A continuous-flow ATP amplification system for increasing the sensitivity of quantitative bioluminescence assay

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Abstract

We constructed a novel ATP amplification reactor using a continuous-flow system, and this allowed us to increase the sensitivity of quantitative bioluminescence assay by controlling the number of ATP amplification cycles. We previously developed a bioluminescence assay coupled with ATP amplification using a batch system. However, it was difficult to control the number of amplification cycles. In this study, ATP amplification was performed using a continuous-flow system, and significant linear correlations between amplified luminescence and initial ATP concentration were observed. When performing 4 cycles of continuous-flow ATP amplification, the gradient of amplification was 1.87^N. Whereas lower quantifiable level was 500 pM without amplification, values as low as 50 pM ATP could be measured after amplification. Sensitivity thus increased 10-fold, with further improvements expected with additional amplification cycles. The continuous-flow system thus effectively increased the sensitivity of quantitative bioluminescence assay.

Keywords: Bioluminescence assay; ATP amplification; Continuous-flow system

Introduction

ATP participates in various intracellular metabolic processes as energy transfer "currency". Therefore, detection and quantification of ATP are important tasks in biochemistry. The firefly luciferase-based (bioluminescence) assay for detection of ATP is a well-established technique [1]. Because the bioluminescence assay is useful for rapidly detecting and quantifying a broad range of ATP, it has been employed in many industrial areas, such as clinical analysis and hygiene monitoring [2]. Furthermore, bioluminescence assay has also become a key technology allowing rapid DNA sequencing [3,4]. In pyrosequencing, incorporation of nucleotides in extension reactions release pyrophosphate, which is converted to ATP, and quantification of ATP is necessary to perform accurate sequencing.

In order to fully utilize the advantages and the potential of the bioluminescence technology, several problems must be overcome. One of the most important problems is the relatively low sensitivity of the bioluminescence assay. At low concentrations of ATP, bioluminescence is too weak to be detected by a luminometer. This problem hinders development of technologies employing bioluminescence assay. Hence, increasing the sensitivity is very important.

Several new methods to increase sensitivity have recently been reported [5-8]. For example, Fujii *et al*., developed a modified-luciferase, which generates more than 10-fold greater luminescence intensity than the wild-type, and could be used to detect amol ($\times 10^{-18}$ mol) levels of ATP [8].

We have also enhanced the sensitivity of ATP detection by developing an ATP amplification reaction employing (i) adenylate kinase (ADK) to convert AMP + ATP into two molecules of ADP, (ii) pyruvate kinase (PK) or polyphosphate kinase (PPK) to convert ADP back to ATP (ATP

amplification), and (iii) a commercially available firefly luciferase (Fig. 1). This technique was effective for detecting low levels of ATP and bacterial contamination [9]. ATP was amplified depending on initial ATP concentrations and the sensitivity of bioluminescence assay increased 10,000-fold. The bioluminescence assay coupled with ATP amplification was able to detect amol levels of ATP and 1 colony forming unit (CFU) of bacteria, providing sufficient sensitivity for some industrial applications. However, achieving reproducibility requires precise control of amplification cycles. The number of amplification cycles depends on reaction time and enzyme activity, and the amount of exponentially amplified ATP varied greatly with small changes in these parameters. Therefore, ATP amplification reaction in the batch system required careful manipulation and precise control of reaction time.

A continuous-flow system has several advantages over a batch reaction system, for example, rapid analysis, reusing of catalysts and automated liquid handling [10-13]. Several biochemical reactions have been improved by using continuous-flow systems. For example, Tabata *et al*., developed several biochemical analyses using two to three types of immobilized enzyme [13]. They connected immobilized columns upstream to downstream in order to perform sequential enzymatic reactions in a continuous-flow system, thereby improving sensitivity and allowing easy manipulation of the analyses in the continuous-flow system.

In this study, we constructed a novel ATP amplification reactor using a continuous-flow system (Fig. 2), which enabled us to control the number of ATP amplification cycles without careful manipulation and precise control of reaction time. Separately immobilized enzymes can catalyze their reactions independently and the reaction is controlled by flow rate to reach equilibrium. ATP

was amplified 2-fold by passing immobilized ADK and PK zones under conditions driving the ADK and PK equilibrium toward ADP and ATP formation. Moreover, ATP could be further amplified by adding ADK and PK zones, which allowed us to control the number of amplification cycles. Following the continuous-flow ATP amplification reaction, ATP could be detected and quantified with high reproducibility and sensitivity.

