUSTAL W (1.81) program [25]. Tree topologies were evaluated by 1000 bootstraps of the original data sets. Tree visualisation and drawings were carried out using TreeView version 1.5 [26].

2.4. Immune stimulation

Each shrimp was injected with 50 µl of laminarin (2 mg ml⁻¹, Sigma, from *Laminaria digitata*) dissolved in shrimp saline, 50 µl shrimp saline [27] was used as mock challenge control. A third shrimp group receiving no injection was used as blank control. Ten shrimps were used for each group. Every treatment was composed of two replications. Haemolymph of each group was collected at 45 min and 3 h at which time the total haemocyte counts and protein concentration showed significant change in the laminarin stimulation group.

2.5. Haemolymph collection and plasma preparation

Haemolymph was collected from the ventral sinus, diluted 1:2 in precooled sterile shrimp anti-coagulant (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 7.3) as described by Vargas-Albores et al. [28] with the addition of a protease inhibitor cocktail (Roche), and transferred to a sterile centrifuge tube. The cold haemolymph was centrifuged at 800g for 5 min at 4 °C to remove haemocytes and then frozen at -80 °C until used.

2.6. Two-dimensional electrophoresis

2.6.1. Preparation of 2D electrophoresis protein samples

The plasma protein was extracted by adding five volumes of extracting solution (acetone, 10% trichloroacetic acid (TCA), 0.07% dithiothreitol (DTT), precooled at -20 °C) immediately and incubating at -20 °C overnight to protect the protein from degradation. The sample was centrifuged for 25 min at 12,000g and 4 °C. The pellet was washed with precooled (-20 °C) acetone and 0.07% DTT several times and then resuspended in rehydration solution (urea 7 M, thiourea 2 M, CHAPS 4% (w/v), DTT 65 mM,

Fig. 1. Nucleotide sequence and translated amino acid sequence of Chinese shrimp AK (GenBank accession number [AY661542](#)). The start and stop codons of the open reading frame are indicated using * and #, respectively. The active site is between 271–77:CP.TNLGT.

2.6.2. Two-dimensional electrophoresis

2.6.3. Computer analysis of 2DE pattern

Gels were scanned with a Gel Doc. Imaging system (BioRad, USA). Computer analysis was carried out using PDQuest 2-D Analysis Software (BioRad, USA)

405	GGT CGC TCC ATG GAG GGT TAC CCC TTC AAC CCC TGC CTC ACT GAG	
	G R S M E G Y P F N P C L T E	142
450	GAT CAG TAC AAG GAG ATG GAG TCC AAG GTT TCC TCT ACC CTT TCT	
	D Q Y K E M E S K V S S T L S	157
495	AGC CTT GAG GGC GAG CTC AAG GGT ACC TAC TAC CCC CTC ACT GGC	
	S L E G E L K G T Y Y P L T G	172
540	ATG GGC AAG GAA GTC CAG CAG AAG CTG ATC GAC GAC CAC TTC CTC	
	M G K E V Q Q K L I D D H F L	187
585	TTC AAG GAG GGT GAC CGC TTC CTG CAG GCC GCC AAC GCT TGC CGC	
	F K E G D R F L Q A A N A C R	202
630	TAC TGG CCC AGC GGC CGT GGC ATC TAC CAC AAC GAC AAC AAG ACT	
	Y W P S G R G I Y H N D N K T	217
675	TTC CTT GTC TGG GTC AAC GAG GAG GAT CAC CTC CGC ATC ATC TCC	
	F L V W V N E E D H L R I I S	232
720	ATG CAG ATG GGC GGT GAT CTC GGC CAG GTC TTC CGC CGC CTC ACC	
	M Q M G G D L G Q V F R R L T	247
765	TCC GCT GTG AAC GAG ATT GAG AAG AGG ATT CCT TTC TCT CAC CAC	
	S A V N E I E K R I P F S H H	262

Fig. 1 (continued).

2.7. Mass spectrometry

2.7.1. Trypsin digestion

The spots of interest were excised with a sterile scalpel and digested in gel with trypsin according to Shevchenko's method [30].

