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Author(s)	INOUE, Botaro; SASAKI, Shoji
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Separation of NADP-pyrophosphatase and 5'-nucleotidase of *Proteus vulgaris*

Botaro INOUE* and Shoji SASAKI

NADP-pyrophosphatase in *Proteus vulgaris*, which was previously reported to have both NADP-pyrophosphatase (EC 3.6.1. x) and 5'-nucleotidase (EC 3.1.3.5) activities, was shown to consist of the above two enzymes which were separable from each other by an improved DEAE-cellulose column chromatography.

Both at least two types of NADP-pyrophosphatase and 5'-nucleotidase were found to exist in *P. vulgaris*. One of the NADP-pyrophosphatases hydrolyzes 5'-ADP, besides NADP, at a pyrophosphate linkage to release β -phosphate, and one of the 5'-nucleotidases acts on 5'-AMP and also nicotinamide mononucleotide (NMN).

Nucleotide pyrophosphatases have been found in many organisms. One of the characteristics of these enzymes is that the activity associates with 5'-nucleotidase activity. Recently, nucleotide pyrophosphatases freed from 5'-nucleotidase activity were isolated from some higher organisms (MATSUDA *et al.*, 1967; BACHORIK and DIETRICH, 1972; DECKER and BISHOFF, 1972; EVANS, 1974). In lower organisms, it has been reported that some nucleotide pyrophosphatases have 5'-nucleotidase activity (SWARTZ *et al.*, 1958; GLASER *et al.*, 1967; MAUCK and GLASER, 1970) and the other enzymes have not (GLASER, 1965; GLASER *et al.*, 1967; VILLELA, 1967; HAROZ *et al.*, 1972; RAETZ *et al.*, 1972).

We previously reported that NADP-degradative enzyme was purified at about 700-fold from *Proteus vulgaris* (NAKAJIMA *et al.*, 1973). Although NMN and 2', 5'-ADP should be produced from NADP by NADP-pyrophosphatase, 2'-AMP was detected instead of 2', 5'-ADP, because the enzyme could hydrolyze 2', 5'-ADP and 5'-AMP to release inorganic orthophosphate, but not hydrolyze NMN. Thus, in the previous work, the purified enzyme preparation had both the activities of NADP-pyrophosphatase and 5'-nucleotidase, but we could not define whether both the activities were attributed to a single enzyme or different enzymes.

In the present paper, we report that the NADP-degradative enzyme in *P. vulgaris* consists of NADP-pyrophosphatase and 5'-nucleotidases which

* Present address: Hokusei-Gakuen Women's High School, Sapporo.

are separable from each other, and that the pyrophosphatase catalyze the cleavage of 5'-ADP at the pyrophosphate linkage.

Materials and Methods

Chemicals

NADP⁺ was obtained from Boehringer, Mannheim. 5'-AMP, 5'-ADP and DL-isocitrate (trisodium salt) were obtained from the Sigma Chemical Co. Brij-58 was purchased from Nakarai Chemicals, Ltd. Sodium deoxycholate and DEAE-cellulose were the products of Difco Co. and Pharmacia Fine Chemicals, Ind., respectively.

Bacteria

Proteus vulgaris was cultivated at 37°C for 18 hr in the ordinary nutrient-broth medium by vigorous shaking. The cells were harvested, washed by centrifugation, and subjected to preparation.

Enzyme

Isocitrate dehydrogenase (EC 1.1.1.42) was prepared from *P. vulgaris* by the method described previously (NAKAJIMA *et al.*, 1973).

Assay of phosphatase activities

Assay procedure of NADP-pyrophosphatase was the same as described previously (NAKAJIMA *et al.*, 1973): Standard reaction mixture for the assay of NADP-pyrophosphatase consisted of 0.3 μ mole of NADP⁺, 170 μ moles of Tris-HCl buffer (pH 7.0) and a suitable amount of the enzyme in a final volume of 3.0 ml. After 20 min of incubation at 30°C, residual NADP⁺ was determined by the isocitrate dehydrogenase system.

Standard reaction mixture for the assay of 5'-nucleotidase consisted of 1.0 μ mole of 5'-AMP, 340 μ moles of Tris-HCl buffer (pH 7.0), and a suitable amount of the enzyme in a final volume of 4.0 ml. Inorganic orthophosphate released for 12~20 min of incubation was determined by the method of Allen as modified by NAKAMURA (1950).

