活性化硫酸,アデノシン5´-ホスホサルフェート (APS) の単離と同定

誌名	日本水産學會誌
ISSN	00215392
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巻/号	53巻10号
掲載ページ	p. 1867-1870
発行年月	1987年10月

Nippon Suisan Gakkaishi 53(10), 1867–1870 (1987)

Isolation and Identification of Adenosine 5'-phosphosulphate (ASP) as a Reaction Product of ATP-sulphurylase

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(Accepted February 9, 1987)

APS formation catalyzed by purified ATP-sulphurylase of *Porphyra yezoensis* was demonstrated. APS accumulated in the incubated enzyme mixture was separated on a DEAE-Toyo pearl 650 M column, and identified by anion-exchange HPLC and TLC. UV absorption spectra, acid-lability, and equimolar contents in adenine, ribose, phosphate and sulphate also substantiated the identity. The low accumulation of APS suggested the presence of some difficulties in the APS synthesizing reaction.

ATP-sulphurylase (EC 2.7.7.4.) catalyzes the first step in the sulphate activating reaction:

$$SO_4^{2-} + ATP \rightleftarrows APS + PPi$$

The synthesis of active sulphate, APS,*2 is recognized to be the physiological function of this enzyme.[1] It is, therefore, necessary to demonstrate the APS production, in the study on this enzyme. However, because of the extremely unfavorable equilibrium for the APS synthesis,[2,3] the amount of APS accumulated in the ATP-sulphurylase reaction is remarkably small. Thus far the APS production has been demonstrated only by the radioactive tracer method using [^{35}S]-sulphate followed by chromatographic or electrophoretic separation.[1,4-10] For studies on this enzyme, several convenient methods for the enzyme assay are available.[8,11,12] However, these methods do not provide the direct evidence of the APS production.

In the previous paper,[13] the occurrence of ATP-sulphurylase in the marine alga *Porphyra yezoensis* was examined by the molybdolysis assay,[3] which was one of the convenient methods for the enzyme assay using molybdate as the substrate in the place of sulphate. It is valuable to demonstrate the production of APS by the enzyme reaction, for evaluating the sulphate activation in this alga, and also for providing the validity of the molybdolysis assay for the *Porphyra* enzyme.

This work was designed to confirm the APS synthesizing activity of the purified *Porphyra* ATP-sulphurylase.

Materials and Methods

Chemicals

APS and PAPS were purchased from Sigma Chemicals. APS was also synthesized, as described by Baddiley *et al.*,[14] from pyridine-sulphur trioxide and AMP. The APS was separated by DEAE-Sephadex A 50 column chromatography. Other nucleotides and inorganic pyrophosphatase were obtained from Boeringer Mannheim. DEAE-Toyo pearl 650M and Toyo pearl 55S were from Toyo Soda Manufacturing Co., Ltd. The Hitachi gel 3013-N packed column (4×150 mm) was from Gasukuro Kogyo Co. Ltd. The pre-coated cellulose plates (0.10 mm thick) for TLC were from E. Merck. All other reagents used were of the best purity commercially available.

ATP-Sulphurylase

ATP-sulphurylase was purified from *P. yezoensis* collected at Sanriku, Iwate, Japan, after essentially the same procedure reported in the previous paper[13] using a Toyo pearl 55S and DEAE-Toyo

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*2 Abbreviations used: AMP, 5'-adenosine phosphate; ADP, 5'-adenosine diphosphate; ATP, 5'-adenosine triphosphate; ASP, adenosine 5'-phosphosulphate; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; PAP; 3'-phosphoadenosine 5'-phosphate; APMo, molybdenum analog of APS; PPi, pyrophosphate; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; AUFS, absorption unit, full scale.

pearl 650M as substitutes for the Sephadex G200 and DEAE-Sephadex A50 respectively to shorten the purification time. The enzyme thus obtained had a specific activity of about 13.7 units per mg protein in the molybdolysis assay,[13] and gave a single protein band on polyacrylamide gel electrophoresis performed on 7% tube gel at pH 8.3.

