Note

Preparation of Highly Concentrated Super-hot [γ - ^{32}P]ATP Using Small-scale Ion-exchange Chromatography[†]

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In order to obtain a highly concentrated, pure and super-hot [γ - ^{32}P]ATP, we improved the purification method of super-hot [γ - ^{32}P]ATP which was synthesized by the method of Johnson and Walseth (1979). The super-hot [γ - ^{32}P]ATP was synthesized in a relatively large volume (2 ml) of reaction mixture and purified using semi-micro scale anion exchange chromatography (Dowex 1 $\times$ 2, 60-70 μl column volume). In combination with washing the reaction product with certain organic solvents, this chromatography technique makes it possible to obtain a highly concentrated and pure super-hot [γ - ^{32}P]ATP (approx. 7 000 Ci/mmol; 20-30 mCi/ml) from [^{32}P]P_i of any commercial source in a good yield.

Key Words: γ -phosphorus-32 adenosine triphosphate, ion exchange chromatography

1. Introduction

The super-hot [γ - ^{32}P]ATP has recently been used in various research fields. Synthesizing method of super-hot [γ - ^{32}P]ATP was investigated by Johnson and Walseth^(1,2), and the method has currently being used with minor modifications. However, this method is not always successful with the use of [^{32}P]P_i from commercial sources other than New England Nuclear (NEN). Furthermore, Johnson and Walseth employed anion exchange chromatography of 1 ml column volume, which was originated by Nakai and Brooker⁽³⁾, to purify the labeled ATP, so that the final volume of the ATP solution amounted to several ml. In order to get a more concentrated sample, we reduced

the volume of the anion exchange column and succeeded in getting concentrated [γ - ^{32}P]ATP very conveniently. A part of this work was briefly communicated in Japanese⁽⁴⁾.

2. Materials

Carrier-free [^{32}P]-orthophosphate ([^{32}P]P_i) was purchased from NEN (NEX-053) and Japan Atomic Energy Research Institute (JAERI; P-32-1). The enzymes used for [^{32}P]P_i incorporation into [γ - ^{32}P]ATP were purchased from Boehringer / Mannheim / Yamanouchi (BMV). D- α -Glycerol phosphate and β -NAD⁺ were purchased from BMV, and Na-ADP, Na-pyruvate and cystein-HCl hydrate were from Sigma. Dowex 1 $\times$ 2 (200-300 mesh) was purchased from Muromachi Chemicals. PEI-cellulose plates for thin-layer chromatography were purchased from Macherey-Nagel Co.. Other reagents were purest grade commercially available.

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3. Reagents

3.1 Enzyme/cysteine mixture

Stock enzyme mixture was prepared by mixing five enzymes as indicated in Table 1. A 50 μ l aliquot was centrifuged ($10\,000\times g$, 5 min) in order to eliminate $(\text{NH}_4)_2\text{SO}_4$. Precipitate was dissolved in 60 mM cysteine/Tris buffer (pH 7.5–8.0). We prepared the cysteine/Tris buffer just before use by adding solid Trizma base to 50 mM cysteine-HCl solution.

Table 1 Enzyme mixture

Enzyme	μ l
Glycerol-3-phosphate dehydrogenase	100
Triosephosphate isomerase	1
Glyceraldehyde-3-phosphate dehydrogenase	20
3-phosphoglycerate kinase	2
Lactate dehydrogenase	20

3.2 Reagent mixture

The reagent mixture was prepared as indicated in Table 2. ADP was purified by using DEAE-sephadex column chromatography, because cold P_i in unpurified ADP reduced the specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Except for 40 mM Na-pyruvate, all the other reagents were stored at -25°C .

Table 2 Reagent mixture

Reagent	μ l
0.5 M Tris/HCl (pH 9.0)	300
0.3 M MgCl_2	120
0.1 M Dithiothreitol	180
2.4 mM L- α -Glycerolphosphate	150
10 mM $\beta\text{-NAD}^+$	150
2 mM 2Na-ADP	75
40 mM Na-pyruvate	75

4. Results and Discussion

4.1 Preparation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

$[\text{}^{32}\text{P}]\text{P}_i$ was incorporated into $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by the method of Johnson and Walseth²⁾ with a minor modification. $[\text{}^{32}\text{P}]\text{P}_i$ solutions purchased from commercial sources were used without further purification. Neutralization was not required even in case of $[\text{}^{32}\text{P}]\text{P}_i$ in 0.1 N HCl (JAERI). A 0.8 ml reagent mixture was

