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Expression, purification, and characterization of arginine kinase from the sea cucumber *Stichopus japonicus*

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Abstract

The arginine kinase gene of sea cucumber *Stichopus japonicus* was cloned and inserted into the prokaryotic expression plasmid pET-21b. The protein was expressed in a soluble and functional form in *Escherichia coli* and purified by Blue Sepharose CL-6B, DEAE-32, and Sephadex G-100 chromotography with a final yield of 83 mg L^{-1} of LB medium. The specific activity, electrophoretic mobility, and isoelectric focusing were all identical with those of arginine kinase that was purified from sea cucumber muscle. The fluorescence emission spectrum of arginine kinase had a maximum fluorescence at a wavelength of 330 nm upon excitation at 295 nm. These results are the first report of this purified protein.

Keywords: Sea cucumber *Stichopus japonicus*; Arginine kinase; Gene clone; Expression; Purification; Characterization

Arginine kinase (ATP: arginine *N*-phosphotransferase, EC 2.7.3.3) in invertebrates catalyzes the reversible phosphorylation of arginine by MgATP to form phosphoarginine and MgADP, thereby regenerating ATP during bursts of cellular activity [1,2]. Although AK[1] is a phosphagen kinase, widely distributed in the lower and higher invertebrate groups, and plays a similar physiological role as creatine kinase (CK) does, it has not been investigated as extensively as CK.

Some cDNA sequences of arginine kinase were obtained in recent years. In 1993, Dumas and Camonis [3] determined the arginine kinase cDNA sequence of lobster *Homarus vulgaris*. Strong and Ellington [4] subsequently published the gene sequence of arginine kinase from the chelicerate arthropod *Limulus polyphemus*. Suzuki et al. [5] isolated the arginine kinases of sea cucumber *Stichopus japonicus*, sea anemone *Anthopleura japonicus* [6], and that of two molluscan: the chiton *Liolophura japonica* and the turbanshell *Battilus cornutus* [7].

Much research has focused on the evolution of phosphagen kinases and the evolutionary relationships between arginine kinase and creatine kinase [5–10]. There was a proposal that AKs have appeared independently twice in the evolution of phosphagen kinase: first at an early stage of phosphagen kinase evolution (its descendants are molluscan and arthropod monomeric AKs) and second from CK later in metazoan evolution [5]. A crystal structure for the transition state analog complex (TSAC) of AK, which is a monomer from the horseshoe crab *Limulus*, has been reported [11], while the crystal structures of other arginine kinase have not been determined.

Arginine kinase of sea cucumber *S. japonicus* exists as a dimer with a molecular weight of 84 kDa [5]. In the present study, we successfully cloned the arginine kinase gene and expressed it in *Escherichia coli*, purified it, and characterized several of its physiological properties for the first time.

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[1] *Abbreviations used:* AK, arginine kinase; CK, creatine kinase; CD, circular dichroism; DTT, dithiothreitol.

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Materials and methods

Materials

Escherichia coli expression vector pET-21b and the strains BL21(DE3) were from Novagen. The Trizol reagent, one-step RT-PCR with Platinum *Taq*, restriction enzyme, T_4 DNA ligase, and Platinum *Taq* DNA polymerase were purchased from Invitrogen. Blue Sepharose CL-6B and DEAE-cellulose 32 were Pharmacia products. Calibration kits for p*I* determinations and ampholine used in IEF were Amersham Pharmacia Biotech products. All other reagents were local products of analytical grade. Sea cucumber *S. japonicus* was purchased from the seafood market in Beijing, China.