Preparation of Reaction Product Solution

To identify the products, the following reaction mixtures were prepared in 0.1 M Tris-HCl buffer (pH 8.2) and a final volume of 10 m*l*: for the forward reaction, 10 mM ATP, 50 mM Na_2SO_4, 10 mM $MgCl_2$, 5 units of inorganic pyrophosphatase, and 1 mg of the purified enzyme; for the reverse reaction, 1 mM APS, 2 mM Na^+-PPi, 2 mM $MgCl_2$, and 50 μg of the enzyme. All incubations were carried out in the dark at 30°C for 30 min under N_2. They were stopped by immersing in boiling water for 2 min. Controls were prepared by omitting the enzyme or either of the substrates from the reaction mixture.

The nucleotides were recovered from the incubation mixture using acid-washed Norit A charcoal. The charcoal was incubated with the heat-stopped mixture for 30 min in an ice-bath, collected by filtration, washed with ice-cold water, and then eluted with 50% ethanol containing 2% ammonia. The eluate was evaporated to dryness at 40°C, and redissolved in water. The nucleotide solution thus obtained was submitted to the analysis.

Analytical Methods

Nucleotides were analyzed by anion-exchange HPLC and TLC on cellulose plates. HPLC was performed on Hitachi gel 3013-N packed column. Elution was carried out with 60 m*l* of linear gradient from 0.06 M NH_4Cl/0.01 M KH_2PO_4/0.01 M K_2HPO_4 in 6% CH_3CN to 0.6 M NH_4Cl/0.1 M KH_2PO_4/0.1 M K_2HPO_4 in 6% CH_3CN at a flow rate of 1 m*l*/min. Nucleotides were detected by the absorption at 260 nm. Under these chromatographic conditions, AMP, ADP, PAP, ATP, APS, and PAPS gave well-resolved peaks (Fig. 1-A).

TLC was carried out in two solvent systems: solvent A, n-propanol/concentrated NH_4OH/water (6: 3: 1, v/v); solvent B, iso-butyric acid/0.5 N NH_4OH (5: 3, v/v). Spots were detected by a short-wave UV lamp. The *Rf* values obtained from AMP, ADP, PAP, ATP, APS, and PAPS were 0.31, 0.21, 0.15, 0.11, 0.47, and 0.24 respectively in solvent A, and 0.61, 0.43, 0.41, 0.29, 0.35, and 0.24 respectively in solvent B.

The UV absorption spectrum was measured with a Shimadzu UV-200 recording spectrophotometer.

Ribose was estimated by the orcinol-Fe^{2+}-hydrochloric acid method, phosphate was by the method of Horecker *et al.*,[15] and sulphate was by the chloranilate method.[16]

Results and Discussion

HPLC chromatograms of the nucleotides from the forward reaction and from the control reaction performed in the absense of sulphate are shown in Fig. 1-B. When ATP and sulphate were present in the mixture, 4 species of nucleotides were detected: traces of AMP and ADP, ATP added as the substrate, and a minor product showing an identical retention time with that of APS. This APS-like compound was not accumulated in the absence of the enzyme or sulphate.

The isolation of the APS-like compound was attempted by using a DEAE-Toyo Pearl 650M column (1 × 20 cm, formate form) chromatography. The nucleotide mixture was applied to the column equilibrated with water. The column was washed with water, and then eluted with a linear gradient of $(NH_4)HCO_3$ (0–0.4 M, 400 m*l*). The eluate was monitored for the elution of nucleotides by changes in absorbance at 260 nm, and collected in 2 m*l* fractions. The column chromatography gave 3 main peaks (Fig. 2). The fractions falling under the same peak were pooled, desalted by repeated evaporations to dryness, and finally redissolved in water. HPLC and TLC analysis of the three fractions showed that the APS-like compound recovered in the third peak was perfectly separated from the other nucleotides: the first peak was AMP, and the second was ATP containing a trace of ADP.

The isolated APS-like compound gave a single peak on HPLC (Fig. 3-B), and its behavior on TLC in solvents A and B was identical to that of the authentic APS. Furthermore, the identification was substantiated by the following criteria. The UV absorption spectrum of the compound showed a single adsorption peak at 260 nm with a sharpe very similar to that of AMP.[14] The molar ratio of adenine to ribose to phosphate to sulphate was 1: 1: 0.97: 0.92, which is consistent with the molar ratio of 1: 1: 1: 1 expected for APS. Treatment with 0.1 N HCl at room temperature for 1 h yielded a single product identified chromato-