2.7.2. Mass spectrometry

A 20 μ l sample of trypsin digest was analysed using an ion-trap mass spectrometer model LCQ DECA XP^{plus} MS (ThermoFinnigan, San Jose, CA, USA). The results were correlated with the sequence database using the SEQUEST database and analysed by Bioworks software. Simply, eluting peptides were introduced into the MS and ionised to be further transferred online to a heated capillary of an ion trap mass spectrometer in positive mode. In each experiment, 20 μ l of protein extract was injected. Spectra were collected in the positive ion mode and mass at data-dependent mode. The liquid chromatography (LC) separation was carried out at a flow-rate of 120 μ l min⁻¹ on a BioBasic-18 column, 150 $\times$ 0.18 mm, particle Sz (μ)5 (Thermohypersil-Keystone, No. 72105-100265). The gradient was developed according to 2% A and 98% B for 15 min, 65% A and 35% B for 45 min, 95% A and 5% B for 10 min, and finally 2% A and 98% B for 15 min, where A is acetonitrile with 0.1% formic acid and B is water with 0.1% formic. The total acquiring time was 90 min.

810	GAC CGC CTG GGC TTC CTC ACT TTC TGC CCC ACC AAC CTT GGC ACC	
	D R L G F L T F C P T N L G T	277
855	ACT GTG CGC GCC TCC GTC CAC ATC AAG CTG CCC AAG CTT GCC GCT	
	T V R A S V H I K L P K L A A	292
900	AAC CGC GAT AAG CTT GAG GAG GTC GCT GGC AAG TAC AAC CTG CAG	
	N R D K L E E V A G K Y N L Q	307
945	GTG CGC GGC ACC CGC GGT GAG CAC ACT GAG GCT GAG GGC GGC ATC	
	V R G T R G E H T E A E G G I	322
990	TAC GAT ATC TCC AAC AAG CGC CGC ATG GGT CTG ACT GAG TTC CAG	
	Y D I S N K R R M G L T E F Q	337
1035	GCT GTC AAG GAG ATG CAG GAC GGT ATC CTT GAG CTC ATC AAG ATG	
	A V K E M Q D G I L E L I K M	352
1080	GAG AAG GAG ATG TAA GAA GCT TAC TTT TAG TTA CTA CAG TCA TTT	
	E K E M #	356
1125	CCG GCT CTC AAC CCC CTA TGG GTG GGG GCG AGC TGG CTT TAA CTG	
1170	TGG CGG TGT AGG AGG	

Fig. 1 (continued).

3. Results

3.1. Determination of the cDNA and amino acid sequences of shrimp AK

A segment of *F. chinensis* AK cDNA with a length of 1184 bp and an open reading frame of 1068 bp (Fig. 1) was obtained. The deduced protein is comprised of 356 amino acid residues, with a calculated molecular mass of 40,129.73 Da and an isoelectric point of 5.92. *F. chinensis* AK is closely related to other shrimp AK proteins, and at the amino acid level, it has the highest homology with *P. monodon* (96% identity), *P. japonicus* (94% identity) and lobster *H. gammarus* (90% identity). The percentage amino acid identity of *F. chinensis* AK and other shrimp AK and human CK sequences are shown in Fig. 2. The profile analysis using Prosite on the ExPASy web site suggests it was ATP:guano phosphotransferases. The active site is between 271–277:CP.TNLGT.

3.2. Phylogenetic analysis

The phylogenetic relationships of AK among some invertebrate and vertebrate species were shown with the NJ method based on the partial amino acid sequences (Fig. 3). The tree topologies revealed the relationships of the *F. chinensis* AK with other invertebrate AKs and vertebrate CKs.