Phosphatase activities for 5'-ADP, inorganic pyrophosphate, and NMN were determined under similar conditions as for 5'-AMP.

Results and Discussion

Washed cells were suspended in 0.1 M Tris-HCl buffer (pH 7.4) containing 0.5% deoxycholate and exposed to sonic oscillation for 7 min in an ice-bath. Brownish supernatant obtained after centrifugation was treated with ammonium sulfate and three times with cold ethanol by similar manner as

described previously (NAKAJIMA *et al.*, 1973), except that 0.1 M and 10 mM Tris-HCl buffer (pH 7.4) were used instead of 83 mM and 20 mM phosphate buffer (pH 8.0), respectively.

The third ethanol fraction was concentrated by polyethylene glycol No. 6000, and dialyzed against 5 mM Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58. After dialysis, the enzyme solution was exposed to sonic oscillation for 5 min, and applied to DEAE-cellulose column previously equilibrated with 5 mM Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58. Then the enzyme was eluted stepwise with different series of NaCl concentration (25 mM to 0.4 M), nevertheless both the activities of NADP-pyrophosphatase and 5'-nucleotidase could not be separated, but seemed to be separable if only suitable procedure has been chosen.

After prolonged dialysis (2 days), the enzyme solution was exposed to sonic oscillation without Brij-58 and applied to DEAE-cellulose column chromatography. Both activities were almost separated from each other, but NADP-pyrophosphatase freed from 5'-nucleotidase could not be obtained (Fig. 1).

The third ethanol fraction was dialyzed against 50 volumes of 5 mM

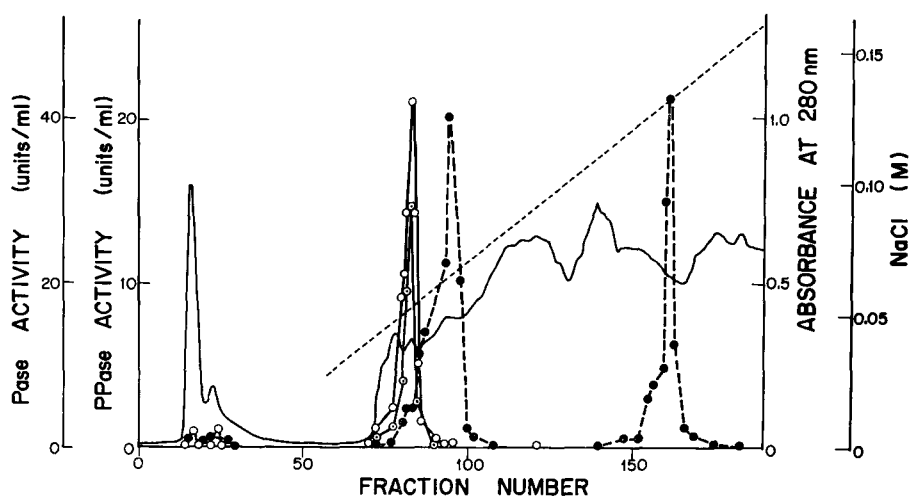


Fig. 1. Elution profile of NADP-pyrophosphatase and 5'-nucleotidase from DEAE-cellulose column. 25 ml of the third ethanol fraction (11 mg/ml) was applied to a column (1.5×30 cm) equilibrated with 5 mM Tris-HCl (pH 7.4) containing 0.2% Brij-58. After washing, the enzyme was successively eluted with the same buffer containing 25 mM NaCl and with a linear gradient of NaCl concentration as indicated in the figure. Fractions of 5 ml were collected. ○—○, NADP-pyrophosphatase (PPase) activity; ●—●, 5'-nucleotidase (Pse) activity for 5'-AMP; ⊙—⊙, ADP-hydrolyzing activity; —, absorbance at 280 nm; ·····, concentration of NaCl.

Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58, and adsorbed on DEAE-cellulose by batch method. After washing with the same buffer, the enzyme was eluted with the same buffer containing 0.2 M NaCl. The enzyme solution was concentrated, dialyzed and applied to DEAE-cellulose column chromatography. As shown in Fig. 2, both the activities of NADP-pyrophosphatase and 5'-nucleotidase were completely separated. However, the yield of the NADP-pyrophosphatase was too poor to be subjected to further purification. Consequently, some improvements were adopted to do further procedure.