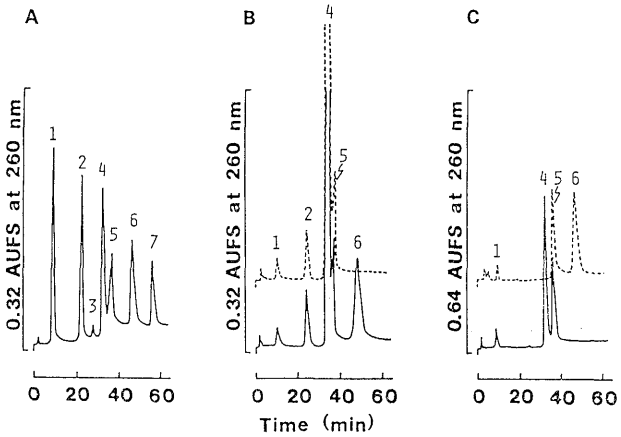

Fig. 1. HPLC chromatograms of the nucleotides recovered from ATP-sulphurylase reactions. Authentic adenosine nucleotides (A), nucleotides recovered from the forward (B), and the reverse (C) reaction mixture were chromatographed on a Hitachi gel 3013-N column (4×150 mm). Chromatograms of nucleotides from control tubes omitting the inorganic substrate (SO_4^{2-} or PPi) were presented by a dotted line. The other experimental conditions are in the text. 1, AMP; 2, ADP; 3, PAP (main degradative contaminant of PAPS); 4, ATP; 5, background peak; 6, APS; 7, PAPS.

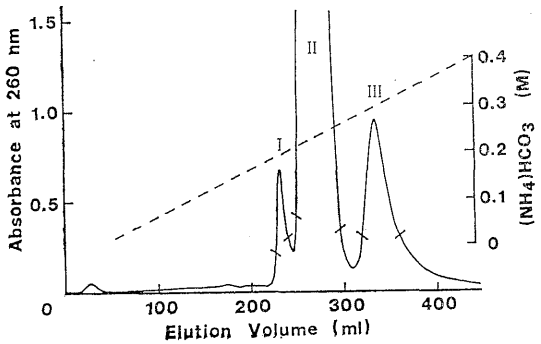

Fig. 2. DEAE-Toyo Pearl column (1×20 cm, formate form) chromatography of the nucleotides prepared from the mixture in which the purified *Porphyra* ATP-sulphurylase was incubated with ATP and sulphate. The flow rate was 0.5 ml/min and fractions of 2 ml were collected. The other experimental conditions are in the text.

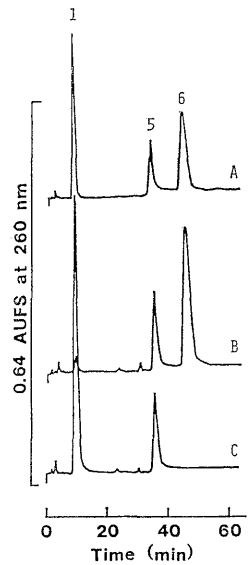

Fig. 3. HPLC chromatograms of APS isolated from ATP-sulphurylase reaction by DEAE-Toyo Pearl 650M column chromatography. HPLC conditions and the figures are the same as Fig. 1. A, authentic AMP and APS; B, isolated APS; C, hydrolysate of the isolated APS.

graphically as AMP (Fig. 3-C). It is known that phospho-sulphate link of APS is unstable at lower pH, while stable at neutral and higher pH. Consequently, the APS-like compound was identified as APS.

The reaction product of the reverse reaction was also investigated by HPLC and TLC. *Porphyra* ATP-sulphurylase catalyzed the formation of ATP from APS and PPi (Fig. 1-C). APS added as the substrate was completely consumed. The ATP production and APS consumption were not observed in the absence of PPi or the enzyme.

The presented result shows that *Porphyra* ATP-sulphurylase surely catalyzes the APS production from ATP and inorganic sulphate. However, the amount of APS accumulated in the ATP-sulphurylase reaction was markedly small even in the presence of inorganic pyrophosphatase, which was generally considered to pull the APS formation

via the hydrolysis of PPi formed in the enzyme reaction.[1] This suggests that the physiological reaction, APS formation, proceeds with some difficulty, which is probably the very unfavorable equilibrium constant for the forward reaction, and the inhibition by the product, APS, in general agreement with studies on the enzyme from other organisms.[2-4,17,18] The enzymic characterization of the enzyme elucidates it clearly. The details will be reported in near future.

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