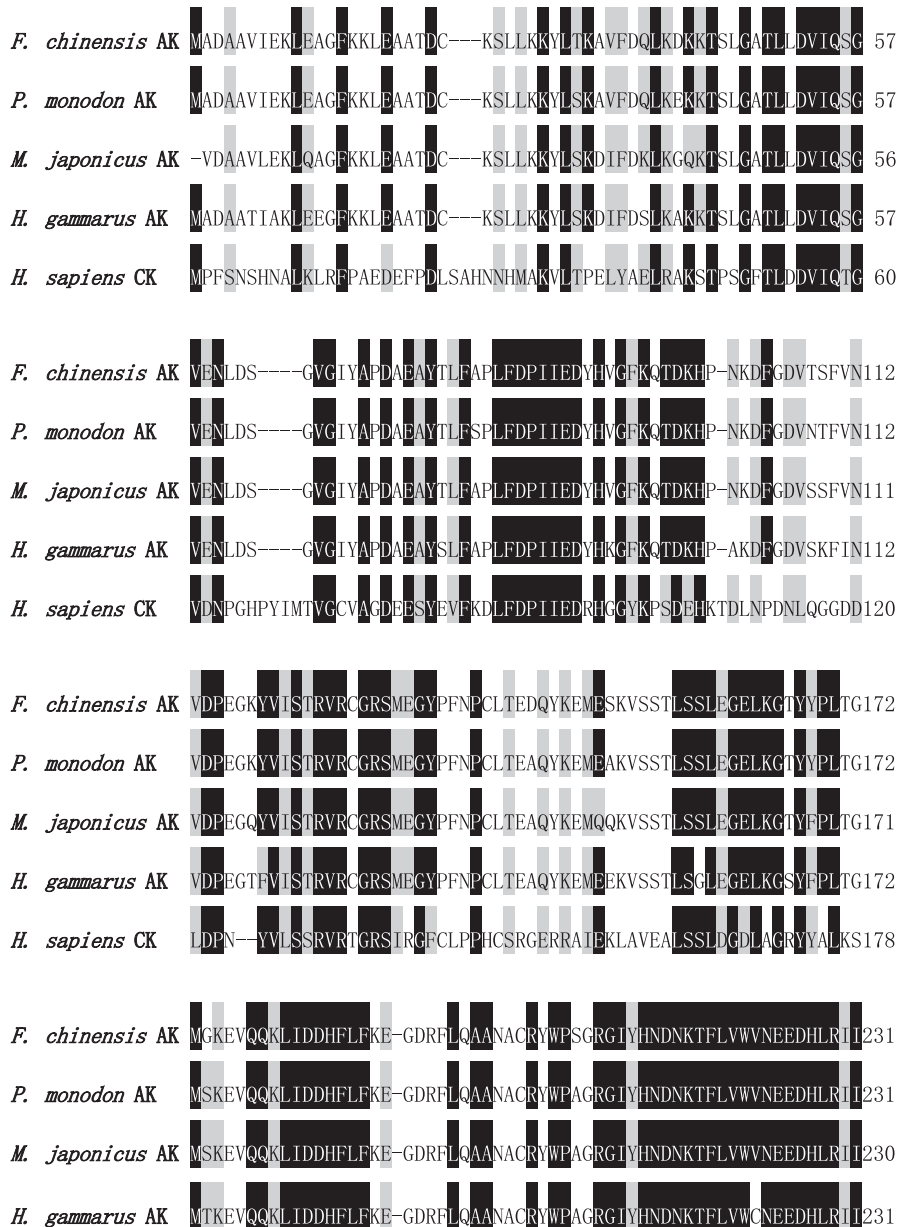


Fig. 2. Alignment of *F. chinensis* AK amino acid sequence with *P. monodon* AK (AF479772), *M. japonicus* AK (P51545), *Homarus gammarus* AK (P14208) and human, *Homo sapiens* CK (AAC31758). Amino acid sequences start with the first methionine. Residues that are identical in all five sequences are enclosed in a black box (in web version). Residues identical in two or three sequences are enclosed in a grey or white box (in web version).

