Full Length Research Paper

Cloning, purification and characterization of recombinant silkworm arginine kinase expressed in *Escherichia coli*

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Arginine kinase (AK) is a major invertebrate phosphagen kinase that catalyzes the reversible transfer of high energy phosphoryl group of adenosine triphosphate (ATP) to arginine, yielding phosphoarginine and adenosine diphosphate. In this study, the 1068 bp open reading frame of a putative *Bombyx mori* arginine kinase gene (BmAK) was amplified from a pool of *B. mori* cDNAs and inserted into the prokaryotic expression plasmid pET-28a(+). The recombinant His-tagged BmAK protein was expressed in soluble form in *Escherichia coli* Rosetta and purified by metal chelating affinity chromatography. The amino acid sequence of recombinant protein was confirmed by mass spectroscopic analysis and the enzyme activity assay that indicated the recombinant protein was able to transfer the gamma phosphoryl group of ATP to arginine.

Key words: Arginine kinase, recombinant protein, enzymatic activity.

INTRODUCTION

Phosphagen kinases catalyze the reversible transfer of the gamma phosphoryl group of adenosine-5'-triphosphate (ATP) to naturally occurring guanidine compounds, such as arginine, creatine, lombricine, glycocyamine and taurocyamin generating adenosine-5'-diphosphate (ADP) and phosphagen (phosphoarginine, phorsphocreatine, etc.). Members of this enzyme family play a key role in maintaining ATP concentration by interconversion of ATP to phosphagen according to the variable energy demands of cells (Kenyon and Reed, 1983; Schlattner et al., 2006;

Wyss et al., 1992). In vertebrates, phosphocreatine is the only phosphagen and the corresponding phosphagen kinase is creatine kinase (CK). In invertebrates, arginine kinase (ATP: L-arginine phosphotransferase EC 2.7.3.3, AK) and CK are two major phosphagen kinases (McLeish and Kenyon, 2005; Morrison 1973, van Thoai, 1968; Watts, 1968). Although, arginine kinase (AK) is widely distributed in the lower and higher invertebrate groups and plays a similar physiological role as CK does, it has not been investigated as extensively as CK.

In two major invertebrate groups, arthropods and mollusks, AK is the only phosphagen kinase (Tanaka et al., 2007). Many researches focusing on the evolutionary relationship between arginine kinase and creatine kinase has been conducted (Anosike et al., 1975; Suzuki et al., 1997a; Suzuki et al., 1997b; Suzuki et al., 2000; Suzuki et al., 1999). Recent phylogenetic analysis revealed that they can be separated into two distinct lineages, a CK group (CK, glycocyamine kinase, taurocyamine kinase and lombricine kinase) and an AK group (AK and hypotaurocyamine kinase) (Uda et al., 2005).

Some arginine kinases have been characterized in recent years, such as the crab *Carcinus maenas* and *Callinectes sapidus* (Kinsey and Lee, 2003; Kotlyar et al.,

Abbreviations: AK, Arginine kinase; **CK,** creatine kinase; **DEPC,** diethylpyrocarbonate; **IPTG,** isopropyl β-D-1-thiogalactopyranoside; **PBS,** phosphate buffered saline; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **ORF,** open reading frame; **PVDF,** polyvinylidene fluoride; IgG, immunoglobulin G; PMF, peptide mass fingerprinting; **BSA,** bovine serum albumin; **NADH,** nicotinamide adenine dinucleotide; **BmAK,** *Bombyx mori* arginine kinase gene.