Cloning of the arginine kinase gene

Total RNA of the sea cucumber *S. japonicus* was isolated using Trizol reagent and the arginine kinase cDNA was amplified using a one step RT-PCR system. The primer was: 5′ GGAATTCCATATGGCAAACTT AAACCAGA-3′ (upstream) and 5′ CGGGATCCTT ACTTGG GGACAAGGTCA-3′(downstream). *Nde*I and *Bam*HI endonuclease sites (underlined) were included in these two primers to facilitate subsequent cloning steps. There was a termination codon at the 5′ end of the downstream primer, so the subsequently expressed arginine kinase was not a fusion protein. The mixture for RT-PCR was incubated at 50 °C for 30 min and then denatured at 94 °C for 2 min. The subsequent PCR cycles were as follows: denaturation at 94 °C for 15 s, annealing at 56 °C for 30 s, and extension at 72 °C for 2.5 min for 35 cycles. The obtained fragment was digested with *Nde*I and *Bam*HI and inserted into vector pET-21b between the *Nde*I and *Bam*HI cloning sites. The ligation product was transferred into *E. coli* JM110 and selected with 100 μg/ml ampicillin pressure. The arginine kinase gene in the recombinant plasmid was confirmed by DNA sequencing. The resulting construct containing the entire arginine kinase gene was designated as pET21AK.

Expression and purification of arginine kinase

The recombinant plasmid pET21AK was transformed into *E. coli* BL21(DE3) for expression of the arginine kinase protein. Positive colonies were identified by 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 0.6–0.8 absorbance units. The expression was then induced at 25 °C for about 14 h by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were collected by centrifugation (5000*g*, 10 min) and resuspended in lysis buffer (20 mM Tris–Ace, pH 8.3; 0.1 mM DTT; and 0.1 mM PMSF). The cells were lysed by ultrasonication and the debris was removed by centrifugation (15,000*g*, 20 min, 4 °C). The supernatant was loaded on a Blue Sepharose CL-6B column preequilibrated with buffer contained 20 mM Tris–acetate, pH 8.3, and 0.1 mM DTT. The active fractions flowing through the above column were pooled and then loaded on a preequilibrated DEAE-32 column for ion-exchange chromotography. The column was washed with 4–5 column volumes of 20 mM Tris–acetate, pH 8.3, and 0.1 mM DTT before the arginine kinase was eluted with gradient elution using 0–0.15 M NaCl. After that the active fractions were concentrated and passed through a column of Sephadex G-100 preequilibrated with the above buffer. All purification steps were carried out at 4 °C.

The purified fractions were characterized by SDS–PAGE [12] and isoelectric focusing (IEF) electrophoresis. IEF electrophoresis of arginine kinase was performed using a 5% nondenaturing polyacrylamide gel [13] (50 μg AK). The fluorescence measurement was carried out using a fluorescence spectrophotometer F-2500 (Hitachi) using 1 cm path-length cuvettes. The excitation wavelength for instrinsic fluorescence emission spectra was 295 nm. CD spectra were recorded on a Jasco 725 spectrophotometer with a 2 mm path-length cell over the wavelength range of 200–250 nm. The secondary structure was estimated using the estimation program of Yang [14].

Activity and concentration assay

The activity assay was an improved version of the phosphate determination method [15]. Enzyme (10 μL) was added to 290 μL assay mixture (10 mM arginine, 2 mM ATP, and 3 mM magnesium acetate, dissolved in 0.1 M Tris–acetate, pH 8.1) and incubated for 0.5 min at 25 °C. The reaction was stopped by the addition of 250 μL of 2.5% TCA, after which the mixture was put in boiling water for 1 min to hydrolyze the phosphoarginine, then immediately cooled on ice for another minute, and equilibrated at 25 °C for 5 min. Four hundred fifty microliters of phosphate determination reagent (44 mM bismuth nitrate, 1.33 M nitric acid, 31 mM ammonium molybdate, and 0.11% ascorbic acid) was added to the above equilibrated solution to give 1 mL final volume of a color developing system. After 3 min, the absorbance was measured at 700 nm. The protein concentration was determined by Bradford method [16] with bovine serum albumin as the standard. The activity and concentration were measured with an Ultrospec 4300 pro UV–Visible spectrophotometer.

Results and discussion

Cloning of the arginine kinase gene

The fragment encoding of the sea cucumber arginine kinase was obtained using the method described under

Table 1
Summary of the purification steps for arginine kinase from recombinant *E. coli* cultures

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Lysis supernatant	287	5010	17.5	100
Blue Sepharose flowthrough	172	4850	28.2	97
DEAE-32 elution	75.8	1910	25.2	38
Sephadex G-100	74.4	2000	26.8	40

The activity is defined as microliters of phosphate mL^{-1} min^{-1} and the specific activity as the activity per milligram of protein.

