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# A rapid and sensitive colorimetric assay for the determination of adenosine kinase activity

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ABSTRACT

Adenosine kinase (ADK) plays an important role in the growth and development of organisms. A convenient, quick, reliable, sensitive and low-cost assay for ADK activity is of great significance. Here, we found the reaction system with bromothymol blue as the pH indicator had a maximum absorption peak at 614 nm. The absorbance change in 614 nm was positively correlated with the generated hydrogen ions in the reaction catalyzed by ADK. Then, we demonstrated this assay was feasible for ADK activity. Further, we analyzed the effects of buffer, bromothymol blue concentrations on the sensitivity of the assay, and investigated the sensitivity of ADK contents and adenosine concentration on the assay. Finally, we calculated the $K_m$ and Vmax of ADK from *Bombyx mori* with this assay. Our results suggested this assay was quick, convenient, reliable, sensitive and economic for the activity of ADK. It is an excellent alternative for the conventional ADK assays.



## 1. Introduction

Adenosine kinase (ADK; ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20), is isolated from yeast cells by Kornberg for the first time in 1951 [1]. ADK belongs to the ribokinase protein family and is one of the most abundant nucleoside kinases in mammalian tissues [2,3]. As the first identified enzyme in the purine salvage pathway, ADK could catalyze the transfer of γ-phosphate group from adenosine triphosphate (ATP) to adenosine to produce AMP (Adenosine + ATP→AMP + ADP). ADK plays the role of potent cardioprotective agent and neuromodulator *via* regulating both intracellular and extracellular adenosine concentration [2,4,5]. In the diabetes model, the decrease of ADK expression impairs the proliferation of T lymphocytes [6]. ADK also plays a significant role in facilitating intracellular methylation. The absence of ADK in the $ADK^{-/-}$-lesioned mice leads to the acute hepatic steatosis in the newborn infants and the death at postnatal day 8 [7]. However, the overexpression of ADK in brain results in frequent electrographic seizures with a frequency of four times per hour [8], and the $ADK^{-/-}$-lesioned mice awakes earlier compared with the wild-type [9]. In addition, ADK also can catalyze the phosphorylation of other nucleosides and their analogs to generate the corresponding monophosphate [10].

Two methods have been developed to measure the enzymatic activity of ADK. One is a coupled multi-enzyme system assay, another one is a radioactive labeling assay [11]. The coupled multi-enzyme system contains three kinds of enzymes: ADK, pyruvate kinase and lactate dehydrogenase. In this assay, ADP formed in the reaction catalyzed by ADK is reconverted to ATP in the presence of excess P-enolpyruvate and pyruvate kinase. At the same time, the produced pyruvate is catalyzed to generate lactate in the presence of excess NADH and lactate dehydrogenase, which will result in the decrease of the absorbance at 340 nm because of the consumption of NADH. It is a time consuming multi-steps process subject to errors at each step, and other two enzymes have different optimal

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reaction conditions, thus it is difficult to balance the optimum conditions for the three enzymes system. Moreover, it could not accurately determine the initial velocity of ADK reaction. Another assay is dependent on the direct measurements of radioactivity of reaction products after separation of the radioactive nucleoside substrates from nucleotides by chromatograph. It needs specific equipment, and the whole process takes up to 18 h. Also, the radioactive nucleoside substrate is harmful to the human body, and it could not accurately determine the real initial rate of ADK reaction. Therefore, it is necessary to develop a convenient, quick, sensitive and low-cost assay to determine the enzymatic activity of ADK.

Acid-base indicators are usually applied to enzymatic assay for their extraordinary sensitivity to pH change. In 2002, Yu et al. have established an assay of arginine kinase activity based on the light absorption of a complex acid-base indicator [12]. Recently, Dhale et al. have developed a rapid and sensitive assay to measure L-asparaginase activity using methyl red as the indicator [13]. Bromothymol blue (BTB) is a good indicator in pH ranges of 6.0–8.0. Its color changes from yellow to blue as pH increases from 6.0 to 8.0, because it can form a highly conjugated structure while deprotonated in alkaline solution [14]. Here, we developed a one-step method to determine ADK activity, using bromothymol blue as the pH indicator, ATP and Adenosine as the substrates. Using this method, we analyzed the activity of adenosine kinase of *Bombyx mori* (BmADK), and evaluated the effect of buffer and pH indicator on the activity of ADK. The results indicated that this assay was time-saving, convenient, reliable and sensitive. This assay has shown a great potential as an alternative for the conventional ADK activity assay.