2000), shrimp *Marsupenaeus japonicus* (Furukohri et al., 1994), chelicerate arthropod *Limulus polyphemus* (Strong and Ellington, 1995), sea cucumber *Stichopus japonicus* (Guo et al., 2003) and silkworm *Bombyx mori* (Liu et al., 2009). AK activity has been identified even in protozoa (Noguchi et al., 2001); furthermore, AK encoding genes have been found in the genomes of some kinds of bacteria, *Paramecium and Tetrahymena*, suggesting that AK has an ancient origin (Tanaka et al., 2007). Moreover, insect AKs from cockroach *Periplaneta americana* (Sookrung et al., 2006), Indian meal moth *Plodia interpunctella* (Binder et al., 2001) and Atlantic shrimp *Pandalus borealis* (Crespo et al., 1995) have been reported as one kind of the major indoor allergens to cause strong symptoms, including allergic asthma, in atopic individuals. In the present study, the mRNA expression profile and genomic structure of *B. mori* arginine kinase (BmAK) gene were also characterized (Hua-bing and Yu-song, 2006) and this protein was identified as a major silkworm allergen in silkworm larvae (Liu et al., 2009). However, the enzymatic activity of BmAK has not been well characterized. Pure and functional BmAK is needed for investigations.

In this investigation, the open reading frame (ORF) of BmAK was cloned and the recombinant enzyme was expressed in *Escherichia coli*. The amino acid sequence of recombinant BmAK protein was verified by mass spectroscopic analysis. The phosphoryl group that transfers the activity of the recombinant BmAK protein was assayed.

MATERIALS AND METHODS

The expression vector, pET-28a (+), and *E. coli* strain, Rosetta, were obtained from Novagen (CA, USA). All primers, Ex Taq polymerase, restriction enzymes, T4 DNA ligase and the subcloning vector pMD18-T were purchased from TaKaRa (Dalian, China). Chemicals were all from Sigma (MO, USA) or a domestic provider in China if not stated otherwise. All other reagents were local products of analytical grade. *B. mori* strain C108 was reared in our laboratory.

Bioinformatics analysis

In order to figure out similarity between BmAK and other AKs, multiple sequence alignment of proteins was performed with the Clustal W program (Thompson et al., 1994) and edited with Genedoc. A phylogenetic tree was constructed using MEGA version 4.1 (Tamura et al., 2007). The neighbor-joining method (Saitou and Nei, 1987) was used to construct the tree.

RNA extraction and cDNA synthesis

The fifth larvae of *B. mori* were dissected and washed with cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4). Then, the tissue samples were frozen immediately in liquid nitrogen and stored at $-80°C$ until extraction of RNA. Total RNA was extracted from frozen samples

with RNeasy® mini kit (Qiagen, Hilden, Germany). DNA was digested with RNase-free DNaseI at $37°C$ for 20 min. The RNA was further extracted with phenol-chloroform and precipitated with ethanol. The RNAs dissolved in diethylpyrocarbonate (DEPC)-treated ddH_2O were used to make cDNAs with the M-MLV RTase (Promega) and an oligo-dT primer following the manufacturer's instructions.

Cloning of the arginine kinase gene

The BmAK specific primers, forward primer (5'-CGGAATTCATG GTCGACGCCGCAACC-3') with a EcoRI site (underlined) and reverse primer (5'-CCAAGCTTCAGAGACTTCTCGATCTTGATGA GC-3') with an HindIII site(underlined) were designed to amplify the ORF of the putative BmAK gene (GenBank accession no. DQ272299). The PCR reaction was carried out with 30 amplification cycles ($94°C$ for 30 s, $58°C$ for 30 s and $72°C$ for 90 s) in a Gene Amp 2400 system thermocycler. The PCR product was ligated into pMD18-T vector using T4 DNA ligase and then, transformed into *E. coli* TG1. A fragment between EcoRI and HindIII containing the BmAK gene was excised from the recombinant plasmid. The purified fragment was subcloned into the pET-28a (+) expression vector. The resulting construction containing the entire ORF of arginine kinase gene was designated as pET28AK. DNA sequencing confirmed that the BmAK was correctly fused to the N-terminal 6 ×His-tag.

