Colorimetric Determination of Pyrophosphate Anion and Its Application to Adenylation Enzyme Assay

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A colorimetric pyrophosphate assay based on the formation and reduction of the 18-molybdopyrophosphate ($[(P_2O_7)Mo_{18}O_{54}]^{4-}$) anion in an acetonitrile–water mixed solvent was modified and improved. The $[(P_2O_7)Mo_{18}O_{54}]^{4-}$ anion is precipitated from the acetonitrile–water solution containing MoO_4^{2-} and HCl, and is re-dissolved in neat acetonitrile or propylene carbonate. This separation process decreases the interference by ATP, and prevents a yellow coloration of the reducing agent, ascorbic acid, due to excess Mo(VI) species. In the organic solvent, the $[(P_2O_7)Mo_{18}O_{54}]^{4-}$ anion is reduced to a more intense blue molybdopyrophosphate species. The application of the colorimetry to the assay of adenylation enzymes is also described in this note.

Keywords Colorimetry, pyrophosphate, molybdopyrophosphate, adenylation enzyme

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Introduction

In a previous paper,¹ we presented a colorimetric method for the determination of the $P_2O_7^{4-}$ anionic species (PPi). In the colorimetric method, first, the yellow 18-molybdopyrophosphate ($[(P_2O_7)Mo_{18}O_{54}]^{4-}$) anion^{2,3} is formed in an acidic acetonitrile–water (AN–W) solution containing MoO_4^{2-} , that is, the Mo(VI) AN–W reaction mixture, according to: $P_2O_7^{4-} + 18MoO_4^{2-} + 36H^+ \rightarrow [(P_2O_7)Mo_{18}O_{54}]^{4-} + 18H_2O$. Next, by the addition of ascorbic acid (ASC), the $[(P_2O_7)Mo_{18}O_{54}]^{4-}$ anion is reduced to give a more readily distinguishable blue coloration of the AN–W solution. The blank sample shows a remarkable yellow background due to the oxidation of a large amount of ASC by the excess Mo(VI) species. However, PPi can be determined by the color change from yellow to blue; this method has been applied to an assay for an AMP+PPi forming enzyme, acetyl-CoA-synthetase.

This note describes a modification of the PPi assay. The $[(P_2O_7)Mo_{18}O_{54}]^{4-}$ anion is separated from the Mo(VI) AN–W reaction mixture by precipitation with the bis(triphenylphosphoranylidene)ammonium (BTPPA⁺) cation. The separation process prevents yellow coloration and interference by ATP, which gives the colored molybdopyrophosphate species in the AN–W reaction mixture through the hydrolysis of triphosphate ester to phosphate anion (Pi). The BTPPA⁺– $[(P_2O_7)Mo_{18}O_{54}]^{4-}$ precipitate was dissolved in neat AN, in which 8-electron-reduced molybdopyrophosphate is formed by the addition of ASC. The reduced species renders the solution a stronger blue than that in the previous method. Thus, PPi can be detected more sensitively.

Nonribosomal peptide synthetases (NRPSs) are multifunctional

enzymes consisting of semiautonomous domains that synthesize numerous peptide products in microorganisms.^{4,6} Adenylation (A) domains of NRPS activate the carboxyl groups of amino acid substrates with ATP, forming an aminoacyl-adenylate (aminoacyl-*O*-AMP) and PPi, according to the following reaction:

Traditionally, this enzymatic activity has been measured by radioactive ATP- ^{32}P PPi exchange assays. However, most facilities are not equipped to handle radioactive materials. Therefore, we also examined whether the present PPi colorimetric assay is available for NRPS A-domains.

Experimental

Disodium molybdate(VI) dehydrate and L(+)-ascorbic acid were obtained from Wako. Bis(triphenylphosphoranylidene)-ammonium chloride (BTPPACl) was obtained from Aldrich. Sodium pyrophosphate decahydrate was used to prepare the sample solutions of PPi. These were used without further purification. Other chemicals were of reagent grade, and were used as received. The 20 mM Na_2MoO_4 , 0.6 M HCl, and 60% (v/v) AN solution and the 0.5 M ASC, 2 M HCl, and 60% (v/v) AN solution were prepared fresh each day.

The UV-Vis absorption spectra were recorded by a spectrophotometer (JASCO V-530) with a temperature controller. In the spectrophotometric measurement, the path length was 1 cm, and distilled water was used as the blank solution. The absorbance of the reaction mixtures in a microtiter plate was recorded with a microplate reader (Molecular Devices SpectroMax M2). All experiments were performed at 25°C.