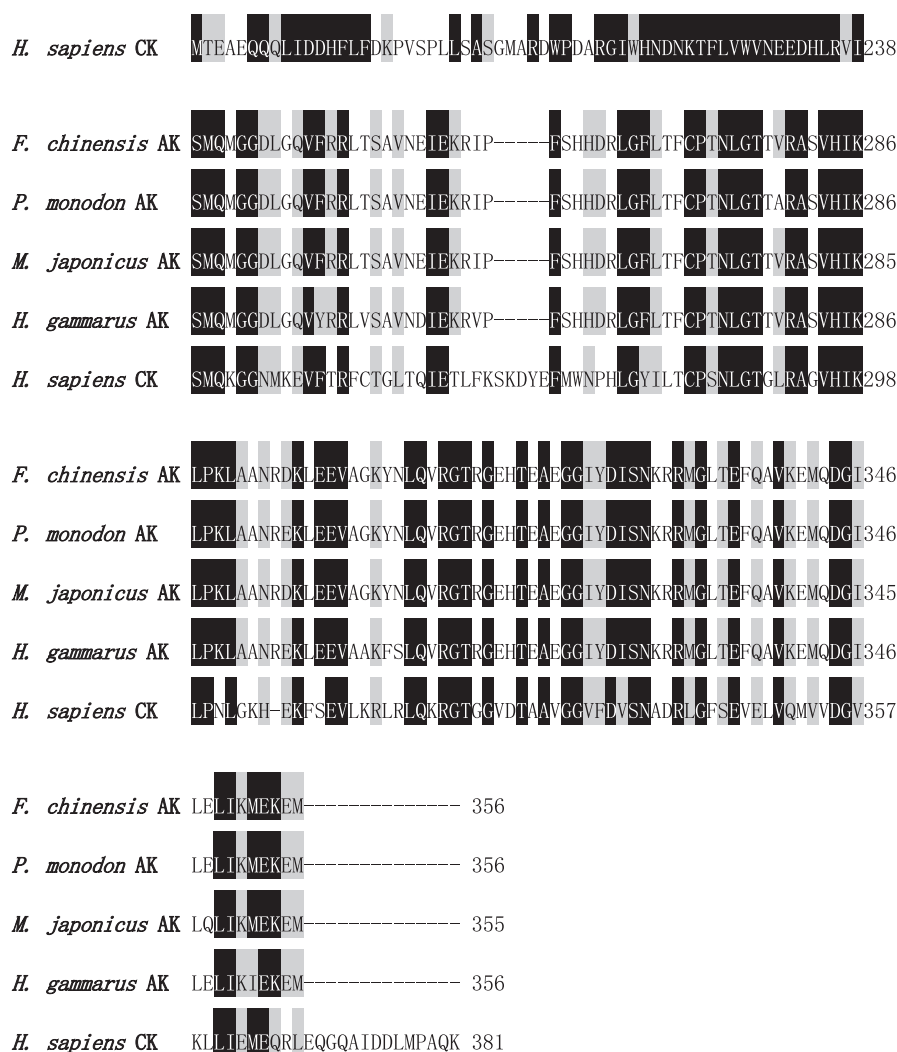


Fig. 2 (continued).

3.3. Identification of arginine kinase on the two-dimensional gel and the change after laminarin injection

Six 2D gels were run for each sample. According to the calculated molecular weight and pI, the spot with molecular weight and pI about 40 kDa and 6.0 on the 2D gel was excised from the gel, digested with trypsin and identified using ESI-MS. The ESI-MS fragments corresponded with the deduced amino acid sequence of AK and matched AK from *P. monodon* (Tables 1 and 2). Computer analysis showed that arginine kinase significantly decreased at 45 min and obviously recovered at 3 h after laminarin injection. Fig. 4 shows part of the 2D-PAGE separation map at 45 min and 3 h after laminarin injection and their control group. The samples (5 mg of total protein) were prepared from shrimp plasma. After electrophoresis, the gel was stained with Coomassie Blue R-250. There was, however, no significant difference between the mock injection group and the non-injection group.

