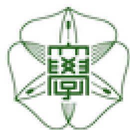
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Solubilization and purification of a NADP degradative enzyme in *Proteus vulgaris*

By

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NADP degradative activity was associated with cell-structures and could not be extracted by Triton X-100 alone, but could be extracted easily after the removal of lipids by the ethanol-ether (3 : 1) solution.

Two peaks of NADP degradative activity were found at pH 4.6 and pH 4.9, respectively, when the enzyme extracted by Triton X-100 was analyzed by electrofocusing column chromatography. When the enzyme supplied with $CoCl_2$ was analyzed, the peaks were observed at pH 4.6 and pH 5.3, respectively. The supplement of $CoCl_2$ ensured the adsorption of the enzyme to phosphocellulose at pH 7.4.

The enzyme concerned with NADP degradation was purified from the ethanol-ether powder by an improved simple method containing phosphocellulose column chromatography.

One of the characteristic properties of nucleotide pyrophosphatases is the membrane-bound enzymes and some of them are activated by treatment with lipase[1] or detergents[2]. BACHORICK *et al.* reported that the enzyme from rat liver required some detergents for its activity and the activity was lost with removal of the detergents[3]. The membrane-bound forms make their purification difficult[4~6], so, different techniques to solubilize them have been used, namely, treatment with some detergents[7,8], delipidization by organic solvents[9~11] and treatment with lipase in the presence of Triton X-100[12]. As for the intracellular distribution of pyrophosphatases, Kuriyama reported an interesting fact that the enzyme of rat liver microsomes was inactive and immunologically indetectable without the treatment with detergents[1]. He concluded that this enzyme existed in the inner compartments of microsomes and was usually separated from the substrates.

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As described above, many reports about pyrophosphatases of higher organisms have been published, but the report on the enzyme of bacteria is scant. GLASER *et al.* purified nucleotide pyrsophosphatases from *E. coli* and *Salmonella weslaco*, and reported that a pyrophosphatase specific toward UDP-glucose was present as soluble form in *E. coli*, while, in *S. weslaco* both a soluble and a particle-bound pyrophosphatase were present and the particle-bound enzyme had broad substrate specificity. But they did not examine about the cleavage of pyridine nucleotides[13]. Mauck and Glaser have reported the presence of a periplasmic pyrophosphatase in *Bacillus subtilis* and found that both activities of nucleotide pyrophosphatase and 5'-nucleotidase were present on the same protein[14].

In our laboratory, it was suggested that the activities of NADP degradation might be present in the extracts of *Proteus vulgaris*[15], and partial purification of a NADP-degradative enzyme and some of its properties were reported[16]. In this report, studies on the solubilization, an improved procedure of the purification and some physiological properties of the enzyme concerned with NADP degradation are presented and some physiological roles of the enzyme will be discussed.

Materials and Methods

Bacteria. *Proteus vulgaris* was grown at 37 C for 18 h on agar plates containing 1% each of peptone and meat extract, or for 15 h in a liquid medium of the same composition by vigorous shaking.

Chemicals. $NADP^+$ was obtained from Boehringer Mannheim. Brij-58 was purchased from Nakarai Chemicals, Ltd. Sodium deoxycholate was a product of Difco Co. Triton X–100 was purchased from Wako Pure Chemical Ind., Ltd. Carrier ampholite was obtained from LKB-Produkter AB. Phosphocellulose was a product of W. R. Balston Ltd. All other reagents were of analytical grade and all bivalent cations were used as chlorides.

Enzymes. Isocitrate dehydrogenase (EC 1.1.1.42) was prepared from *P. vulgaris* by the method described previously[16].

Assays. The concentration of protein was measured by the method of WARBURG and CHRISTIAN[17].

NADP degradative activity was assayed using the reaction mixture containing 0.2 μ mole of $NADP^+$, 170 μ moles of Tris-HCl buffer (pH 7.4) and an appropriate amount of the enzyme which made the reaction to proceed linearly for the initial 30 min in a final volumc of 3.0 mℓ. After incubation for 20 min at 30 C, 0.1 mℓ of isocitrate dehydrogenase solution

and 0. 1mℓ of 50 mM sodium isocitrate were added to the reaction mixture. The amounts of reduced NADP were determined by measuring the absorbance at 340 nm in a Hitachi Perkin-Elmer spectrophotometer Type 139 and calculated based on an extinction coefficient of 6.22×10^6 cm^2/mole. The amounts of NADP degraded during incubation were calculated from the difference of the concentration of reducible NADP before and after incubation. When the enzyme treated with EDTA was used, 1 μmole of $CoCl_2$ was added to the reaction mixture. The units of enzyme activity were defined as μmoles of NADP degraded per h.

Results

Solubilization of NADP Degradative Activity. The condition of the solubilization of the enzyme(s) was studied to simplify the purification procedure. Fig. 1 shows a series of the procedures to examine the effects of chelating agent, osmotic shock, high salt condition, sonic vibration and detergent on the extraction of the enzyme. The cells (about 0.7 g) harvested from 100 mℓ of the culture medium were used. The samples obtained at each step were supplemented with EDTA to 25 mM and dialyzed against 10 mM Tris-HCl buffer (pH 7.4) overnight at 4 C. These results (see Table 1) show the ineffectiveness of EDTA, osmotic shock, sonic vibration, Triton X-100 and high salt condition on the extractability

TABLE 1. Degrees of extraction of NADP degradative activity by some treatments

Fraction	Total volume (mℓ)	Total activity (units)	Distribution (%)
Sup. I	18.0	0	0
Sup. II	14.4	0	0
Sup. III	35.2	0	0
Sup. IV	20.2	0	0
Sup. V	17.0	0.12	1.8
Sup. VI	34.0	1.16	16.6
Pellet	21.2	5.75	81.6

NADP degradative activity of each fraction was assayed under the standard assay method described in Materials and Methods. Sup. I, supernatant fraction obtained after EDTA-treatment; Sup. II, fraction obtained after treatment with EDTA and 1% Triton X-100; Sup. III, fraction obtained after osmotic shock; Sup. IV, fraction obtained after LiCl-treatment; Sup. V, fraction obtained after sonic vibration; Sup. VI, fraction obtained after 1% Triton X-100 treatment; Pellet, disrupted cells obtained after 1% Triton X-100 treatment.

