Fluorometric Measurement of Adenosine 5’-Triphosphate Using Exonuclease V Activity

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A new method of a fluorometric measurement of adenosine 5’-triphosphate (ATP) based on the coupling of exonuclease V and a fluorescence-labeled DNA probe was developed. Quantification of micromolar-order ATP and real-time measurement of the activity of the ATP-generating enzyme were performed. This method provides a simple fluorometric assay of ATP and ATP-related enzymes using easily available materials.

Keywords: Fluorometry, ATP, Exonuclease V, DNA probe, Kinase, Femtoliter

1. Introduction

Adenosine 5’-triphosphate (ATP) plays important roles as the major energy source molecule in various cellular processes. ATP is measured to detect microorganisms [1–3], assay the catalytic activity of kinases [4,5], and analyze metabolic processes [6–8]. Conventionally, ATP is quantified by chemiluminescence assay using luciferases. Chemiluminescence assay has the advantages of a lower background noise level and a wider dynamic range than fluorescence assay [9]. However, the emission signal intensity in chemiluminescence assay is quite low and hence it requires a long exposure time for detection [9]. When chemiluminescence assay is applied to recently developed miniaturized assay methods that use femtoliter-scale volumes in microwells or microdroplets to achieve ultrahigh-throughput analysis, low signal intensity is no longer acceptable [10–13].

To complement a chemiluminescence-based assay, many methods have been proposed for fluorescence-based detection of ATP using ATP-binding compounds [14,15], proteins [16,17], and aptamers [18], as well as a glucose detection system composed of multiple enzymes [19].

DNA probes, which contain an aptamer as a sensing probe, are suitable for use on microdevices because they can be easily modified with various molecules, such as fluorophores or redox molecules, and with functional groups for further modification or immobilization on solid phases [20–22]. Although the use of ATP-binding aptamers as probes on microdevices has also been proposed by many researchers [18], their practical use has been limited by the low specificity of existing ATP-binding aptamers to ATP; the already available ATP-binding aptamers also bind to adenosine 5’-diphosphate (ADP) and adenosine 5’-monophosphate (AMP), which coexist with ATP in real analysis samples [18,23,24].

Here, we report a fluorometric method for the detection of ATP using exonuclease V and a fluorophore-labeled single-strand DNA (ssDNA) probe. Exonuclease V (RecBCD) is a DNA repair enzyme and requires ATP to catalyze the degradation of DNA [25]. The scheme of exonuclease V-based ATP detection assay is shown in Fig. 1. When the ssDNA probe labeled with a fluorophore and a quencher is used as a substrate of exonuclease V, the quenched ssDNA probe is cleaved by exonuclease V dependent on the presence of ATP, and fluorescence is generated upon the release of the fluorophore. In this method, accurate discrimination of ATP from ADP and AMP is realized by specific ATP recognition by
exonuclease V compared with previously reported exonuclease-based fluorometric ATP detection methods using ATP-binding aptamers [26,27].

Fig. 1. Scheme of the fluorometric detection of ATP using exonuclease V. Exonuclease V degrades the ssDNA probe, which is labeled with a fluorophore and a quencher, dependent on the presence of ATP. The fluorescence of the fluorophore is quenched by the closely located quencher dye, and the quenched fluorescence is recovered via the cleavage of the ssDNA probe.

2. Experimental

Exonuclease V and adenosine 5’-triphosphate (ATP) were purchased from New England Biolabs. A dual-labeled DNA probe, 5’-(6-FAM)-CCCTGTCCTTTTACCAGACAACCATTACCT-(BHQ1)-3’, was custom-synthesized by Eurofins Genomics. Pyruvate kinase from rabbit muscle and luciferase from *Photinus pyralis* were purchased from Sigma-Aldrich. Phosphoenolpyruvic acid, adenosine 5’-monophosphate (AMP), and D-luciferin were purchased from Wako Pure Chemical Industry. Ultrapure adenosine 5’-diphosphate (ADP) was purchased from Cell Technology.