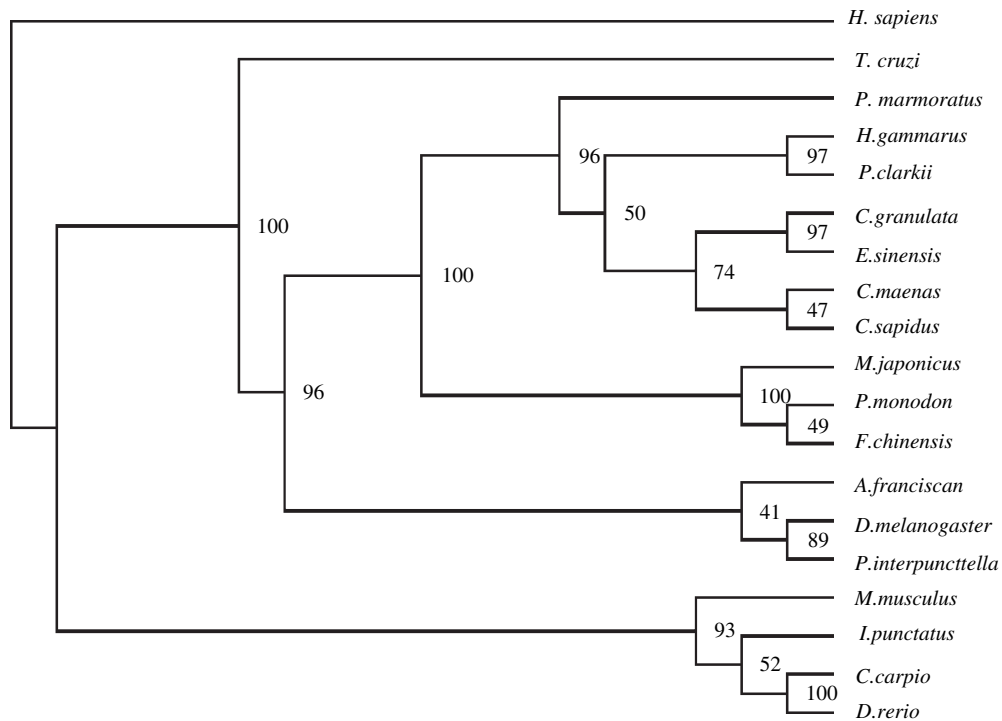


Fig. 3. Phylogenetic relationship among *F. chinensis* and other invertebrate AK and vertebrate CK amino acid sequences. The sequence alignment was performed and the trees were drawn as described in Fig. 4. *Pachygrapsus marmoratus* (marbled crab, AF288785), *Eriocheir sinensis* (Chinese mitten crab, AF233356), *Callinectes sapidus* (blue crab, AF 233355), *Carcinus maenas* (green crab AF167313), *Chasmagnathus granulata* (AF233357), *Procambarus clarkii* (red swamp crayfish, 2020435A), *Artemia franciscana* (Q95V58), *Drosophila melanogaster* (fruit fly, NP_729446), *Plodia interpunctella* (Q95PM9), *Trypanosoma cruzi* (AAC82390), *Cyprinus carpio* (common carp, AAC96092), *Danio rerio* (zebrafish, NP_571007), *Ictalurus punctatus* (channel catfish, AAO25756), *Mus musculus* (house mouse, BAB26603) were also used.

4. Discussion

Expressed sequence tags (ESTs), generated from partial sequencing of randomly selected cDNA clones, is an effective approach for gene cloning and gene expression analysis in various species where knowledge about the genome under investigation is not available or rather limited [31]. This technique is relatively simple and powerful for identification of useful genes in a particular application [11,32–34]. More than 10,000 EST sequences from *Fenneropenaeus chinensis* were generated in this laboratory [35]. The data from *F. chinensis* EST showed that the expression of arginine kinase (AK) was correlated to shrimp health (unpublished). Another analysis of EST showed that AK was an important enzyme correlated with immune capability and shrimp disease of *Penaeus stylirostris* [11].