Materials and methods

Chemicals

PK, ADK, ATP and phosphoenolpyruvate (PEP) were obtained from Sigma-Aldrich (Missouri, U.S.A.). AMP was obtained from Tokyo Chemical Industry (Tokyo, Japan). Bioluminescence assay mixture contained 25 μg of luciferase (Bioenex, Hiroshima, Japan), 2 mM luciferin, 1 mM $(CH_3COO)_2$ Mg, 5% (w/v) trehalose and 50 mM HEPES-NaOH (pH 7.8).

Enzyme immobilization

PK and ADK were each suspended in 0.5 M NaCl and 0.2 M $NaHCO_3$ (pH 8.3), and immobilized on 1-ml HiTrap NHS-activated HP columns (GE Healthcare Ltd., Amersham, U.K.). Immobilization of PK and ADK were performed as follows. The columns were washed with 6 ml of ice-cold 1 mM HCl. Immediately after this, 1 ml of PK solution (144 μg/ml) and ADK solution (30 μg/ml) were injected into the columns. After 30 min of incubation at 25°C, 6 ml of a blocking buffer containing 0.5 M ethanolamine and 0.5 M NaCl (pH 8.3) was injected into each column in order to inactivate the excess NHS-active groups. Following additional incubation at 25°C for 30 min, the columns were washed with 6 ml of washing buffer containing 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4).

Removal of ATP and ADP in substrates

AMP was purified by using a DEAE column (Tosoh, Tokyo, Japan) with buffer A: 10 mM NaCl and 10 mM citrate buffer (pH 2.85), and buffer B: 1 M NaCl and 10 mM citrate buffer (pH 4.40). AMP was eluted after 30 min with a linear gradient of 0%-100% buffer B. Next, 50 nM PEP was manually injected into the column immobilized with 10 U apyrase according to the above method. After 2 h of incubation at room temperature in the column, injection of 1 ml of buffer containing 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4) extruded PEP from the column.

Continuous-flow enzymatic reaction and bioluminescence assay

PK and ADK columns produced by the above method were connected to an HPLC system (ÄKTA explorer 10S, GE Healthcare Ltd., Amersham, U.K.). Each column was equilibrated with 10 ml of running buffer containing 5 mM $MgCl_2$, and 50 mM HEPES-NaOH (pH 7.4) at 1.0 ml/min. After equilibration, 2 ml of injection mixture containing 100 nM ADP, 1 mM PEP, 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4) for PK, and 50 nM ATP, 0.1 mM AMP, 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4) for ADK, were injected into columns with flow rates ranging from 0.1 to 4.0 ml/min at room temperature. For each reaction, 5 ml of reaction mixture was collected in 0.1-ml fractions from the outlet.

The amount of ATP and ADP in each fraction was measured as follows. For ATP, 20 µl of each fraction was mixed with 20 µl of bioluminescence assay mixture, and bioluminescence was measured using a multiplate luminometer (PerkinElmer. Inc., Massachusetts, U.S.A). For ADP+ATP, 40 µl of each fraction was mixed with 20 µl of a solution containing 10 U/ml PK, 1 mM PEP, 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4), followed by incubation at 37°C for 1h.

When the PK reaction reached equilibrium, 40 μl of reaction mixture was added to 20 μl of the bioluminescence assay mixture, and bioluminescence was measured using a multiplate luminometer. The amount of ADP in each fraction was calculated by subtracting the amount of ATP from that of ATP+ADP.

Continuous-flow ATP amplification reaction

Four sets of ADK and PK columns were prepared as described above. PK columns were washed with buffer containing 1 mM PEP, 5 mM $MgCl_2$, and 50 mM HEPES-NaOH (pH 7.4) at a flow rate of 1.0 ml/min. Each set of ADK and PK columns (ADK-PK column) was connected in the order of the reaction steps (ADK to PK from upstream to downstream), and washed with 10 ml of running buffer using an HPLC system. Reaction mixtures containing various amounts of ATP, 1 mM PEP, 0.1 mM AMP, 5mM $MgCl_2$, and 50 mM HEPES-NaOH (pH 7.4) were injected into the ADK-PK column at 0.5 ml/min at room temperature. The number of amplification cycles increased two-fold by connecting four ADK-PK columns in tandem. Following amplification, 20 ml of each reaction mixture from the outlet were collected in 0.2-ml fractions. From each fraction 20 μl was mixed with 20 μl of bioluminescence assay mixture, and bioluminescence was measured using a multiplate luminometer.