ATP-dependent fluorescence emission was examined using a fluorescence microplate reader (Infinite M1000 PRO, Tecan) at an excitation/emission of 495/518 nm at 30 °C in 50 μL of 1× NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9) containing 0.02 unit/μL exonuclease V, 0.2 μM DNA probe, and 0–100 μM ATP.

Substrate selectivity was examined as follows: 50 μL of 1× NEBuffer 4 containing 0.02 unit/μL exonuclease V, 0.2 μM DNA probe, and with or without 50 μM ATP, ADP, or AMP was incubated at 30 °C for 60 min. The fluorescence intensity of the solution was measured using the fluorescence microplate reader at an excitation/emission of 495/518 nm.

The ATP generation activity of pyruvate kinase was measured using the fluorescence microplate reader at an excitation/emission of 495/518 nm at 30 °C in 30 μL of 50 mM Tris-HCl (pH 7.5) containing 5 mM magnesium sulfate, 1 mM phosphoenolpyruvic acid, 0.2 mM ADP, 0.02 unit/μL exonuclease V, 0.2 μM DNA probe, and 0–200 pg/μL pyruvate kinase.

The detectability of ATP in femtoliter volume when using an ATP detection system based on the exonuclease V reaction was compared with that based on the luciferase/luciferin reaction. A pyruvate kinase reaction solution (20 μL) combined with the exonuclease V-based ATP detection system containing 5 mM magnesium sulfate, 1 mM phosphoenolpyruvic acid, 0.2 mM ADP, 0.02 unit/μL exonuclease V, 0.2 μM DNA probe, and 0 or 100 pg/μL pyruvate kinase in 50 mM Tris-HCl (pH 7.5) was prepared. Another pyruvate kinase reaction solution (50 μL) combined with the luciferase/luciferin-based ATP detection system containing 5 mM magnesium sulfate, 1 mM phosphoenolpyruvic acid, 0.2 mM ADP, 0.5 mM D-luciferin, 0.05 mg/mL luciferase, and 0 or 100 pg/μL pyruvate kinase in 50 mM Tris-HCl (pH 7.5) [13] was also prepared. Both reaction solutions were incubated at 30 °C for 30 min. Fluorescence intensity was measured using the fluorescence microplate reader at an excitation/emission of 495/518 nm at 30 °C, and luminescence intensity was measured using a microplate luminometer (Glomax, Promega) at the same temperature. The pyruvate kinase reaction solutions after the 30 min incubation were applied onto a glass microreactor array chip, on which microwells with a diameter and a depth of both 4 μm were fabricated [12,28]. The solutions in the microwells were covered with silicone oil (KF96-100cs, Shin-Etsu Chemical). The fluorescence and luminescence images of the solutions in the microwells were captured using an inverted microscope system (Eclipse Ti, Nikon) equipped with an objective (CFI Plan Fluor 10×/0.30 NA, Nikon), a B-2A filter cube (excitation filter, 450–490 nm; dichroic mirror, 505 nm; emission filter, 520 nm; Nikon), and an electron multiplying CCD (EMCCD) camera (C9100-14, Hamamatsu Photonics). A xenon lamp was used for fluorescence imaging.
3. Results and discussion

3.1. Fluorescence detection of ATP based on exonuclease V reaction

Fluorescence detection of ATP through the degradation of the DNA probe by the ATP-dependent activity of exonuclease V was examined. Fluorescence was successfully generated and its intensity increased with time depending on the concentration of ATP (Fig. 2A). As shown in Fig. 2B, fluorescence intensity as a function of the concentration of ATP fitted well with a sigmoidal function, which can be used as a calibration curve. This result matches a previous report that the activity of exonuclease V (RecBCD) is allosterically regulated [29,30]. The specificity of the exonuclease V-based assay was examined. As shown in Fig. 3, only ATP was recognized by this assay system.