Arginine kinase plays a key role in the coupling of energy production and utilisation in animals [36–39]. A large amount of AK is present in shrimp muscle tissue [6,9]. Some cDNA sequences and deduced proteins of AK had been obtained from shrimps. An open reading frame of 1068 bp that codes for arginine kinase in *F. chinensis* shrimps was obtained in this study. The AK protein shows a high degree of similarity to known AK in shrimps and to other predicted proteins belonging to the conserved phosphagen kinase family. Phylogenetic analysis indicated a close affinity of *F. chinensis* and *P. monodon* AK. Invertebrate AK and vertebrate CK belong to two major clusters and they all belong to the conserved family of ATP:guanidino

Table 1

Identification of AK fragments by ESI-MS analysis: matched tryptic peptide sequences of AK in *F. chinensis* with allergen Pen m2 in *P. monodon* in ESI-MS

Scan(s)	Sequence	MH +	XC	Delta Cn	Sp
Allergen Pen m 2 (<i>Penaeus monodon</i>)				180.17	1708615.0
1981	-.LTSAVNEIEK.-	1104.24	1.73	0.20	421.3
1985	-.LTSAVNEIEK.-	1104.24	2.22	0.24	707.8
2012	-.LTSAVNEIEKR.-	1260.42	2.52	0.17	942.2
2045	-.RLTSAVNEIEK.-	1260.42	2.32	0.05	528.9
2052	-.RLTSAVNEIEK.-	1260.42	2.58	0.05	637.2
2225	-.LIDDHFLFK.-	1148.34	2.47	0.05	638.3
2275	-.VSSTLSSLEGELK.-	1350.50	3.43	0.38	1031.2
2279	-.VSSTLSSLEGELK.-	1350.50	3.24	0.32	701.3
2387	-.LIDDHFLFK.-	1148.34	2.74	0.24	706.5
2391	-.LIDDHFLFK.-	1148.34	3.17	0.07	708.4
2396	-.LIDDHFLFK.-	1148.34	2.15	0.07	241.8
2428	-.TFLVWVNEEDHLR.-	1658.84	3.09	0.00	1203.3
2436	-.TFLVWVNEEDHLR.-	1658.84	2.86	0.12	456.9
2439	-.TFLVWVNEEDHLR.-	1658.84	3.46	0.12	1276.6
2443	-.TFLVWVNEEDHLR.-	1658.84	3.00	0.15	309.3
2601	-.LAANRDKLEEVAGK.-	1514.71	1.23	0.21	298.1
2696	-.ASVHIKLPK.-	993.23	2.53	0.28	198.9
2724	-.ASVHIKLPK.-	993.23	2.30	0.00	197.8
2763	-.ASVHIKLPK.-	993.23	2.66	0.00	609.8

phosphotransferases. This finding supports a number of previous studies that provided evidence for an excellent conservation of both primary and secondary structures amongst phosphagen kinases [6,40,41].

Proteins are the final products manufactured in living cells according to the “blueprint” contained in the genome. Explaining how these gene products cooperate in complex physiological processes is a scientific challenge [42]. Two of the main proteomic tools are two-dimensional electrophoresis (2DE) and mass spectrometry. Two-dimensional electrophoresis is a unique method for large-scale protein characterisation and, combined with mass spectrometry, allows for identification of the protein repertoire of specific tissues [43]. In this experiment, protein was extracted from plasma at 45 min and 3 h after laminarin injection. It was found that 45 min and 3 h were the critical time points at which obvious changes of haemocyte counts and protein concentration occurred (data not shown) in the laminarin injected group. One important finding was that arginine kinase (AK) was one of the most significantly changed proteins between control group and treatment group. Many other obviously changed proteins in plasma after laminarin injection

Table 2

Identification of AK fragments by ESI-MS analysis: the entire sequence of allergen Pen m 2 [*Penaeus monodon*]

Protein	Protein sequence
Reference: gi/27463265 allergen Pen m 2 (<i>Penaeus monodon</i>) gi 27463265 gb AAO15713.1 /arginine kinase, AK, Database: no. Fasta	VDAAVLEKLQAGFKKLEAATDCKSLLKKYLSKDIFDKLK GQKTSLGATLLDVIQSGVENLDSGVGIYAPDAEAY TLFAPLFDPIIEDYHVGFKQTDKHPNKDFGDVSSFVNVD EGQYVISTRVRCGRSMEGYPFNPCLTEAQYKEMQQK VSSTLSSLEGELKGTYPPLTGMSKEVQQKLIDDHFLFK EGDRFLQAANACRYWPAGRGIYHNDNKTFLVWVNEEDHLR IISMQMGGDLGQVFRRLTSAVNEIEKRIPFSSHDLRGLFTFCPTNLGTTVR ASVHIKLPKLAANRDKLEEVAGKYNLQVRGTRGEHTEAEGGIYDISNK RRMGLTEFQAVKEMQDGILQLIKMEKEM

Bold letters represent tryptic peptides identified via mass sequence analysis from arginine kinase of *F. chinensis*.

