**Improved Activity Assay Method for Arginine Kinase Based on a Ternary Heteropolyacid System**

CHEN Baoyu ( ), GUO Qin ( ), GUO Zhi ( ), WANG Xicheng ( )

1 Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China;
2 Protein Science Laboratory of the Ministry of Education, School of Life Science and Engineering, Tsinghua University, Beijing 100084, China

**Abstract** This paper presents a new system for the activity assay of arginine kinase (AK), based on the spectrophotometric determination of an ascorbic acid-reduced blue ternary heteropolyacid composed of bismuth, molybdate and the released phosphate from N-phospho-L-arginine (PArg) formed in the forward catalysis reaction. The assay conditions, including the formulation of the phosphate determination reagent (PDR), the assay timing, and the linear activity range of the enzyme concentration, have been tested and optimized. For these conditions, the ternary heteropolyacid color is completely developed within 1 min and is stable for at least 15 min, with an absorbance maximum at 700 nm and a molar extinction coefficient of 15.97 (mol/L)\(^{-1}\)cm\(^{-1}\) for the phosphate. Standard curves for phosphate show a good linearity of 0.999. Compared with previous activity assay methods for AK, this system exhibits superior sensitivity, reproducibility, and adaptability to various conditions in enzymological studies. This method also reduces the assay time and avoids the use of some expensive instruments and reagents.

**Key words** arginine kinase (AK); activity assay; phosphate determination; heteropolyacid; bismuth; molybdate

**Introduction**

Arginine kinase (EC 2.7.3.3, AK) in invertebrates plays a central role in both temporal and spatial ATP buffering in cells with high, fluctuating energy requirements (muscle, nerves, etc.) by catalyzing the magnesium-dependent reversible phosphorylation between L-arginine and ATP according to the following reaction \(^{[1, 2]}\):

\[
L^-\text{arginine} + \text{Mg}^+\text{ATP} \rightarrow N^-\text{phospho-L-arginine} + \text{Mg}^+\text{ADP}
\]

AK is an ideal paradigm for the classical enzymology of bimolecular reactions \(^{[3, 4]}\) and activity assay methods are needed which provide adequate accuracy, fidelity and adaptability. In previous works, AK activity was usually detemined using: 1) the phosphate determination method \(^{[5-7]}\), 2) a pH-stat assay measuring proton released \(^{[8]}\), 3) the arginine diacetyl colorimetric reaction \(^{[7]}\), 4) an enzyme-coupled reaction derived from the activity assay method of creatine kinase \(^{[9]}\), and 5) the isotopic assay method \(^{[10]}\).

The phosphate determination method has been extensively used for AK activity assay because of its simplicity. The underlying principle is the chemical detemination of the inorganic phosphate released by hydrolysis of the acid-labile PArg formed in the forward catalysis reaction, see Reaction (1). Traditional methods of chemical determination of the phosphate employed for AK activity assays are based on the reaction of phosphate to form a binary heteropolyacid with molybdate, which is then reduced to develop colors suitable for spectrophotometric assay \(^{[7]}\). However, in our studies of AK, we were often frustrated by this system’s inadequate sensitivity and reproducibility \(^{[11, 12]}\). Therefore, the method was modified by introducing into the AK activity assay an ascorbic acid-reduced blue ternary
heteropolyacid system using bismuth, molybdate and phosphate. This ternary heteropolyacid system was then optimized and compared with the original binary method.

1. Materials and Methods

1.1 Materials

A K was prepared from the muscle of sea cucumber Stichopus japonicus according to the procedure of A nosike et al. and proved to be homogeneous on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. ATP, Arg, and Fiske/Subbarow Reducer were purchased from Sigma. All other reagents were local products of analytical grade and used without further purification.