![Image 1](image1.png)

**Fig. 2.** Fluorometric measurement of ATP by exonuclease V-based assay. (A) Time course of fluorescence intensity as a function of ATP concentration: (a) 0, (b) 2, (c) 4, (d) 6, (e) 8, (f) 10, (g) 20, (h) 40, (i) 60, (j) 80, and (k) 100 μM. (B) Fluorescence intensity at 60 min as a function of the ATP concentration. The solid line indicates the four-parameter logistic curve fit.

![Image 2](image2.png)

**Fig. 3.** Specificity of exonuclease V-based assay for ATP. H2O instead of the nucleotides was used as a control. The error bars represent mean ± SD (n=5 for ATP, ADP, and AMP; n=4 for H2O).

![Image 3](image3.png)

**Fig. 4.** Fluorometric measurement of ATP-generating activity of pyruvate kinase by exonuclease V-based assay. (A) Time course of fluorescence intensity as a function of concentration of pyruvate kinase: (a) 0, (b) 5, (c) 10, (d) 25, (e) 50, (f) 100, and (g) 200 pg/μL. (B) Fluorescence intensity change during 5–10 min as a function of concentration of pyruvate kinase. The solid line indicates the four-parameter logistic curve fit.
3.2. Fluorescence measurement of pyruvate kinase activity by the exonuclease V-based ATP detection assay

The applicability of the exonuclease V-based ATP detection assay to the measurement of ATP-related enzyme activity was examined using pyruvate kinase as an enzyme model. Pyruvate kinase catalyzes the generation of ATP from ADP via the transfer of a phosphate group from phosphoenolpyruvate to ADP in glycolysis. The catalytic activity of pyruvate kinase was measured by detecting the generated ATP. As shown in Fig. 4, fluorescence emission dependent on the concentration of pyruvate kinase was successfully observed. This result indicates that the exonuclease V-based ATP detection assay can also be applied to the measurement of ATP-related enzyme activity in the presence of ADP.

3.3. Fluorescence detection of pyruvate kinase activity in femtoliter-volume wells.

The detectability of ATP in femtoliter volume when using the exonuclease V-based method was confirmed. The pyruvate kinase reaction solutions combined with the ATP detection systems based on
the exonuclease V and luciferase/luciferin reactions were prepared. The ATP generation activity of pyruvate kinase was measured using a conventional microwell plate and a microreactor array chip, on which 50-fL-volume microwells with a diameter and a depth of both 4 µm were fabricated. The ATP generated via the pyruvate kinase reaction in microliter volume was easily detected by both the exonuclease V- and luciferase/luciferin-based methods (Fig. 5A). On the other hand, the ATP in 50 femtoliter volume was detected by the exonuclease V-based method (Fig. 5B). No luminescence signals were detected by the luciferase/luciferin-based method even with 5 min exposure using the EMCCD camera at the maximum gain level (data not shown).

The method of fluorometric ATP detection using exonuclease V and DNA probes with high specificity and high sensitivity was thus developed. It is considered that this method can be applied to ATP sensing on microdevices in combination with technologies for highly sensitive nuclease assay using DNA probes [31].

4. Conclusions

Fluorometric detection of ATP using the coupling of exonuclease V and the DNA probe labeled with a fluorophore and a quencher has been developed. By this method, micromolar-order ATP was quantitatively detected. As a model experiment for the assay of enzyme reactions, ATP production was examined using pyruvate kinase and successfully measured. Using this method, the ATP production in femtoliter-volume wells was also detected, which is difficult by conventional chemiluminescence assay. Although optimization is required for practical use, this method provides a simple fluorometric measurement of ATP using easily available materials.

Acknowledgements

The authors thank Dr. Shusuke Sato and Shoichi Tsuchiya for support in preparation of the microwell array chip. This work was supported by Research Complex Program of the Japan Science and Technology Agency (JST), and JSPS KAKENHI Grant Numbers JP15K13313, JP16K16644.

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