Results and discussion

Construction of ADK and PK columns

In the proposed continuous-flow system, unbound enzymes may cause unwanted reactions downstream of the column, resulting in poor reproducibility of ATP amplification. Therefore, ADK and PK immobilized columns should be washed until unbound enzymes are removed from the

columns. The HiTrap NHS-activated HP column contains a carrier that covalently couples the enzymes. No enzymatic activity was observed in efflux solutions after washing ADK and PK columns, thus indicating covalent immobilization of enzymes.

In addition, enzymes for batch reactions should be treated with apyrase and further purified using HPLC to reduce ATP and ADP contamination, which causes low S/N in the ATP amplification and detection reactions [9]. This laborious preparation of enzymes resulted poor reproducibility of enzyme activity. However, treatment and purification were unnecessary in the continuous-flow system, as simple washing with buffer containing substrate for PK, as well as with running buffer for ADK and PK, also significantly reduced ATP contamination.

Removal of ATP and ADP in substrates

Commercially available AMP and PEP are often contaminated with ATP and ADP. A 1 mM solution of commercial AMP was found to contain 7.6 nM ATP and 200 nM ADP, and a 1 mM solution of commercial PEP was found to contain 12 pM ATP and 25 pM ADP. ATP and ADP contamination were reduced to less than 1 pM as described in Experimental. Thus, they had to be removed from each substrate. Following purification and treatment, ATP and ADP contamination in the 1 mM solutions of AMP and PEP was reduced to less than 1 pM.

Optimization of flow rate in continuous-flow enzymatic reactions

To achieve full and reproducible ATP amplification, ADK and PK reactions in each column must reach equilibrium. Therefore, the reaction time of immobilized ADK and PK must be sufficient to reach equilibrium in each enzymatic reaction. Manipulating the flow rate allowed us to control the

reaction time in continuous-flow enzymatic reactions, as flow rate is inversely proportion to residential substrate time.

On each immobilized column, reactants reached equilibrium with a flow rate of less than 0.5 ml/min (Fig. 3). Thus a flow rate of 0.5 ml/min was employed in subsequent experiments.

ATP amplification using ADK-PK columns

Typical hygiene monitoring gives a solution of several milliliters from a swab sample, while bioluminescence assay requires only several tens of microliters of sample solution. Bioluminescence assay then depends on concentration, and it is preferable to amplify ATP without dilution.

A flow chart of the reaction sample gave a peak ATP concentration with a plateau area at the top in each ADK-PK column, thus indicating no dilution (Fig. 4).

The maximum number of ATP amplification cycles was four, as the back pressure must be less than the durability of the column. The initial ATP concentration of the plateau area at the peak increased to approximately 100 nM from 50 nM (ATP concentration in injection mixture) in the first ATP amplification cycle using an ADK-PK column. ATP concentration could be further increased by adding ADK-PK columns. An exponential correlation was observed between the height of the plateau area at the peak tops and the number of ADK-PK columns in each amplification reaction (Fig. 5). The gradient of amplification was 1.87^N (N=number of ADK-PK columns). These data suggest that the number of ADK-PK columns determines the number of ATP amplification cycles.

Continuous-flow ATP amplification for bioluminescence assay

Various amounts of ATP were amplified by continuous-flow ATP amplification composed of four ADK-PK columns (Fig. 6).

The 50 pM ATP sample was not detectable without amplification due to the detection limit of the luminometer. However, the amplified 5 pM sample was detectable after amplification. The sensitivity of bioluminescence assay increased with continuous-flow ATP amplification system to a comparable degree as with the batch system.

In addition to the increase in sensitivity, a significant linear correlation was observed between initial ATP concentration and luminescence after ATP amplification. Because the number of ATP amplification cycles was controlled, each luminescence value was amplified quantitatively. Whereas lower quantifiable level was 500 pM without amplification, values as low as 50 pM ATP could be measured after amplification.

The reproducibility of this assay was high without careful manipulation and precise control of reaction time. The coefficient variation (CV) of this assay was approximately 10 % with three amplifications using different lots of columns, enzymes and substrates (CV= 10.5 % for 5 nM ATP, 9.6 % for 0.5 nM ATP and 11.7 % for 0.05 nM ATP).

Therefore, the continuous-flow amplification method was useful for not only the detection of, but also the quantification of levels of ATP below the detection limit of the luminometer.