## 2. Materials and methods

### 2.1. Chemicals and materials

ATP and adenosine were purchased from Aladdin (Shanghai, China) as the sodium salt formulation. Magnesium acetate ($MgAC_2$) was a product of Sigma (St Louis, MO). Bromothymol blue sodium, glycine and other reagents were from Sangon Biotech (Shanghai, China). Plastic cuvettes were purchased from Centome (Chengdu, China). *Bam*H I and *Hin*d III DNA restriction enzymes were purchased from TaKaRa (Dalian, China). Ni Sepharose excel was from GE Health (USA). Primers were synthesized by Sangon Biotech (Shanghai, China).

### 2.2. BmADK preparation and concentration determination

*BmADK* gene was cloned from the cDNA of silkworm, and digested by *Bam*H I and *Hin*d III, then inserted into pSKB2 expression vector, expressed in *Escherichia coli* at 25 °C, and purified by nickel column affinity chromatography and gel filtration chromatography. BmADK's concentration was determined on a NanoDrop 2000C spectrophotometer (ThermoFisher, USA) according to the extinction coefficient of 12, 950 $L \cdot M^{-1}$ $cm^{-1}$ at 280 nm.

### 2.3. ADK activity assay

In this assay, we used ATP and adenosine as the substrates to measure the activity of ADK. The reaction system included 1 mL 0.1 M $MgAC_2$, 1 mL 1 M Gly-NaOH (pH 9.0), 2 mL 0.1% bromothymol blue, 48 mg ATP and 53 mg adenosine, and had a total volume of 20 mL after the addition of MiliQ water. The reaction system was adjusted with 0.5 M NaOH to have an absorbance of about 2.2 at 614 nm. The reaction mixture was freshly prepared before the reaction started. The reaction began only after adding BmADK (5 μL, 3.68 mg/mL) into the reaction mixture (995 μL) in a plastic cuvette with a light-path of 1 cm. The absorbance of the reaction system at 614 nm within 180 s was recorded on a DU800 nucleic acid/protein analyzer (Beckman, USA) at 30 °C. The reaction velocity was defined as the slope of the absorbance reduction at 614 nm in the first 10 s of the reaction.

### 2.4. Optimizing the assay

Buffer and pH indicator may affect the sensitivity of this assay. We used 2-cyclohexylamino ethanesulfonic acid (CHES) buffer (1 M, pH 9.0) to replace Gly-NaOH buffer, then determined the influence of different concentrations of buffer on the sensitivity of this assay. Similarly, we analyzed the effect of different concentrations of pH indicator on the sensitivity of this assay *via* the addition of different volume of 0.1% bromothymol blue. Each test was replicated at least three times.

### 2.5. The assay for BmADK activity

The minimum amount of BmADK was determined by addition of different concentrations of BmADK solution. The reaction system contained 5 mM $MgAC_2$, 50 mM Gly-NaOH, 0.01% bromothymol blue, 4.7 mM ATP, and 10 mM adenosine. BmADK (3.68 mg/mL) was gradiently diluted with Tris-buffer (20 mM Tris-HCl, 500 mM NaCl, 5% glycerol, pH 8.0). Then, the serial dilutions of BmADK (5 μL) were added into the reaction system (995 μL) at 30 °C. The absorbance at 614 nm was recorded to determine the minimum of BmADK content. Similarly, we determined the minimum of adenosine concentration *via* fixing enzyme concentration. Each test was replicated at least three times.

## 3. Results

### 3.1. Purification of BmADK

BmADK gene was cloned, expressed in *Escherichia coli* and then purified as previously described. The purity of BmADK was up to 99% as judged by 12.5% SDS-PAGE and Coomassie blue staining. The final concentration of BmADK was 3.68 mg/mL.

### 3.2. The theory of the assay for ADK activity

The schematic diagram of the assay for ADK activity was shown in Fig. 1. The generated hydrogen ions in the reaction catalyzed by ADK with ATP and adenosine as the substrates changed the pH of the reaction system, thus resulted in the color change of the reaction system, which could be easily indicated by the absorbance change. The absorbance change was proportional to the concentration of hydrogen ions. Thus, we could determine ADK activity and the initial reaction velocity according to the absorbance change.

### 3.3. Determining the maximum absorption wavelength

To determine the maximum absorption wavelength of the reaction system, we used hydrochloric acid to change the pH of the system and performed wavelength scan for the reaction system. Serial dilutions of hydrochloric acid (10 μL) were added into the reaction system (990 μL) and well mixed. After 3 min of balance, we performed full wavelength scan from 450 to 800 nm for each sample. A maximum absorption peak at 614 nm was observed. The absorbance at 614 nm decreased with the increase of hydrochloric acid concentration (Fig. 2A), indicating the correlation between the absorption at 614 nm and the concentration of hydrogen ions.

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