Materials and methods. The expression vector pET-21AK was constructed by inserting the fragment between the *Nde*I and the *Bam*HI restriction sites and then transformed into *E. coli* strain BL21(DE3). Since the reading frame begins at the initiation codon ATG in the *Nde*I site, and terminates at the termination codon before the *Bam*HI site, the expressed arginine kinase was a nonfusion protein. The cloned arginine kinase gene containing 1113 base pairs in the recombinant plasmid was confirmed by DNA sequencing. The sequence was not absolutely identical with the previously published sequence of sea cucumber *S. japonicus* arginine kinase. But the amino acid sequence translated from it was completely identical with the published one [5].

Expression and purification of arginine kinase

Protein expression was induced in early log phase cultures by the addition of IPTG. It was found that when the cells were induced at 37 °C, most of the arginine kinase was expressed in an insoluble form (data not shown). Decreasing the inducing temperature to 25 °C leads to a higher yield of soluble AK. A summary of the arginine kinase purification steps is listed in Table 1. Compared to the Blue Sepharose flowthrough, the specific activity was reduced after DEAE-cellulose elution. But the SDS–PAGE electrophoresis (shown in Fig. 1) results show that ion-exchange chromotography was a very efficient step in this purification process. The reduction of specific activity may be due to the long period of ion-exchange chromotography. The unstable enzyme lost some of its activity. The purified arginine kinase presented a single band on SDS–PAGE after it is passed through the column of Sephadex G-100, which indicates its high level of purity.

Under the procedure described above, approximately 75 mg of homogeneous AK was obtained from the 900 mL cell culture.

Activity analysis

The specific activity (26.8 units/mg) of the final purified product is essentially identical to that of tissue-purified arginine kinase (28.1 units/mg).

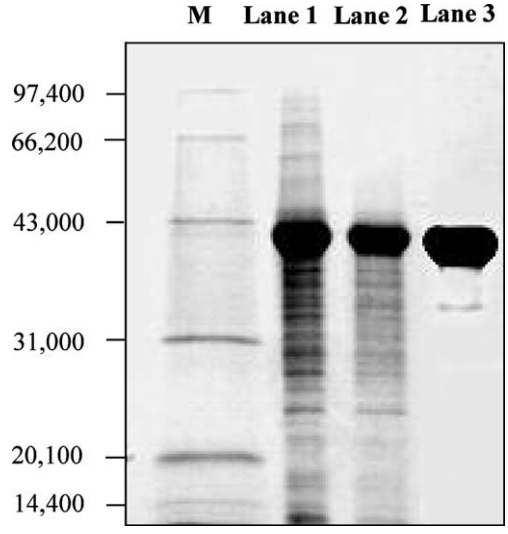

Fig. 1. SDS–PAGE of purified arginine kinases expressed by *E. coli* in each step. SDS–PAGE was performed on a 12.5% polyacrylamide gel and stained using Coomassie brilliant blue. Lane 1, the active fraction through Blue Sepharose CL-6B step. Lane 2, the active fraction eluted from DEAE-32. Lane 3, the active fraction through Sephadex G-100. Lane M contains molecular mass markers in daltons.

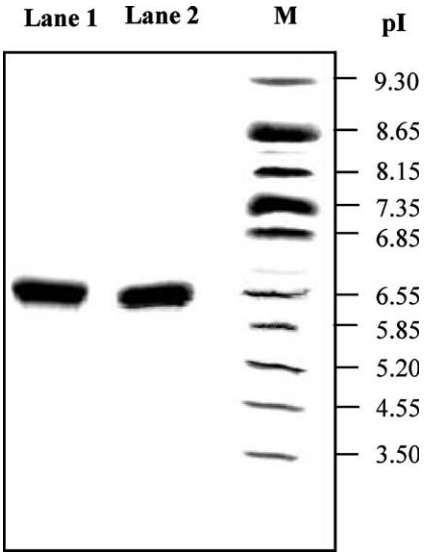

Fig. 2. Isoelectric focusing (IEF) electrophoresis of arginine kinase. Lane 1, arginine kinase purified from muscle of sea cucumber *S. japonicus*. Lane 2, arginine kinase expressed by *E. coli*. Lane M, indicates p*I* marker proteins (Amersham Pharmacia Biotech) in pH values.