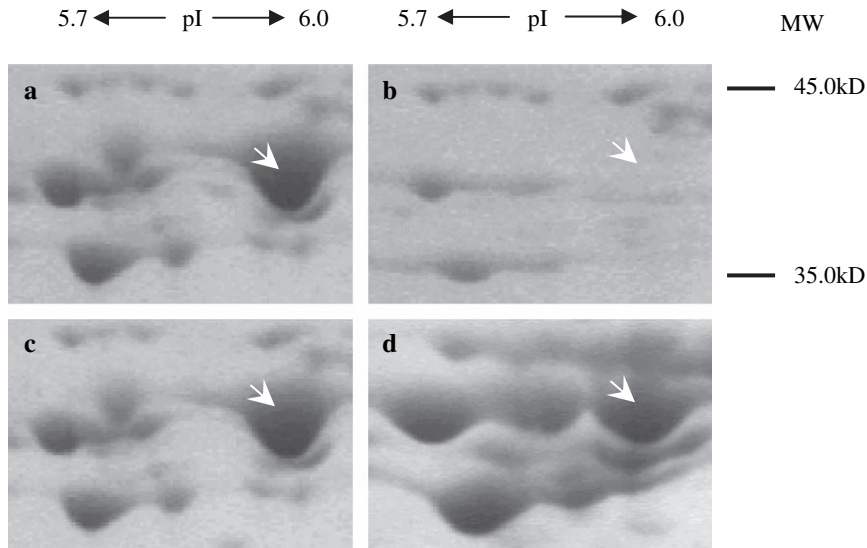


Fig. 4. Part of 2D-PAGE maps (stained by Coomassie Brilliant Blue R-250) of shrimp plasma protein extract 45 min after shrimp saline injection (a), 45 min after laminarin injection (b), 3 h after shrimp saline injection (c), and 3 h after laminarin injection (d). The second dimension was 12% SDS-PAGE. Arginine kinase is indicated by arrows.

were shown on the 2D gel. Unfortunately, because the information in the shrimp database is poor, they were not identified.

Previous research indicated that the variation of AK might be related to some external factors in invertebrates. It was reported that AK increased after hypoxia, sodium azide, or pentachlorophenol exposure in abalone [43]. AK concentration and specific activity increased along the epimastigote growth curve in *Trypanosoma cruzi*, suggesting that the enzyme is regulated by cell density, parasite replication or nutritional stress [44]. Another study demonstrated that the homologous overexpression of *T. cruzi* AK improved the ability of the transfectant cells to grow and resist nutritional and pH stress conditions [45]. Yu et al. [13] demonstrated that glycerol, sucrose, and sorbitol could improve the reactivation of denatured arginine kinase. The present data showed that AK significantly changed at 45 min and 3 h after laminarin injection, suggesting that laminarin affected the level of expression of AK. Johansson et al. [46] once suggested that laminarin could act as an immunostimulant and a source of energy to crustacea.

In summary, in this study, the cDNA of arginine kinase was cloned from haemolymph of *F. chinensis*. Phylogenetic analysis suggested a close affinity of *F. chinensis* and *P. monodon* AK and that AK from *F. chinensis* belongs to the conserved phosphagen kinase family. The first proteomics approach to identify changes in arginine kinase protein in *F. chinensis* plasma is presented using 2D-PAGE and mass spectrometry. As one of the significant protein responses after laminarin injection, AK might play an important role in the coupling of energy production and utilisation in shrimps and an allergen to humans. Therefore, further investigation on the structure and function of AK is necessary.

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