Exponential amplification reactions are difficult to control in a batch system, as small variations in enzyme activity and reaction time can result in significant differences in the degree of amplification. Therefore, a batch system may be used to detect the low levels of ATP, but not to quantify initial ATP concentrations. On the other hand, in continuous-flow ATP amplification, the reaction can be

maintained at equilibrium through flow rate, and the number of amplification cycles can be controlled through the number of columns. The use of a continuous-flow system therefore offers a way to control exponential amplification reactions with sufficient precision for quantitative assays.

Increasing the number of continuous-flow ATP amplification cycles

The maximum number of ATP amplification cycles in this experiment was four due to the pressure drop on the columns. The back pressure with over four cycles of continuous-flow ATP amplification was more than 0.3 MPa, and this exceeded the column durability. The pressure drop was a critical factor in increasing the number of ATP amplification cycles. In this study, enzymes were immobilized on a commercially available column whose protein capacity was more than sufficient to immobilize the enzymes. A smaller column with lower capacity could therefore help to reduce the pressure drop without reducing flow rate. Moreover, the pressure drop can be further reduced by using a circular flow system [14] containing a set of ADK and PK columns.

As an alternative to the continuous-flow system, a repeated injection method was also examined. In this method, the product from a system with a single ADK-PK column was repeatedly injected back into the system. A sample of 50 pmol ATP was exponentially amplified to 92.8, 260.4 and 449.6 pmol with 1 to 3 amplification cycles. However, no comparable increase in ATP concentration was observed due to dilution of the sample with running buffer. While the repeated injection method is impractical for amplification systems that require running buffer, this method is helpful to reduce the pressure drop. Further investigations are needed to examine its use in amplification systems.

Conclusion

We devised and tested a continuous-flow ATP amplification system. Using the continuous-flow system, the number of ATP amplification cycles can be controlled by the number of immobilized columns and significant linear correlations between amplified luminescence and initial ATP concentration are observed. This system was an effective way to perform reproducible ATP amplification and to increase the sensitivity of ATP quantification by bioluminescence assay.

Though only 4 amplification cycles were possible with the system discussed here, further pressure reduction should increase the maximum number of amplification cycles and lead to exponential increase in sensitivity. Our method thus represents a novel approach to quantitative and highly sensitive ATP detection.

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Figure legends

Fig. 1. ATP amplification reaction. ADK, PK and excess AMP and PEP were prepared in the reaction mixture. ATP amplification reaction started when ATP was added to the reaction mixture and ended when endogenous AMP was converged to ATP.

Fig. 2. Principle of continuous-flow ATP amplification reaction. (a) ATP was amplified twice by flowing through ADK and PK immobilized zones under conditions driving ADK and PK equilibrium toward ADP and ATP formation. This was controlled by flow rate, activity of

immobilized enzymes and substrate concentrations. (b) ATP was further amplified by adding ADK and PK zones.

Fig. 3. Reaction rates of ADK and PK at various flow rates. Reaction rates were calculated by measuring the amounts of ADP and ATP. (a) Reaction rates of ADK at 0.1 ml/min, 0.2 ml/min, 0.5 ml/min, 1.0 ml/min, 2.0 ml/min and 4.0 ml/min. (b) Reaction rates of PK at 0.1 ml/min, 0.2 ml/min, 0.5 ml/min, 1.0 ml/min, 2.0 ml/min and 4.0 ml/min.

Fig. 4. ATP concentration gradients in yield products. Concentrations of ATP in the fractions from various numbers of ADK-PK columns are indicated. ATP concentration in each fraction was assayed using luciferase, as described in Experimental.

Fig. 5. Correlation between peak ATP concentrations and number of ADK-PK columns. ATP concentrations in peak top areas were obtained by averaging 5 fractions in each peak.

Fig. 6. Bioluminescence assay with amplification and without amplification. Relative bioluminescence values are the means of three separate experiments.

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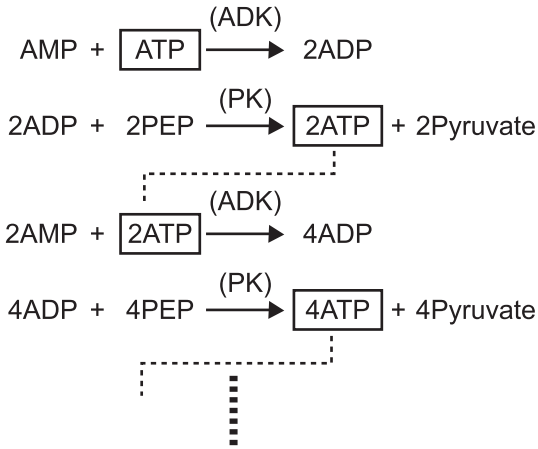
(ADK)
AMP + ATP
2ADP
(PK)
2ADP + 2PEP
2ATP + 2Pyruvate
(ADK)
2AMP + 2ATP
4ADP
(PK)
4ADP + 4PEP
4ATP + 4Pyruvate

Fig. 1.