Physicochemical properties of arginine kinase

The isoelectric point of arginine kinase occurs at pH 6.5 (Fig. 2), quite different from that of monomeric AK which is of pH 5.4 [17], but very similar to that of muscle CK which is of pH 7 [18,19]. This may provide further evidence to support the theory that dimeric arginine kinase originated from creatine kinase [5]. The theoretical p*I* of arginine kinase from sea cucumber *S. japonicus* analyzed by computer tool of EXPASY was 6.51, identical with the present result. To our surprise, the results were quite different from those of arginine kinase purified from sea cucumber *Caudina arenicola*, whose p*I* was 7.8 [20]. There may be some differences between these two types. Since the amino acid sequence of arginine kinase from sea cucumber *C. arenicola* has not been published, no explanation can be offered here.

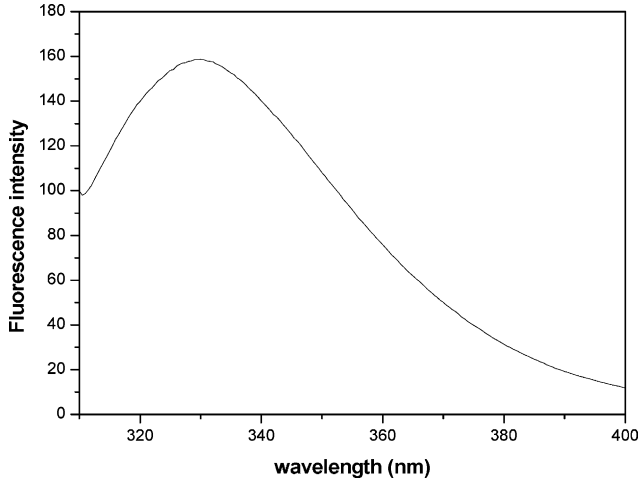

Fig. 3. Fluorescence emission spectrum of arginine kinase. The concentration of the protein was 0.67 mg/mL. The excitation wavelength was 295 nm. The experiment was carried out at 25 °C.

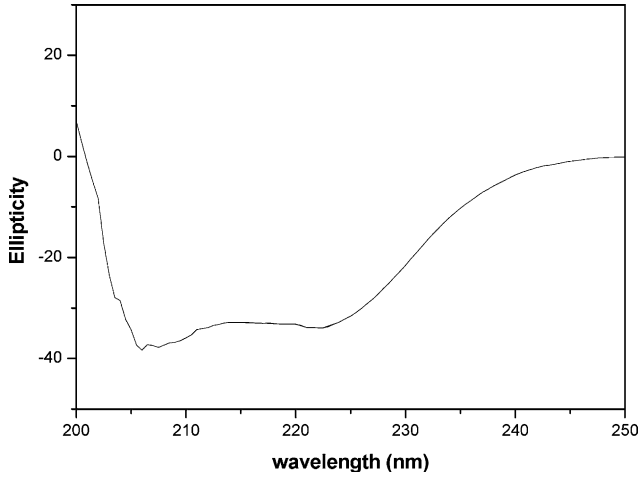

Fig. 4. Far-UV circular dichroism spectrum of arginine kinase. The concentration of the protein was 0.67 mg/mL. The experiment was carried out at 25 °C.

The fluorescence emission spectrum of AK had a maximum fluorescence wavelength of 330 nm upon excitation at 295 nm, as shown in Fig. 3.

Far-UV circular dichroism spectrum of arginine kinase is shown in Fig. 4. The predicted secondary structure shows that each subunit of arginine kinase molecules contains 31% α-helix, 7.7% β-sheet, 20% turn, and 42% random coil.

In conclusion, we successfully cloned the gene for sea cucumber arginine kinase into an *E. coli* expression vector and acquired a highly purified functional enzyme. The purification system makes it easy for one to obtain arginine kinase for enzymic research. The active site and catalytic mechanism of the enzyme are still not clear and its crystal structure has not been determined. The present study may be very useful for further work.

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