Expression and purification of arginine kinase

The recombinant plasmid pET28AK was transformed into Rosetta for expression of the arginine kinase protein. Positive colonies were identified by polymerase chain reaction (PCR). A fresh, isolated colony was chosen and incubated overnight in liquid LB medium at $37°C$. The overnight culture was diluted 1:10 in the same LB medium and grown at $37°C$ until A_{600} reached 0.6 to 0.8 absorbance units. The expression was then induced at $16°C$ for about 14 h by the addition of 0.2 mM isopropyl-β-D-thio-galactoside (IPTG). The cells were collected by centrifugation (5000 g, 10 min) and resuspended in lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 10 mM imidazole pH 8.0). The cells were lysed by ultrasonication and the debris was removed by centrifugation (15,000 g, $4°C$, 20 min). The supernatant was loaded onto a Ni-NTA affinity column (Qiagen, Hilden, Germany) preequilibrated with lysis buffer. After washing the captured column with 50 and 100 mM imidazole, the fusion protein was eluted with 250 mM imidazole. The eluted protein was concentrated using centrifugal ultrafiltration (Millipore, Boston, USA) and dialyzed to remove imidazole. All purification steps were carried out at $4°C$.

Immunoblot

Total cell extracts from *E. coli* with expression of BmAK were resolved by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the gel were blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, USA) and the membrane was incubated with 20 ml phosphate buffered saline Tween 20 (PBST) containing 4% (w/v) nonfat dry milk for 1 h and then, incubated with the mouse anti-6×His monoclonal antibody (1:500 dilution) for 1 h. After washing 4 times with PBST for 5 min, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (1:8000 dilution) for 1 h. After washing 4 times with PBS for 5 min, the bands were visualized by 10 ml PBS containing 0.05% 3, 3'-diaminobenzidine and 0.03% H_2O_2.
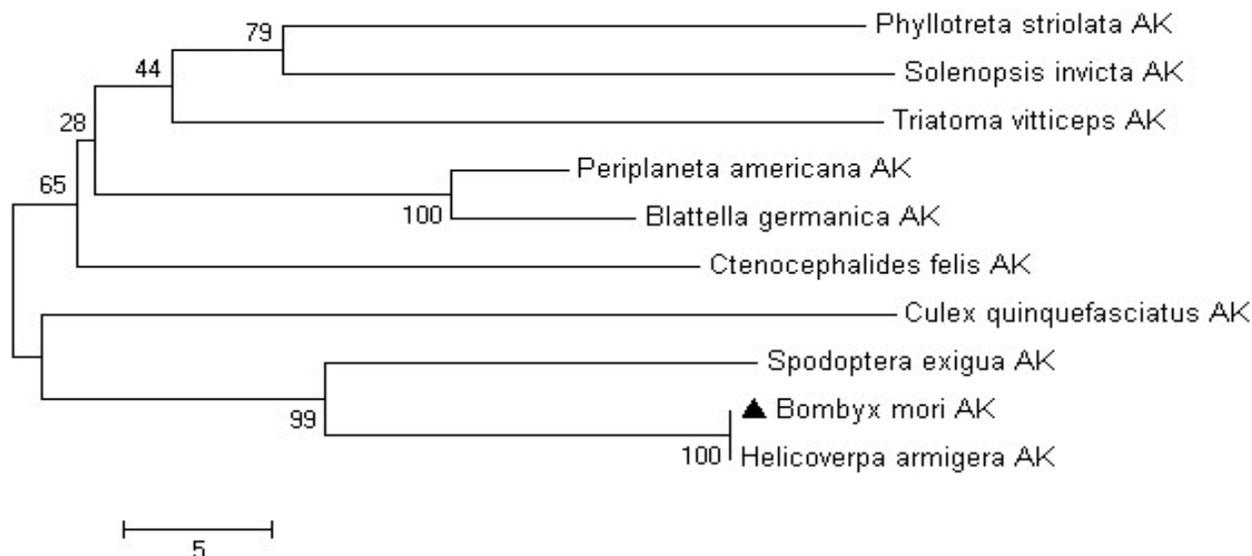
Figure 1. Phylogenetic analysis of BmAK and homologs from various insect species. The phylogenetic tree was constructed using MEGA version 4.1 with neighbor-joining algorithm. AK sequences used in the analysis were from *B. mori* (GenBank accession No. NP_001037402.1), *H. armigera* (ABU98622.1), *S. exigua* (ACU68932.1), *P. americana* (AAT77152.1), *T. vitticeps* (ABJ88949.1), *C. felis* (CAZ65719.1), *S. invicta* (ACF04198.1), *P. striolata* (ABZ10961.1), *B. germanica* (ABC86902.1) and *C. quinquefasciatus* (XP001849654.1).

