Sensitive Electrochemical ATP Assay Combined with Enzymatic ATP Amplification Reaction

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ABSTRACT

We propose an electrochemical, low-concentration adenosine triphosphate (ATP) assay combined with enzymatic ATP amplification. The electrochemical ATP assay consists of four parts: (i) myokinase for converting adenosine monophosphate (AMP) + ATP to two adenosine diphosphate (ADP) molecules, (ii) pyruvate kinase for converting two ADP molecules back to two ATP molecules (ATP amplification) and conversion of phosphoenolpyruvate to pyruvate, (iii) pyruvate dehydrogenase for generating H2O2 coupling with pyruvate oxidation, and (iv) electrochemical detection of H2O2. This system was used to detect ATP with a detection limit of 10⁻⁹ M ATP.

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1. Introduction

Adenosine triphosphate (ATP) plays a central role in all aspects of metabolism, such as in an organism’s energy conservation and utilization, and the ATP assay is very important in various fields. The presence of ATP indicates the possibility of microbial contamination. Cooking utensils may contaminate food with bacteria. This may cause food poisoning. Therefore, an ATP assay is used as an indicator of microbial contamination in hygiene management, especially in food safety and quality control.

The sensitivity of ATP detection required for food sanitation is around 10⁻⁹ to 10⁻¹² M, and the firefly luciferase-based ATP assay is a highly sensitive, well-established technique. ATP is detected through luciferin-luciferase reactions (bioluminescence reaction) in the presence of divalent metal ions. These reactions produce one photon of light at the expense of the hydrolysis of one ATP molecule through a series of intermediates. Bioluminescence determination of ATP is an easy and highly sensitive method suitable for use in food sanitation. However, luminometers are expensive and complicated devices. Moreover, with cloudy samples such as milk or oil in water, the causative substance of the cloudiness must be removed before the assay can be run.

An electrochemical assay is a simple, reliable, and practical method. Because the electrochemical reaction occurs at the electrode/liquid surface, it is especially suitable for colored or turbid samples, usually found among food-bearing samples. Compagnone et al. developed an electrochemical assay for 10⁻⁸ M ATP through a combination of glucose oxidase and hexokinase. Kueng reported that an enzyme electrode with immobilized glucose oxidase and hexokinase was able to detect 10⁻⁸ M ATP. However, these electrochemical assay methods did not have the sensitivity required for use in food sanitation.

In this paper, we propose an electrochemical assay for 10⁻⁹ M ATP combined with enzymatic ATP amplification.

2. Experimental

2.1 Enzyme and reagents

Phosphoenolpyruvate (PEP), pyruvate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and 25% glutaraldehyde solution were obtained from Wako Pure Chemical Industries (Osaka, Japan). Thiamin pyrophosphate (TPP), flavin adenine dinucleotide (FAD), and pyruvate kinase (PK) from rabbit muscle were obtained from Oriental Yeast (Tokyo, Japan). Myokinase (MK) from chicken muscle was obtained from Sigma-Aldrich (St. Louis, MO, USA). Pyruvate dehydrogenase (PDH) from Lactobacillus was obtained from Toyobo (Osaka, Japan).

2.2 Instruments

A three-electrode system was equipped with an Ag/AgCl reference electrode (model RE-1; BAS, Tokyo, Japan), and a Pt counter electrode and Pt electrode (3-mm diameter, BAS) were used as working electrodes for the experiments. The potentiotstat used was obtained from Hokuto Denko (Tokyo, Japan).

2.3 Preparation of PDH electrode

PDH solutions (9 µL) containing 0.1 U of enzyme were deposited onto the electrode surface. After air-drying at 4°C for 4 h, the enzyme electrode was cross-linked under glutaraldehyde atmosphere for 30 min and then incubated in 1 mM Tris-HCl (pH 7.0) for 20 min to block the free aldehyde group. The electrode was immersed in 50 mM potassium phosphate buffer (PPB) (pH 7.0) for 30 min to 1 h and stored at 4°C until use.

2.4 Electrochemical ATP assay by PDH electrode combined with ATP amplification

Amperometric measurements were performed at +600 mV vs. Ag/AgCl in the three-electrode system. A 3-mL reaction mixture containing 0.01 mM FAD; 0.2 mM TPP; 10 mM MgCl₂; 0.33 mM PEP; 0.33 mM AMP; MK; PK and 50 mM PPB (pH 7.0) was used.
for ATP measurement. After a steady current was observed, the ATP solution was added sequentially and the steady current was measured.