1.2 Methods

The enzyme assay procedure was as follows: The reaction mixture consisted of 10.34 mmol/L Arg, 2.07 mmol/L ATP disodium salt, and 3.10 mmol/L magnesium acetate dissolved in 0.1 mol/L Tris acetate, pH 8.1. The ATP was added fresh to the reaction mixture and the solution was available for 4 h. The reaction was carried out at 25°C by adding 10 μL enzyme sample to 290 μL of the reaction mixture to give final concentrations of 10 mmol/L Arg, 2.0 mmol/L ATP and 3.0 mmol/L magnesium acetate. The reaction was allowed to continue for 0.5 - 1.5 min, after which 250 μL of 2.5% trichloroacetic acid (TCA) was added immediately to stop the reaction. The solution was placed in boiling water for 1 min to fully hydrolyze the formed PAArg, then immediately plunged into an ice bath for another minute for quick cooling, and finally left in air to equilibrate at room temperature for 5 min. The liberated inorganic phosphate was determined afterwards with the phosphate determination reagent (PDR). The PDR was prepared fresh by mixing stock solutions of 1 mL 0.2 mol/L bismuth nitrate dissolved in 6 mol/L nitric acid, 1 mL 0.14 mol/L ammonium molybdate, 0.5 mL 1% ascorbic acid and 2 mL distilled water. 450 μL PDR was added to the equilibrated solution to give 1 mL final volume of a system whose color developed over time. After 3 min of color development, the solution absorbance was measured at 700 nm. The control was prepared in parallel with the same conditions except that the 10 μL enzyme sample was replaced by buffer in the enzyme sample. For accurate analysis of the released phosphate and, thus, the formed PAArg, a standard curve was made using the same procedure except that 10 μL standard solutions of various concentrations of KH₂PO₄ were added instead of the enzyme sample.

The method described by France was used to compare the traditional binary heteropolyacid system for phosphate determination with the method described here. The enzyme concentration was determined according to the method of Bradford with bovine serum albumin as the standard A4 absorbance spectrophotometer. Specord 200 UV VIS (Jena, Germany), was used to measure the enzyme concentration and activity.

2. Results and Discussion

2.1 Characterization of the ternary heteropolyacid system

The bismuth-molybdate-phosphate system is reduced by ascorbic acid to a blue ternary heteropolyacid complex with a bismuth-phosphate-molybdate composition equivalent to 1:1:1 mole ratio. As with previous results, the absorbance spectra of the ternary heteropolyacid system containing the inorganic phosphate were characterized by a maximum absorbance peak at 700 nm, which was absent from the control without any inorganic phosphate (Fig 1). The sample containing ATP but no added phosphate exhibited a similar but much weaker absorbance at 700 nm, indicating that spontaneous hydrolyzation of ATP contributing to the total phosphate in the system cannot be overlooked. Therefore, the control must be prepared in parallel during the activity assay process to correct the phosphate background for the spontaneous hydrolyzation of ATP.

![Absorbance spectra of 1 mL activity assay system. The absorbance was recorded as described in Section 2.1 with 0.6 mmol/L ATP and 0.0651 mol/mL KH₂PO₄ (a), 0.6 mmol/L ATP and no phosphate (b), and neither ATP nor phosphate (c).](image-url)
2. 2. Optimization of the PDR formulation

Arg, magnesium acetate, and Tris acetate in the catalysis reaction solution, as well as TCA, showed no significant contribution to the absorbance spectrum and intensity at 700 nm (data not shown). Besides arsenic, many other common substances also do not interfere with the results of the heteropolyacid system. Therefore, this method is adaptable to various conditions in enzymological studies with various different substances.

However, the concentrations of bismuth, molybdate, ascorbic acid, and nitric acid in the PDR affected, to different extents, the sensitivity, rate and stability of the system at the color development end point (Fig. 2). The PDR formulations are listed in Table I.