Mass spectrometry

The specific bands corresponding to BmAK protein were excised manually from the polyacrylamide gel with a sterile scalpel and digested with trypsin according to Li et al. (2006) method. The digested samples were analyzed by an ultraflex MALDI-TOF-TOF instrument (BRUKER, Germany). Peptide mass fingerprinting (PMF) was performed by comparing the masses of identified peptides to the theoretically tryptic peptides of protein from National Center for Biotechnology Information (NCBI) protein database using the MASCOT search engine (http://www.matrixscience.com).

Enzymatic activity assay

The catalyzing activity of the recombinant BmAK was measured by monitoring the consumption of ATP in its forward reaction. Firstly, protein samples were incubated with 75 µl reaction buffer A (7 mM arginine, 7 µM ATP, 0.3 mM magnesium sulfate, phosphor buffered saline, pH 7.5 at 25°C for 10 min. And then, the residue ATP concentration was measured by an ATP assay kit (Beyotime, Jiangsu, China) immediately. Briefly, 100 µl ATP assay buffer was added into aforementioned reaction mixture and ATP standards prepared with the same buffer and the luminescence from fire fly luciferase in ATP assay buffer was measured and imaged by a fluorescence/chemiluminescence imaging system ChemiScope 2850 (Shanghai, China).

RESULTS AND DISCUSSION

Bioinformatics analysis of BmAK

According to phylogenetic analysis (Figure 1), BmAK and *Helicoverpa armigera* AK have the closest genetic relationship and they have the common ancestor with *Spodoptera exigua* AK. It was also found that, AK sequences were highly conserved across insect species (Figure 2). Prediction of protein domains by SMART program revealed that an ATP-gua PtansN and an ATP-gua Ptrans domain were at the position 19 to 95 and 106 to 356, respectively.

Cloning of the arginine kinase gene

Total RNA was isolated from *B. mori* fifth instar larvae and reversely transcribed into cDNA using oligo-dT primers. After amplification with BmAK specific primers, PCR product was separated on agarose gel and one 1068 bp DNA fragment was revealed. Then, the 1068 bp DNA fragment was extracted from the agarose gel and ligated into pMD18-T vector using T4 DNA ligase and the resulting plasmid was transformed into *E. coli* TG1. Restriction analysis of the plasmids from selected colonies using EcoRI and HindIII showed that, an inserted fragment was around 1068 bp. The insert was excised from recombinant plasmid pMD18-T-BmAK and the excised 1068 bp fragment was ligated into pET-28a (+). The resulting expression plasmid pET-28a (+)-BmAK was transformed into *E. coli* Rosetta. Restriction analysis of selected colonies using EcoRI and HindIII showed that a 1068 bp fragment was inserted into pET-28a(+). DNA sequencing confirmed that the right BmAK sequence was correctly fused to N-terminal His-tag.