3. Results and Discussion

We propose a sensitive electrochemical assay of ATP employing (i) MK for converting one AMP molecule and one ATP molecule to two ADP molecules, (ii) PK for converting two ADP molecules back to two ATP molecules (ATP amplification) and simultaneously generating pyruvate from PEP, (iii) PDH for generating H$_2$O$_2$ coupling with pyruvate oxidation, and (iv) electrochemical detection of H$_2$O$_2$. In this series of reactions, excess AMP and PEP were added to the reaction mixture to drive equilibrium of the MK reaction toward ADP formation and that of the PK reaction toward pyruvate formation, respectively (Scheme 1). Theoretically the amount of pyruvate was equal to the amount of amplified ATP, the amperometric pyruvate measurement using the PDH electrode reflected the initial ATP concentration. Figure 1 shows the PDH electrode exhibiting good linearity from 2 to 450 µM pyruvate with sufficient sensitivity of 1.0 nA·µM$^{-1}$·mm$^{-2}$; therefore, we used the PDH electrode for the measurement of amplified ATP.

3.1 Evaluation of MK and PK concentration

The current outputs for 3.3 nM ATP were demonstrated for various PK concentrations in the presence of 0.94 U mL$^{-1}$ MK using an electrode with 0.69 U immobilized PDH (Fig. 2). We observed that the current rapidly increased according to time, indicating that ATP was successfully amplified in this system and could be detected amperometrically. As PK concentration increased, the time taken to reach the maximum current reduced. For ATP amplification, the MK reaction was coupled with the PK reaction; therefore, the MK:PK ratio was critical for the resolution of the assay and time required for ATP measurement.

3.2 Electrochemical ATP assay by PDH electrode combined with ATP amplification

Using an electrode with 0.1 U immobilized PDH, the time course of sensor responses for 0, 3.3, and 33 nM of ATP were compared in a reaction mixture containing several MK:PK ratios. When the MK:PK ratio was 1:2 or 1:4 (data not shown), the current outputs for 0 and 3.3 nM ATP were difficult to distinguish. When the MK:PK ratio was 1:24, 0 nM ATP and 3.3 nM ATP were clearly distinguished. The output current in Fig. 3A is bigger than that in Fig. 3B. The output current depended on the amount of MK because MK plays the main role in the amplification of the ATP molecule. The MK concentration in Fig. 3A (0.56 U mL$^{-1}$) was greater than that in Fig. 3B (0.3 U mL$^{-1}$). We were able to determine the ATP concentration in Fig. 3B by comparing the current readout at 3 min.

Scheme 1. A sensitive electrochemical adenosine triphosphate (ATP) assay combined with an enzymatic ATP amplification reaction.

Figure 1. Response to pyruvate using a pyruvate dehydrogenase (PDH) electrode. PDH (0.1 U) was immobilized on the Pt electrode surface. Various concentrations of pyruvate were added, and the current was recorded at 600 mV vs. Ag/AgCl.

Figure 2. Effect of pyruvate kinase (PK) amount on sensor response when 0.69 U of PDH was immobilized on the Pt electrode surface. In the presence of 0.94 U mL$^{-1}$ of myokinase (MK) and 3.3 nM of ATP, various concentrations of PK were added to the reaction buffer. PK: 3.0 U mL$^{-1}$ (■), 1.5 U mL$^{-1}$ (▲), 1.0 U mL$^{-1}$ (▲), 0.6 U mL$^{-1}$ (∆), and 0.3 U mL$^{-1}$ (○).
These results indicate that $10^{-9}$ M ATP could be detected in less than 3 min, and an electrochemical ATP assay using ATP enzymatic amplification is possible (Fig. 3). In this system, the upper limit of detection is around $10^{-8}$ M; the dynamic range of this detection system must be improved for practical use. In addition, our system includes three kinds of enzyme reaction, and different optimum conditions exist for each enzyme; thus, temperature control may be needed for precise detection.

Possible inhibitors for the reaction include 10 mM ascorbate for PK. Ascorbate may be a possible contaminant in food-related samples and is easily oxidized at the electrode; thus, the elimination of ascorbate is essential. For example, ascorbic acid oxidase enzyme pretreatment is usually used for ascorbate elimination in diagnostic kits.

In this study, we confirmed that $10^{-9}$ M ATP can be electrochemically detected in combination with an enzymatic ATP amplification reaction. We observed that the current rapidly increased according to time, indicating that ATP was successfully amplified in this system. Therefore in principle, this amplification system is applicable to other ATP detection methods such as luciferin-luciferase based ATP assay.

Hygiene inspections using luciferin-luciferase kits involve swabbing a 10 cm $\times$ 10 cm area of a cooking utensil and transferring the swab to 0.1 mL of solution containing luciferin and luciferase, following which luminescence is measured. More than 0.1 pmol ATP in 0.1 mL of swab extract implies that the sample is “relatively contaminated,” and less than 0.1 pmol ATP in 0.1 mL of swab extract implies that the sample is “clean.” Because $10^{-9}$ M ATP in 0.1 mL of swab extract contains 0.1 pmol ATP, our ATP system can clearly distinguish whether cooking utensils are clean or not. This assay is a suitable technique that can determine the presence of low ATP concentrations electrochemically and is applicable for on-site monitoring of ATP.

References