![Graphs showing the effect of different PDR formulations on phosphate determination results.](image)

**Table I. PDR formulations**

<table>
<thead>
<tr>
<th>Formulation No</th>
<th>0.2mol/L bismuth nitrate (mL)</th>
<th>0.14mol/L ammonium molybdate (mL)</th>
<th>1% ascorbic acid (mL)</th>
<th>Nitric acid to dissolve bismuth nitrate (mol/L)</th>
<th>Distilled water (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>6.0</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td>6.0</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
<td>6.0</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
<td>6.0</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>2.0</td>
<td>0.5</td>
<td>6.0</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>6.0</td>
<td>1.5</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
<td>6.0</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td>4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>9</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td>8.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>
The proportion of bismuth nitrate greatly influenced the assay results (Fig. 2a). In 4.5 mL PDR solution, the 0.5 mL (No. 1) and 1 mL (No. 2) bismuth nitrate concentrations induced rapid color development; however, the 1 mL (No. 2) and 1.5 mL (No. 3) bismuth nitrate concentrations caused the system to have a more stable absorbance. 1 mL 0.2 mol/L bismuth nitrate per 4.5 mL PDR was then used for the often tests for the best assay result. In Fig. 2b, the low ammonium molybdate concentration (No. 4) slowed color development, while the higher concentrations of 1 mL (No. 2) and 2 mL (No. 5) molybdate caused the color to develop faster. The concentration of the reducer in the system, ascorbic acid had little effect on the assay result, except that less ascorbic acid in the system led to a slight improvement in the sensitivity (Fig. 2c). The nitric acid concentration used to dissolve the bismuth nitrate strongly affected the system sensitivity. The results in Fig. 2d show that higher concentrations gave better sensitivity with a slight slowing of the color development. From the results of all these tests, the optimized PDR formulation was chosen to be 0.2 mol/L bismuth nitrate dissolved in 6 mol/L nitric acid, 0.14 mol/L ammonium molybdate, and 0.5 mL 1% ascorbic acid in 2 mL distilled water for a ratio of 1:1:0.5:2 (No. 2). Therefore, the final PDR concentrations of bismuth nitrate, nitric acid, ammonium molybdate and ascorbic acid were 44 mmol/L, 133 mol/L, 31 mol/L and 0.11% (mass fraction), respectively. With this formulation, the color development was completed within 1 min and was stable for at least 15 min.

2.3 Timing of phosphate determination procedure

The timing of the method was also evaluated using the method given by France[7]. The results in Fig. 3 show that the absorbance at 700 nm with 3 min of color development showed no difference for incubation time in boiling water exceeding 1 min. Therefore, 1 min in the boiling bath is adequate for full release of the phosphate from the formed PArg. Varying equilibration time at room temperature after quick cooling in the ice bath was found to have no effect on the assay result (data not shown).

2.4 Standard curve

The measured standard curve, Fig. 4, was both linear and reproducible, which guarantees the accuracy and reproducibility of this method for measuring the phosphate in AK assays.

2.5 Standard curve for phosphate concentration with KH2PO4 as standard

The tested upper range of the linearity for the phosphate measurement was at least 0.065 μmol phosphate radicals per 1 mL reaction system, with a linearity of 0.999. The molar extinction coefficient of the reduced ternary polyanionic acid was determined to be 15.97 (mmol/L)·1·cm⁻¹ for the phosphate. Independent experiments with the same conditions reproduced nearly the same results, with an error of less than 5% (data not shown).
2. 5 Comparison to other methods used to assay AK activity

Five methods have been widely used to assay AK activity[10]. The arginine-diacyt colorimetric reaction and enzyme coupled reaction methods utilize the reverse reaction catalyzed by AK so they use PAArg, which is extremely expensive. Expensive reagents and instruments are required for the isotopic assay and pH-stat assay methods. AK assay methods based on phosphate determination are relatively simple, but traditionally utilize a binary heteropolyacid system consisting of phosphate and molybdate.