We thus, confirm the presence of one single AK in the silkworm, which is consistent with observations on most other organisms. As a mater of fact, it is unusual to see
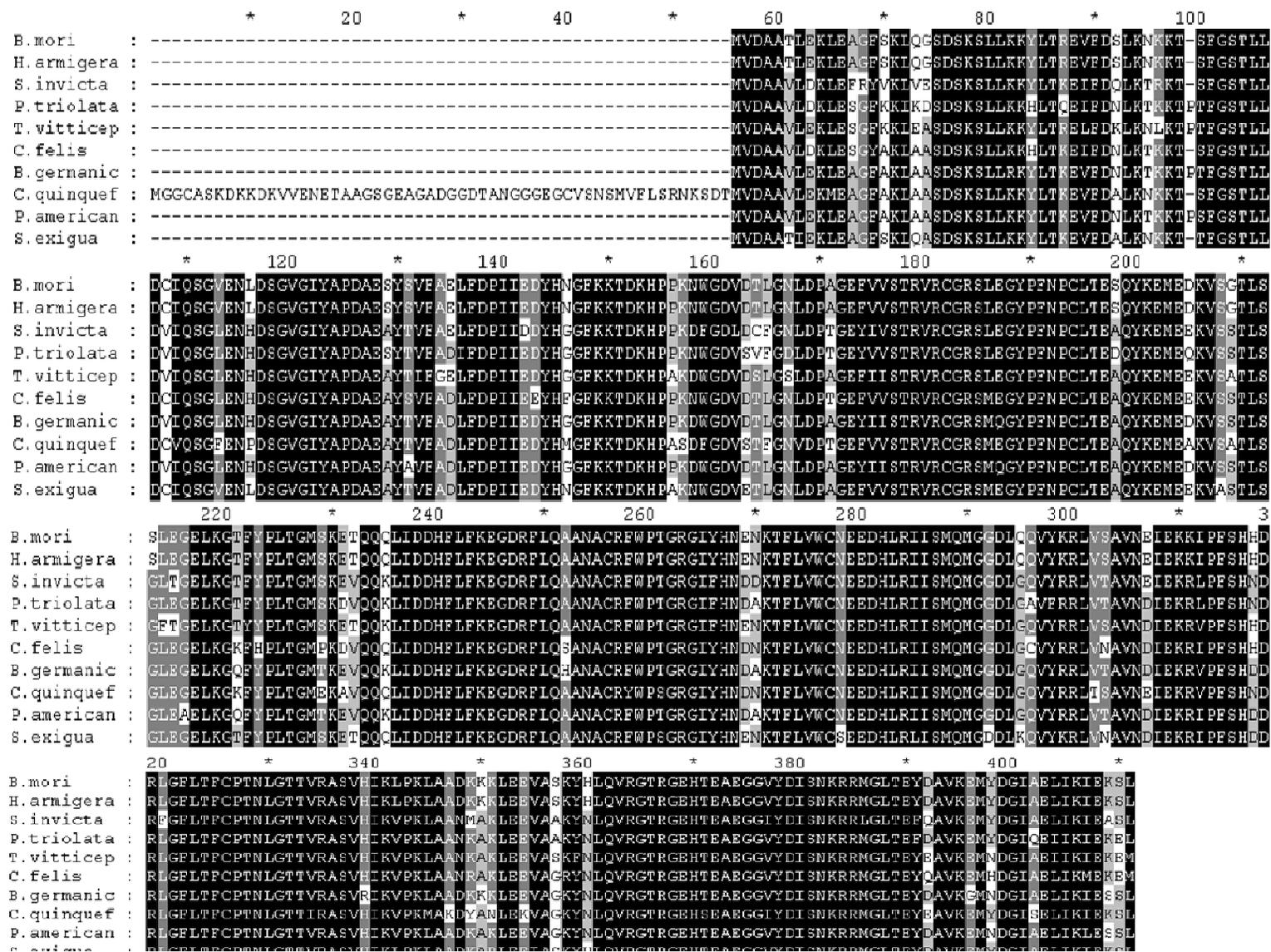
Figure 2. Multiple sequence alignment of amino acids of *Bombyx mori* AK and AKs from other insects. The identical amino acids were shown in black shade and the conserved amino acids were shown in gray shade. The aligned AKs were from *B. mori* (GenBank accession No. NP_001037402.1), *H. armigera* (ABU98622.1), *S. exigua* (ACU68932.1), *P. americana