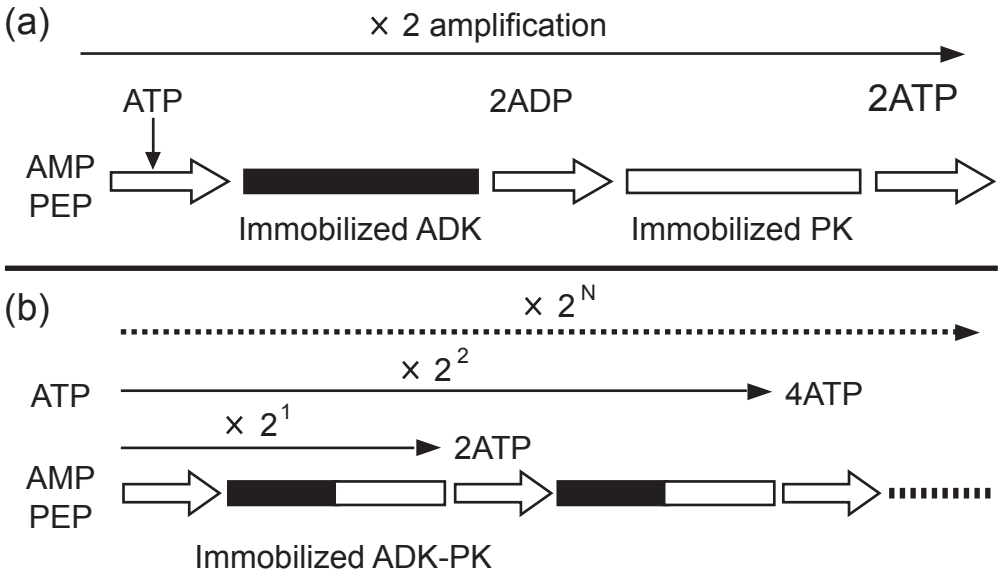
(a)
× 2 amplification
ATP
2ADP
2ATP
AMP
PEP
Immobilized ADK
Immobilized PK
(b)
× 2^N
ATP
× 2^2
4ATP
× 2^1
2ATP
AMP
PEP
Immobilized ADK-PK

Fig. 2.

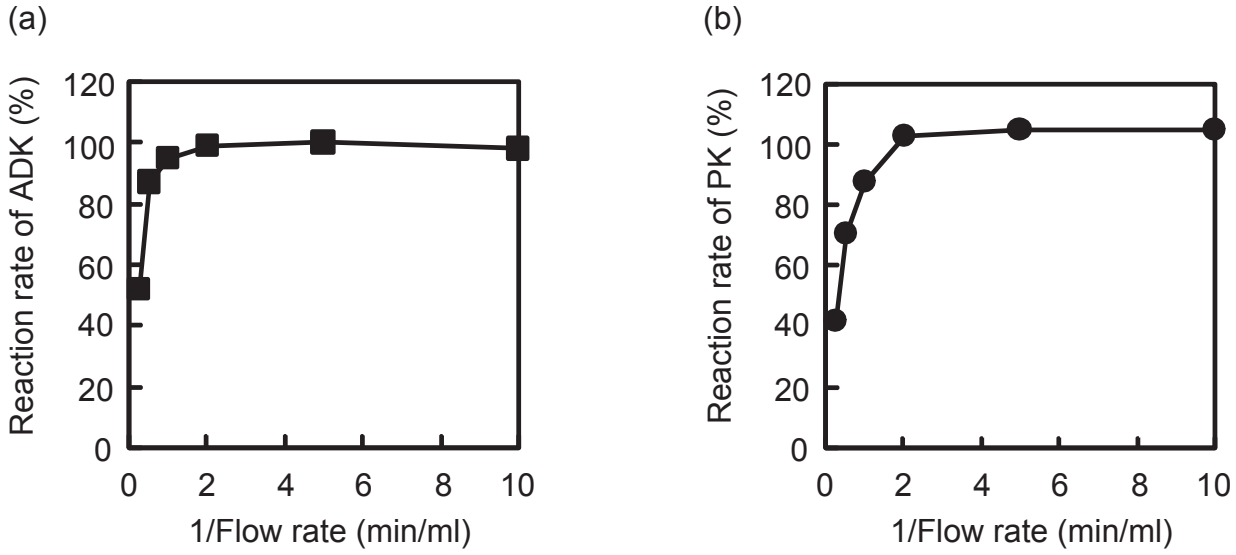
(a)
Reaction rate of ADK (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
1/Flow rate (min/ml)
(b)
Reaction rate of PK (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
1/Flow rate (min/ml)