The third ethanol fraction was dialyzed against 100 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58. After concentration, to the enzyme solution was added Brij-58 to 0.2%, and exposed to sonic oscillation. The enzyme solution was applied to DEAE-cellulose column chromatography. NADP-pyrophosphatase was eluted very broadly (see Fig. 3 A). So, the fractions having NADP-pyrophosphatase activity were combined, concentrated, dialyzed and exposed to sonic oscillation. The resultant solution

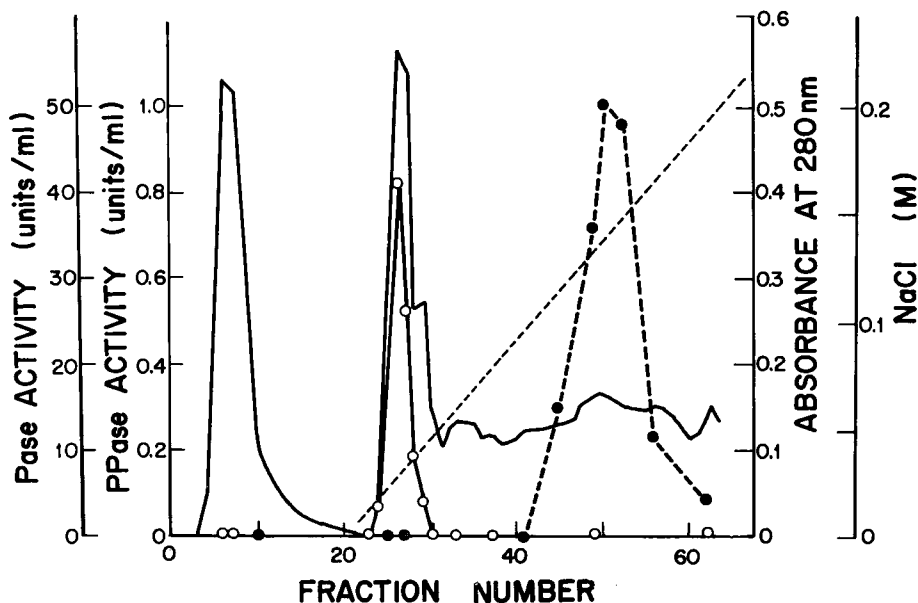


Fig. 2. Separation of NADP-pyrophosphatase and 5'-nucleotidase by DEAE-cellulose column chromatography. Dialyzed ethanol fraction was pre-treated with DEAE-cellulose as described in the text. After concentration and dialysis, about 25 ml of the enzyme solution (1.8 mg/ml) was applied to a column (1.5×30 cm) and eluted with a linear gradient of NaCl concentration. Symbols and abbreviation were the same as in the legend to Fig. 1.