* (AAT77152.1), *T. vitticeps* (ABJ88949.1), *C. felis* (CAZ65719.1), *S. invicta* (ACF04198.1), *P. striolata* (ABZ10961.1), *B. germanica* (ABC86902.1) and *C. quinquefasciatus* (XP001849654.1)
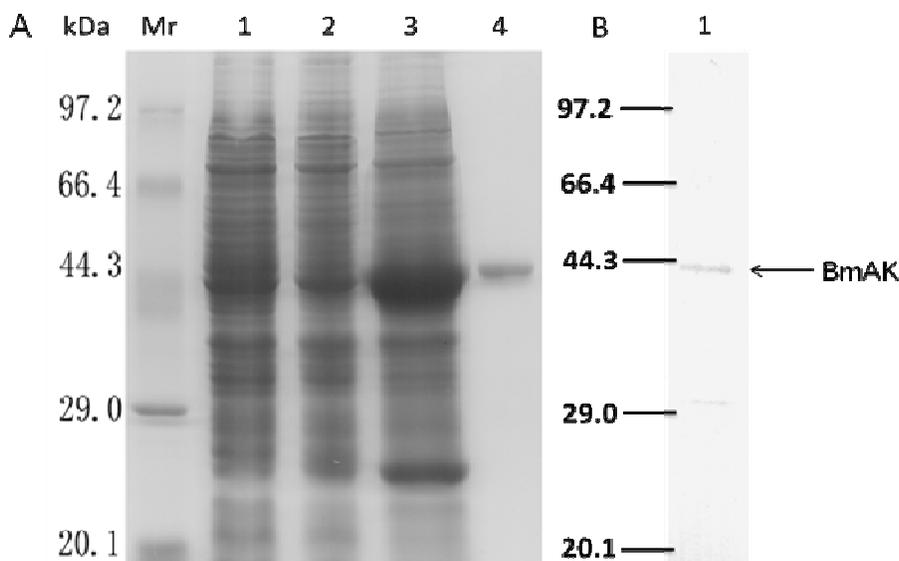
Figure 3. SDS–PAGE stained with Commassie blue and western blotting analysis of recombinant BmAK. (A) Lane 1, Rosetta; lane 2, Rosetta harboring empty vector; lane3, Rosetta with expression of BmAK; lane 4, purified recombinant BmAK; (B) Lane 1: western blotting analysis of recombinant BmAK with anti-6 ×His antibody.