Fig. 3.

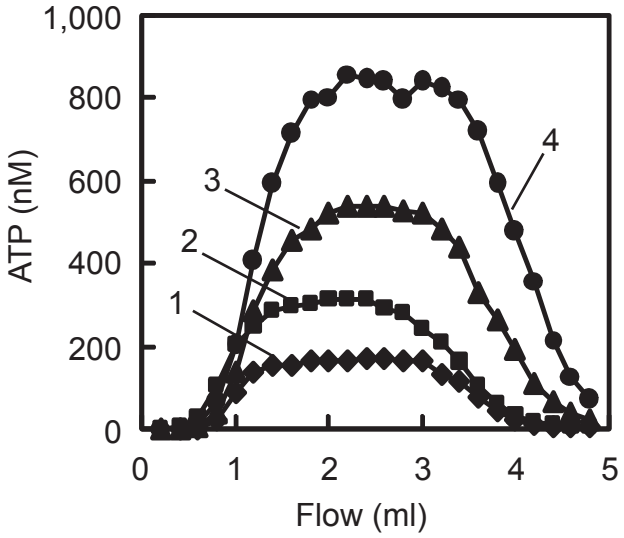

Fig. 4.

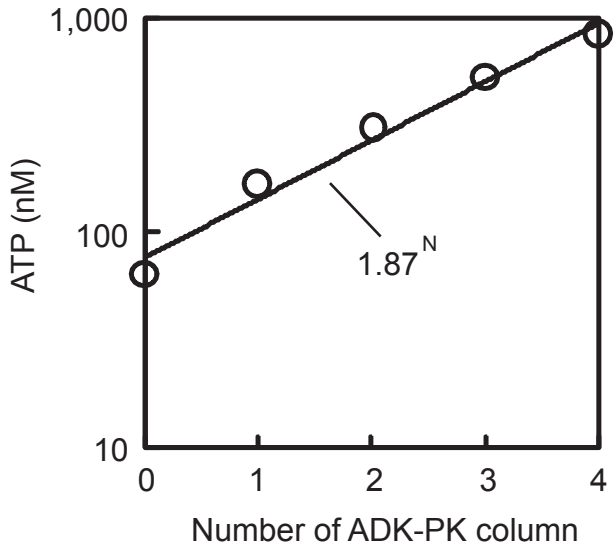

Fig. 5.

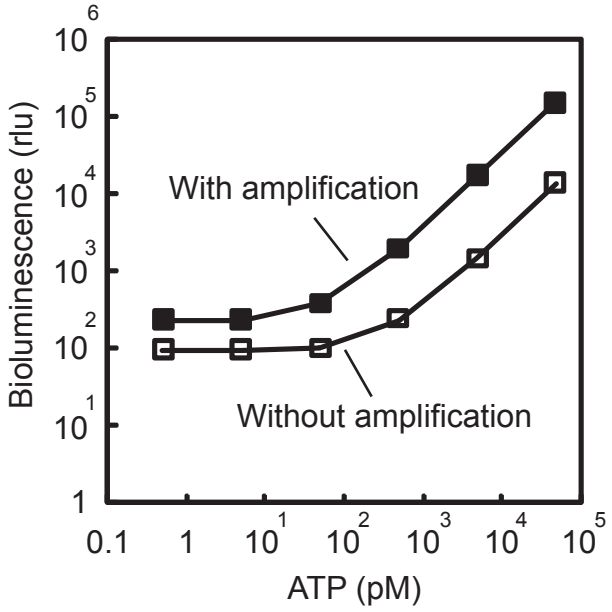
Bioluminescence (rlu)
10^6
10^5
10^4
10^3
10^2
10^1
1
With amplification
Without amplification
0.1
1
10^1
10^2
10^3
10^4
10^5
ATP (pM)

Fig. 6.