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Results and Discussion

In the present method, first, 200 μL of the sample solution was mixed with 2 mL of the 20 mM Na_2MoO_4 , 0.6 M HCl, and 60% (v/v) AN solution to prepare the Mo(VI) AN-W reaction mixture. The 500 μM PPI sample solution gave a yellow Mo(VI) AN-W reaction mixture due to the formation of the $[(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4-}$ anion.¹⁻³ The yellow-color-development was fast, indicating that the $[(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4-}$ -formation reached equilibrium within a few minutes (Fig. S1a, Supporting Information). On the other hand, the 10 mM AMP sample solution gave a colorless mixture, indicating that AMP formed no colored heteropolymolybdate species. However, the 10 mM ATP sample solution resulted in a slow development of yellow color from the AN-W reaction mixture (Fig. S1b). This can be explained by the dissociation of Pi from ATP, facilitated by the formation of the yellow 12-molybdophosphate $[(\text{PO}_4)\text{Mo}_{12}\text{O}_{36}]^{3-}$ anion,¹ according to: $\text{PO}_4^{3-} + 12\text{MoO}_4^{2-} + 24\text{H}^+ \rightarrow [(\text{PO}_4)\text{Mo}_{12}\text{O}_{36}]^{3-} + 12\text{H}_2\text{O}$.^{7,8}

Second, 3 min after the preparation of the Mo(VI) AN-W reaction mixture, 40 μL of a 50 mM BTTPACl AN solution was added to the mixture; then, the mixture was centrifuged, and the supernatant was removed. The blank solution gave no precipitate, indicating that colorless isopolymolybdate species in the Mo(VI) AN-W reaction mixture could not be precipitated with the BTTPA⁺ cation. The 500 μM PPI sample solution gave a yellow precipitate of the BTTPA⁺ salt with the $[(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4-}$ anion. The 10 mM ATP sample solution also gave a yellow precipitate of the BTTPA⁺ salts with the $[(\text{PO}_4)\text{Mo}_{12}\text{O}_{36}]^{3-}$ anion. However, the amount of the precipitate was much less than that given by the PPI sample solution.

Third, 2.2 mL of neat AN was added to the precipitate, and the mixture was centrifuged. Then, 2 mL of the supernatant was transferred, and was used as the AN solution of the heteropolymolybdate anions. The 500 μM PPI sample solution gave a yellow AN solution. The absorbance at e.g. 400 nm of the AN solution was close to that of the Mo(VI) AN-W reaction mixture given by the 500 μM PPI sample solution, indicating that the $[(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4-}$ anion was separated from the AN-W mixture into the AN solution at a high yield. On the other hand, the 10 mM ATP sample solution gave an almost colorless AN solution, indicating again that the amount of the BTTPA⁺- $[(\text{PO}_4)\text{Mo}_{12}\text{O}_{36}]^{3-}$ precipitate was much less than that of the BTTPA⁺- $[(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4-}$ precipitate.

Fourth, 200 μL of the 0.5 M ASC, 2 M HCl, and 60% (v/v) AN solution was added to the AN solution to prepare a heteropolymolybdate-ASC AN reaction mixture. A remarkable blue-color-development was observed in the $[(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4-}$ -ASC AN reaction mixture given by the 100 μM PPI solution. Curve a in Fig. 1 shows the absorption spectrum of the reaction mixture recorded 10 min after preparation. The absorption maximum was observed at around 620 nm, and the absorption rendered the solution blue. Also a developed peak was observed at 790 nm. The spectrum was very similar to that of the 8-electron-reduced molybdopyrophosphate, which was generated by electrolysis of the $[(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4-}$ anion in AN-W.² The absorbance at 620 nm (A_{620}) increased and reached a constant value within several minutes (Fig. S2a, Supporting Information). The blue-color-development was faster than that in the PPI-Mo(VI)-ASC organic-water systems.^{1,3}

On the other hand, the 10 mM ATP sample solution gave an almost colorless reaction mixture. Curve b in Fig. 1 shows the spectrum of the reaction mixture recorded 10 min after the preparation. The A_{620} -value was much less than that given by

Fig. 1 Absorption spectra of the heteropolymolybdate-ASC AN reaction mixtures given by (a) 500 μM PPI and (b) 10 mM ATP sample solutions recorded 10 min after preparation.

the 500 μM PPI sample solution. In the previous method, 5 mM ATP would give more than a 10% positive error in the spectrophotometric determination of 500 μM PPI.¹ The positive error value decreases to less than 2% in the present method. It has been found that the reduction of the $[(\text{PO}_4)\text{Mo}_{12}\text{O}_{36}]^{3-}$ anion in the solvent is much slower than that of the $[(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4-}$ anion (Fig. S2b). This is another reason that ATP gave a much lower A_{620} -value. The slow reduction of the $[(\text{PO}_4)\text{Mo}_{12}\text{O}_{36}]^{3-}$ anion may be due to isomerization between the α - and β -form of the Keggin structure.⁸