multiple AK genes in a given organism like *M. japonicas* (Abe et al., 2007) and *Neocaridina denticulate* (Iwanami et al., 2009).

Expression and purification of arginine kinase

The recombinant BmAK protein was expressed in *E. coli* Rosetta harboring the expression vector pET-28a(+)-BmAK. Upon induction by the final concentration of 0.2 M IPTG at 16°C for approximate 12 h, the highest amount of BmAK protein was produced in soluble form with the N-terminus fused to 6 × His-tag. Approximate 4.515 g of wet cells were harvested from 1 L of medium. The fusion protein was extracted from *E. coli* by sonication and then, purified on a Ni^{2+}-NTA affinity column. Then, the soluble BmAK was purified to 85% purity (Figure 3a). The molecular weight of the fusion proteins was calculated to be around 40 kDa and peptides were detected by SDS-PAGE (Figure 3a). The amount of pure protein product is about 1.767 mg for 1 L *E. coli* culture estimated using the Bradford method with bovine serum albumin as the standard (Bradford, 1976). The expression of recombinant 6 × His-tagged protein was further corroborated by anti-6 × His monoclonal antibody (Figure 3b).

Mass spectrometry

To determine whether the amino acid sequence of purified protein matches the sequence accessed in GeneBank,

the MALDI-TOF–TOF mass spectra of tryptic digests of recombinant peptides was characterized to identify the recombinant protein. Seven peptide fragments were identified in mass spectra (Figure 4). By comparing the masses of identified peptides to the hypothetical tryptic peptides for proteins in non-redundant NCBI database using the MASCOT search engine, BmAK was obviously identified with MOWSE score of 71. The identified 7 peptide fragments were matched against the deduced amino acid sequence of BmAK with 23% sequence coverage.

Enzymatic activity assay

The BmAK can function by consuming ATP, arginine and producing ADP, phosphoarginine, respectively. Here, we assayed the specific activity of the recombine BmAK protein by measuring the rate of consumtion of ATP in its forward reaction. The standard curve of different concentrations of ATP was made using ddH$_2$O as blank. Obviously, with different concentrations, the chemiluminescence changed linearly (Figure 5ai). As for the test, besides employing water as blank, bovine serum albumin (BSA) was used as negative control. The result showed that there was no chemiluminescence in the last two wells since the ATP was completely consumed by the protein added in to the reaction mixture (Figure 5aii). The specific enzyme activity of BmAK expressed in *E. coli* lysate was significantly higher than that of BSA protein with the p value of 0.025 (Figure 5b). The specific activity
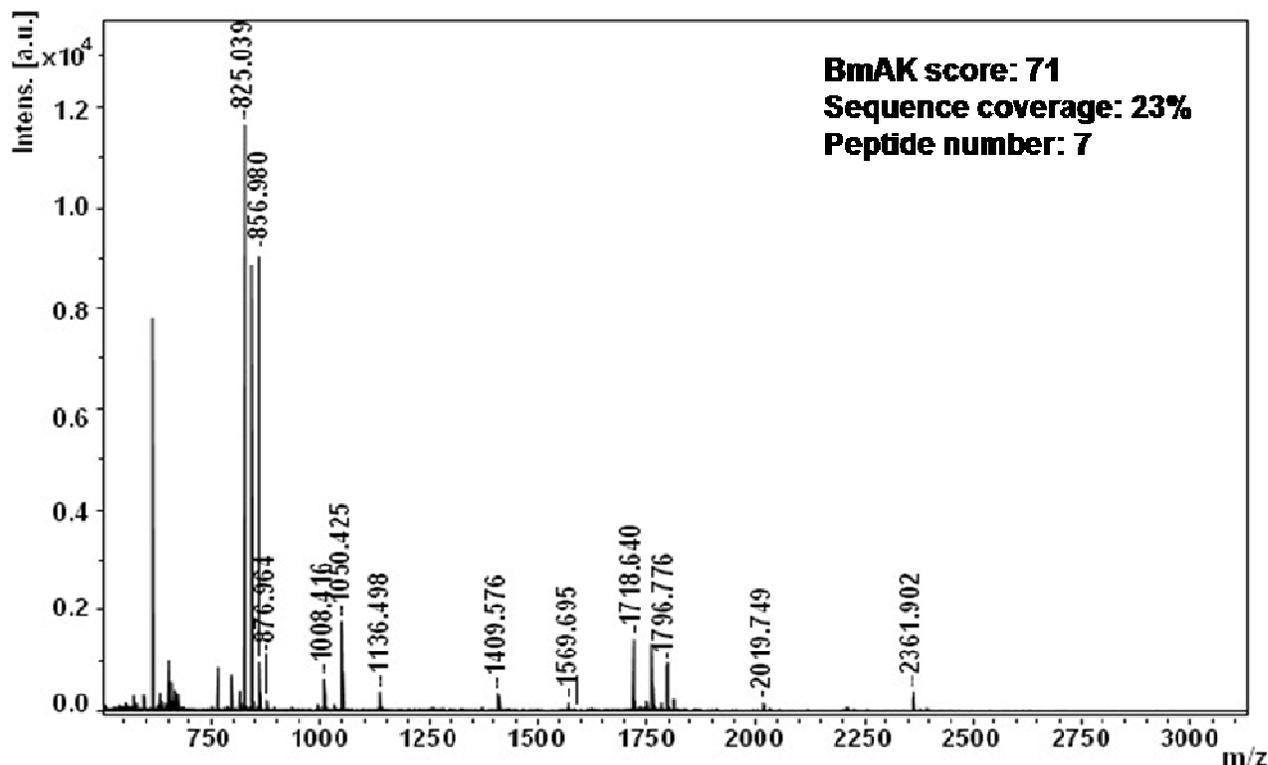
Figure 4. MALDI-TOF-MS spectrum of tryptic digests of recombinant BmAK. The identified protein, score, amino acid sequence coverage and the number of identified peptides are shown. The sequences of identified peptides shown in bold and underlined covered 23% amino acid sequence of BmAK.