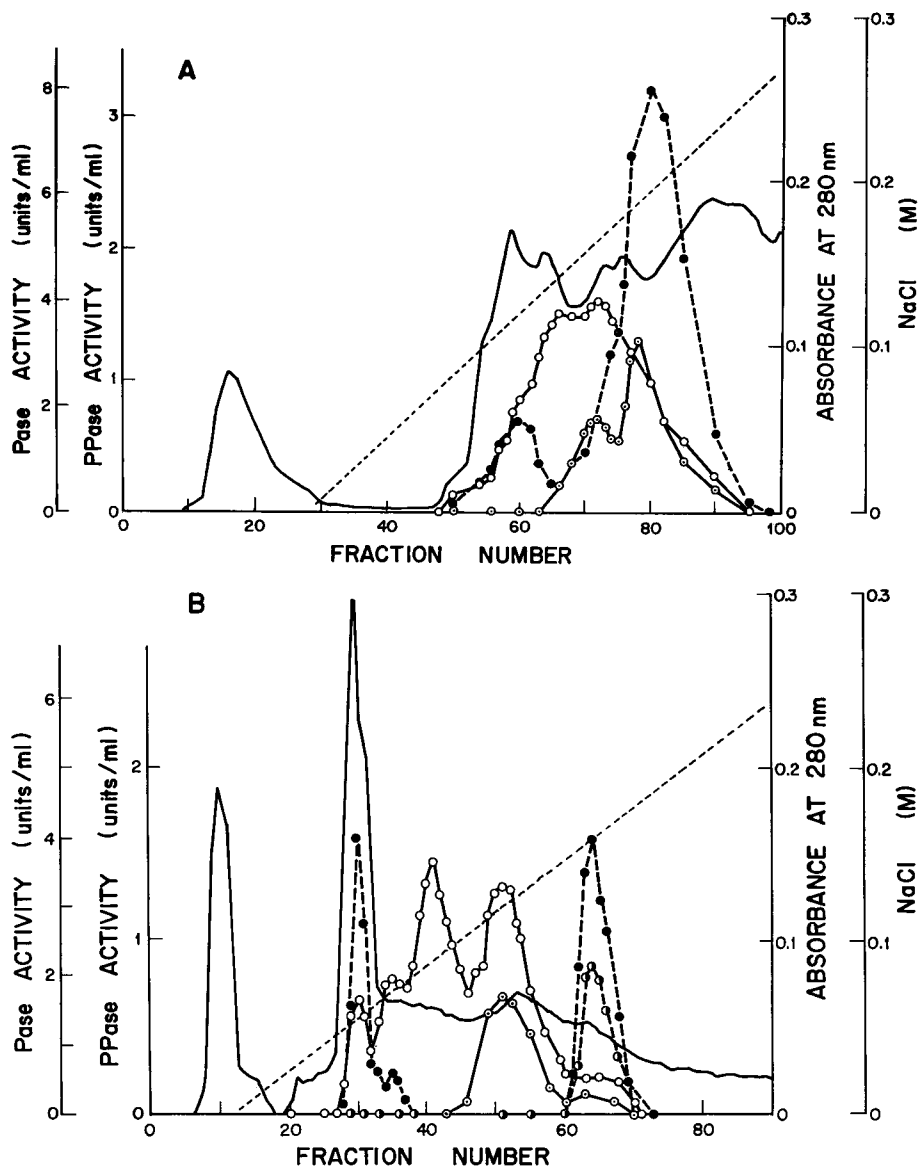


Fig. 3. Separation of both two types of NADP-pyrophosphatases and 5'-nucleotidases by DEAE-cellulose column chromatography. (A) 44 ml of the third ethanol fraction (10.1 mg/ml) was dialyzed and concentrated to about 7 ml. After sonic oscillation in a presence of 0.2% Brij-58, the enzyme solution was loaded on a DEAE-cellulose column (3×20 cm) and eluted with a linear gradient of NaCl concentration. (B) Fractions (No. 60 to 77 in Exp. A) were combined (27 mg/87 ml). After concentration, dialysis, and sonic oscillation, the enzyme solution was applied to rechromatography with DEAE-cellulose column (1.5×30 cm). Symbols and abbreviation were the same as in the legend to Fig. 1, except (●---●), 5'-nucleotidase (Pase) activity for NMN.

was applied to rechromatography. NADP-pyrophosphatase activities were eluted in two regions (Fig. 3B). One of the enzymes could hydrolyze 5'-ADP, besides NADP. Furthermore, 5'-nucleotidase activities were also eluted in two regions (Fig. 3B). Both 5'-AMP and NMN were hydrolyzed by one of the 5'-nucleotidases. Although data were not presented here, inorganic pyrophosphatase (EC 3.6.1.1) was not detected in any fractions tested, owing to removal of the enzyme by the ethanol treatment. Consequently, it is obvious that 5'-ADP was degraded at the pyrophosphate linkage to release β -phosphate by the NADP-pyrophosphatase. If 5'-nucleotidase is present in this enzyme fraction, 5'-AMP produced by the pyrophosphatase must be hydrolyzed by 5'-nucleotidase to release α -phosphate. For instance, the second higher peak of the ADP-degradative activity shown in Fig. 3A seemed to be an apparent one, because these fractions in the region had high activity of 5'-nucleotidase.

From these results, it is concluded that in *Proteus vulgaris* NADP-pyrophosphatase and 5'-nucleotidase are separate enzymes and that NADP is cleaved at the pyrophosphate linkage to produce 2', 5'-ADP and NMN by the pyrophosphatase, and the products are further degraded to 2'-AMP and probably nicotinamide riboside by 5'-nucleotidases. As seen in Fig. 3B, there are both two types of NADP-pyrophosphatases and 5'-nucleotidases in *P. vulgaris*, which are different in respect of substrate specificity.

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