Comparison of the present ternary heteropolyacid system with a representative binary heteropolyacid method[7] using a commercially available phosphate determination reagent, Fiske/Subbarow Reducer, derived from the method of Fiske and Subbarow[16], showed that the ternary method has several advantages. First, the procedure very quickly (about 1 min) reaches full color development, in contrast to at least 30 min for the Fiske method as shown in Fig. 5, which greatly shortens the assay time. Secondly, the ternary system had good reproducibility and linearity of the standard phosphate curve. The Fiske method has less stability possibly due to the formulation of the Fiske/Subbarow Reducer, which consists of 1-amino-2-naphthol-4-sulfonic acid, sodium sulfate, and bisulfite, which are unstable in solution and susceptible to unexpected factors during usage. Finally, the molar extinction coefficient of the ternary heteropolyacid system 15.97 (mmol/L)⁻¹·cm⁻¹ was not only much greater, by two orders of magnitude, but also more stable than that of the Fiske method (about 0.50 (mmol/L)⁻¹·cm⁻¹ after 15 min of color development). These results are consistent with previous findings on the determinations of inorganic phosphate that a ternary heteropolyacid provides greater sensitivity and fidelity than a binary one[11,12]. The lower molar extinction coefficient means less sensitivity to the released phosphate and more samples of the enzyme are required for the activity assay, which increases both the error of the Fiske method and the waste of the precious enzyme.

2. 6 AK activity assay results using the ternary heteropolyacid method

The ternary heteropolyacid system to assay AK activity was tested to determine the linearity limits with respect to assay time, protein concentration and absorbance at 700 nm. The data in Fig. 6a

![Image](image_url)

**Fig 5** Time course of color development of Fiske method. The system was as described by France[7] with 2 μmol/mL KH₂PO₄, 200 μL catalysis solution, 250 μL 2.5% TCA, 500 μL 0.09 mol/L ammonium molybdate dissolved in 2.5 mol/L sulfuric acid and 50 μL Fiske/Subbarow Reducer.

![Image](image_url)

(a) Assay linearity for various AK concentrations for 30 s assay period

![Image](image_url)

(b) Duration of catalysis and assay linearity. AK concentrations were 0.023 mg/mL ( ), 0.056 mg/mL (△), 0.1125 mg/mL (○), and 0.225 mg/mL ( ).

**Fig 6** AK activity assay results using the ternary heteropolyacid method. Activity was defined as μmol·min⁻¹·mg⁻¹ for the PAArg.
show that protein concentrations of 0.02 - 0.3 mg/mL gave linear activities for assay periods of 30 s. The results in Fig. 6b show that the catalysis measurement was linear for reaction time of 30 - 300 s for a protein concentration of 0.023 mg/mL. For all the tested AK concentrations, linearity was guaranteed as long as the protein concentration and catalysis time were adjusted so that the final absorbance at 700 nm was less than 0.8, which was adjacent to the upper linearity limit of the standard curve in Fig. 4.

3 Conclusions

A ternary heteropolyacid system for phosphate detemination was successfully introduced into the assay of AK activity. The experimental conditions optimized to obtain the best assay results had a total assay volume of 1 mL, with 300 μL catalysis reaction mixture, 250 μL 2.5% TCA to terminate the reaction, and 450 μL PDR for color development. The PDR formulation had 44 mmol/L bismuth nitrate, 1.33 mmol/L nitric acid, 31 mmol/L ammonium molybdate, and 0.11% ascorbic acid at least 1 min of incubation in the boiling water gave full hydrolysis of the PAK. The molar extinction coefficient at 700 nm was 15.97 (mmol/L) *cm^-1 for the phosphate after 3 min of color development. The upper range of linearity for the phosphate measurement was at least 0.065 μmol phosphate radicals per 1 mL reaction system. Tests show that the protein concentration and catalysis time should be adjusted to keep the final absorbance below 0.8 for good linearity in the activity assay. Compared with other AK activity assay methods, this system provides better sensitivity, reproducibility, and adaptability to the various conditions in enzymological studies. Moreover, this method reduces the assay time, saves enzyme usage, and avoids use of some expensive instruments and reagents. The ternary heteropolyacid system has potential applications in many other areas besides just AK activity assays, such as the assay of many other kinases or nucleic acids.

References