added to 1.0 ml $[\text{}^{32}\text{P}]\text{P}_i$ solution. A disposable spitts tube made of polypropylene (Eiken Kizai Co. LTD, P-spitts) was used as a reaction vessel. The reaction was initiated by the addition of 0.2 ml enzyme–cysteine mixture and was allowed to proceed at room temperature for 30 min. Progress of the reaction was monitored by determining the amount of charcoal-adsorbable radioactivity in the mixture⁹⁾. That is, 1–2 μ l of samples were withdrawn and diluted with 1 ml of 5 mM KH_2PO_4 , and a 10 μ l aliquot of this was then withdrawn for liquid scintillation counting with the use of Cherenkov radiation. About 100 mg of charcoal (Norit A) was added to the remainder of the KH_2PO_4 solution. After vigorous mixing of the solution with charcoal and then precipitating the Norit A by centrifugation, a 10 μ l aliquot of the supernatant was withdrawn for liquid scintillation counting. The difference between the two values (before and after Norit A treatment) divided by the first value represents the fraction of charcoal adsorbable $[\text{}^{32}\text{P}]\text{P}_i$, i.e. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, to the total ^{32}P (this value is usually more than 0.99).

4.2 Purification of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using small-scale anion-exchange column chromatography

The synthesized $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purified by small-scale anion-exchange column chromatography (Dowex 1×2 , Cl^- form; 3 mm $\times$ 10 mm) at room temperature. The Dowex resin was reactivated by repetitive washing with 1 N NaOH, 1 N $(\text{NH}_4)_2\text{SO}_4$, 3 N HCl and H_2O . We used a disposable Pasteur pipet (Corning, 7095B) for the column by cutting off its tip and shortening it to about 16 cm. The column end was made of fine mesh glass wool (Shimadzu Seisakusho, Tokyo; No. 201-35311). The resin was poured into the pipet very carefully to avoid its adsorption on the inside wall of the column above the resin. Tight packing of glass wool should be avoided since it considerably reduces the elution speed. The most adequate elution speed was 1 drop/2–3 s. A schematic diagram is shown in Fig. 1. A plastic reservoir made from a 5 ml plastic pipet tip was attached to the column top in order to facilitate the sample application and washing the column.

Fig. 1 Small-scale column used for purification of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Lucite cylinder was adapted for shielding.

The column was thoroughly washed with distilled water prior to use. The content of the reaction vessel was directly diluted with 2–3 parts distilled water and allowed to run into a column. When we used $[^{32}\text{P}]\text{P}_i$ from NEN as starting material, no pretreatment of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ solution was required. Successful adsorption of ATP slightly changed the color of the top of the Dowex column to pale dark brown. The column was washed thoroughly with water to eliminate the enzymes contained in the reaction mixture, and then rinsed with 0.5 ml of 30 mM HCl to eliminate inorganic phosphate, AMP, ADP and $\beta\text{-NAD}$.

The labeled ATP was then repeatedly eluted with 100 μl of 250 mM HCl. As indicated in Fig. 2, the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ appeared in the first 0.2–0.5 ml of the 250 mM HCl eluate. The ATP peak was pooled and neutralized by adding a small aliquot of 2 M Tris-base solution (5–7 μl to the 100 μl solution of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$). A small aliquot (0.5–1 μl) of sample was withdrawn and the pH value was checked using fine strips of Universal Indicator

Fig. 2 Elution profile of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ from a semi-micro scale Dowex 1×2 column by 250 mM HCl. Total 444 MBq (12 mCi) $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was applied to the column, and the column was successively washed with 10 ml of H_2O and 0.7 ml of 30 mM HCl. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was eluted with 250 mM HCl by the stepwise elution of 100 μl each. About 1.85 MBq (0.5 mCi) was eluted with 30 mM HCl, and 388.5 MBq (10.5 mCi) was eluted with 250 mM HCl in the case indicated in this figure.

(Merck). The solution was rapidly frozen by dropping it into a vial which was pre-cooled in liquid nitrogen in order to avoid self-decomposition of the purified $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by the condensation during freezing.

The concentration of the labeled ATP amounted to about 30 mCi/ml. The radiochemical purity of this product was over 99.9% when it was checked by PEI-cellulose thin-layer chromatography⁹⁾.

When $[^{32}\text{P}]\text{P}_i$ from JAERI was used as a starting material, resulted reaction mixture containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ should be washed with organic solvents prior to being applied on chromatography. When ATP was synthesized from $[^{32}\text{P}]\text{P}_i$ of the commercial source other than NEN, this washing procedure was essential for adsorbing the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to Dowex 1×2 column. Some organic contamination seems to prevent the adsorption of ATP to

resin. The washing procedure is as follows. The reaction mixture was washed twice with a water-saturated phenol/chloroform-mixture (1:1) and successively washed 3-times with diethylether. The remaining diethylether in the [γ - ^{32}P]ATP solution was removed by flushing the solution with nitrogen gas flow. [γ - ^{32}P]-ATP could be purified by the procedure mentioned above. On addition, when [^{32}P]P_i from JAERI was neutralized and left for several days in a freezer, less than 10% of [^{32}P]P_i was incorporated into ATP.

The procedures described above took a total of approximately 5 to 6 hours, and yielded a very economical and highly concentrated super-hot [γ - ^{32}P]ATP.

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