It has been found that, instead of AN, a low volatile solvent, propylene carbonate (PC), can be used as the solvent for the reduction process. In PC, $[(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4-}$ anion is also reduced to the blue molybdopyrophosphate species, of which the spectrum is similar to that produced in AN. On the basis of these findings, the procedure for the PPI microassay has been designed as follows:

1. Transfer 20 μL of the sample solution into wells of a 96-well plate, and add 200 μL of a 20 mM Na_2MoO_4 , 0.6 M HCl, and 60% (v/v) AN solution.
2. Add 4 μL of a 50 mM BTTPACl AN solution; centrifuge the mixture, and remove the supernatant. These operations should be completed within 10 min after preparation of the AN-W mixture.
3. Add 220 μL of PC; centrifuge the mixture, and transfer 200 μL of the supernatant.
4. Add 20 μL of a 0.5 M ASC, 2 M HCl, and 60% (v/v) AN solution to the PC solution; allow to stand for 15 min, and determine the concentration of PPI in the sample solution (c_{PPI}) colorimetrically with the reference reaction mixtures, as shown in Fig. 2.

Figure 2 shows the reaction mixture prepared with the PPI sample solutions with different c_{PPI} 's. The 100 μM PPI sample solution gave a blue-color, and the blue increased with c_{PPI} . The A_{620} -value of the reaction mixture in the microplate was proportional to c_{PPI} , as shown by Fig. 3. When the procedure was performed with a ten-times volume of the sample solution and the 20 mM Na_2MoO_4 , 0.6 M HCl, and 60% (v/v) AN and 50 mM BTTPACl solutions, 10 μM level PPI could be determined (Fig. S3, Supporting Information). On the other hand, the blank solution and the 10 mM AMP and ATP solutions

Fig. 2 PPI colorimetric assay of (A) 0, (B) 100, (C) 200, (D) 500, (E) 1000, and (F) 2000 μM PPI solutions and 10 mM (G) AMP and (H) ATP solutions.

Fig. 3 Calibration curve of PPI in the present microassay. The A_{620} -value was recorded 15 min after preparing of the PC reaction mixture with a microplate reader.

gave almost colorless reaction mixtures. The A_{620} -values of the mixtures in the microplate were less than 0.005.

The above results indicate that the present PPI assay can be advantageously applied to the screening of AMP+PPI-forming enzymes. We have reported that histidine-tagged recombinant ϵ -poly-L-lysine synthetase containing only the A-domain and the thiolation domain (rPls-AT) catalyzes the adenylation of L-lysine.⁹ Recently, we also reported that the A-domain (ORF 19), which is involved in the biosynthesis of an antibiotic streptothricin, catalyzes the adenylation of β -lysine.¹⁰ In this study, these two A-domains were employed to evaluate our PPI determination method. The enzyme reaction mixture, consisting of 50 mg/L rPls-AT or rORF 19, 5 mM MgCl_2 , 5 mM ATP, and 50 mM Tris-HCl (pH 8.0), was incubated at 30°C for 30 min. A 10- μL aliquot from the reaction mixture was used for the PPI colorimetric assay. Contrary to our expectation, no PPI was detected from the enzyme reaction mixtures. This can be explained by that the equilibrium of Eq. (1) shifted to the left-hand side. Therefore, to depress the reverse reaction, the enzyme reactions were performed in the presence of NH_2OH (32 mM), which reacts irreversibly with acyl-*O*-AMP to produce

Enzymes	Substrate	
	+	-
rPls-AT		
rORF19		

Fig. 4 PPI colorimetric assay of A-domains. rPls-AT and rORF 19 were incubated with and without L-lysine and β -lysine, respectively.

a hydroxamate derivative:

It is noted that 100 mM NH_2OH did not interfere with the present PPI assay, indicating that NH_2OH does not affect the formation equilibria of heteropolymolybdates in the Mo(VI) AN-W reaction mixture.

As shown in Fig. 4, the enzyme reaction mixtures with the substrates gave a positive blue color, indicating that the reverse reaction was successfully depressed. In fact, HPLC/ESI-MS analysis proved the production of the hydroxamate derivatives (data not shown). Also, it was confirmed that Pi was not produced in the enzyme reaction mixtures by a spectrophotometric Pi assay.¹¹ In contrast, the negative controls without the substrates showed no color. These findings were in good agreement with those by the ATP-[^{32}P]PPI exchange assay used in previous studies.^{9,10} A kinetic study on the enzymatic reactions is in progress.

The present PPI assay is simple, rapid and, easy to carry out. Thus, it can be stated that the present method is useful for the assay of AMP+PPI forming enzymes, including adenylation enzymes.

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Supporting Information

This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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