of the final purified product was 1.0 U. One unit will convert 1.0 uM of ATP to ADP per minute at pH 7.5 at 25°C.

There are many ways to determine enzyme activity of arginine kinase, such as nicotinamide adenine dinucle-otide (NADH)-linked spectrophotometric assay (Fujimoto et al., 2005), the phosphate determination method (France et al., 1994) and direct continuous pH-spectro-photometric assay (Yu et al., 2002). The kinetic constants of arginine kinase of several species were obtained by NADH-linked spectrophotometric assay, such as beetle *Cissites cephalotes* (Tanaka et al., 2007), *Migratoria*

manilensis (Uda and Suzuki, 2004) and so on. It was reported that, the specific activity of purified AK of sea cucumber *S. japonicas* by improved version of the phosphate determination method was 26.8 units/mg (Guo et al., 2003) and the specific activity of recombinant *Metapenaeus ensis* greasyback shrimp AK was 12.72 units/mg (Wang et al., 2009). It was recently suggested that, arginine kinase would play a role as a stress resistance factor in *Trypanosoma cruzi* and its specific activity was gradually increased in the transfected parasites along the growth curve because of parasite replication or nutritional stress (Pereira et al., 2003).
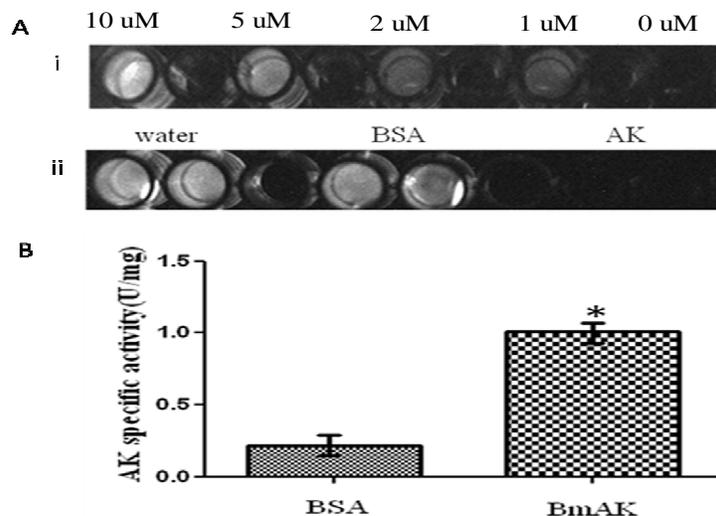
Figure 5. Chemiluminescence of standard curve and test samples and arginine kinase specific activity. A (i) Standard curve with 5 ATP concentrations; (ii) test samples, the left and middle two wells used water and BSA as blank and negative control, respectively. The last two was test sample; B. ATP consumption was measured in purified protein. BSA, acted as control; BmAK, purified BmAK protein.

Conclusions

In this study, we have successfully cloned, optimized the expression and purified arginine kinase of *B. mori* in the *E. coli* Rosetta. The purified recombinant BmAK protein was proved to be capable of catalyzing ATP to ADP, which suggests that the BmAK protein can be correctly folded and suitable for further structural and functional studies.

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