Washed cells (about 0.7 g)

Suspend in 10 mℓ of 100 mM Tris-HCl buffer (pH 7.4) containing 50 mM EDTA and stand for 10 min at room temperature; centrifuge at 12,000×g for 5 min

Pellet — Supernatant I

Suspend in 10 mℓ of 100 mM Tris-HCl buffer (pH 7.4) containing 50 mM EDTA and 1% Triton X-100 and stand for 10 min at room temperature; centrifuge at 12,000×g for 5 min

Pellet — Supernatant II

Suspend in 100 mM Tris-HCl buffer (pH 7.4) containing 50 mM EDTA and 10% sucrose; centrifuge at 12,000×g for 5 min

Pellet

Suspend in 20 mℓ of 100 mM Tris-HCl buffer (pH 7.4) containing 50 mM EDTA and 1% Triton X-100; centrifuge at 12,000×g for 5 min

Pellet — Supernatant III

Suspend in 10 mℓ of 100 mM Tris-HCl buffer (pH 7.4) containing 5 M LiCl; centrifuge at 12,000×g for 5 min

Pellet — Supernatant IV

Suspend in 10 mℓ of 100 mM Tris-HCl bufferr (pH 7.4) containing 50 mM EDTA, then expose to sonic vibration for 7 min in an ice bath; centrifuge at 12,000×g for 20 min at 4 C

Pellet — Supernatant V

Suspend in 10 mℓ of 100 mM Tris-HCl buffer (pH 7.4) containing 50 mM EDTA and 1% Triton X-100 and stand for 30 min with stirring at room temperature; centrifuge at 12,000×g for 20 min at 4 C

Pellet — Supernatant VI

Suspend in 10 mℓ of 100 mM Tris-HCl buffer (pH 7.4)

Pellet fraction

Fig. 1. Flow chart for the examination of the extractability of NADP degradative activity.

of the enzyme. Most of enzyme activity was recoverd in the fraction of the precipitated cell-debris. Triton X–100 had no effects on the solubilization of the enzyme as described above, but from the powder (ethanol-ether powder) obtained from the precipitated cell-debris desribed above by treatment with cold ethanol-ether (3 : 1), the enzyme could be extracted by 1% Triton X–100 containing 50 mM EDTA. These procedures of the solubilization are summarized in Fig. 2. As shown in Table 2, after the

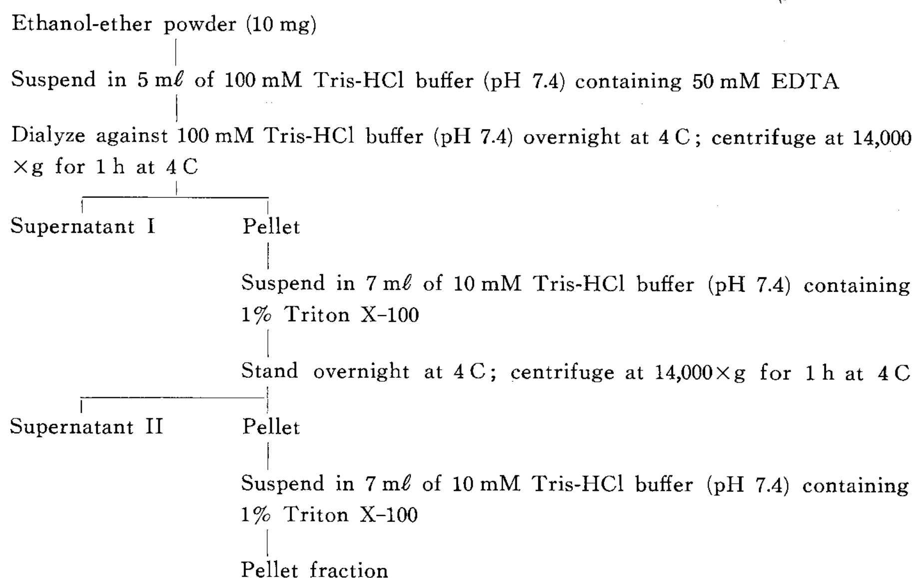

Fig. 2. Flow chart for the extraction of NADP degradative activity from the ethanol-ether powder.

TABLE 2. Extraction of NADP degradative activity from the ethanol-ether powder

Fraction	Total volume (mℓ)	Total activity (units)	Distribution (%)
Sup. I	8.6	1.28	5.0
Sup. II	7.0	19.2	80.3
Pellet	7.0	3.5	14.7

NADP degradative activity of each fraction was assayed under the standard assay method described in Materials and Methods. Sup. I, supernatant fraction obtained by extraction with Tris-HCl buffer; Sup. II, fraction extracted by 1% Triton X–100 from the precipitate fraction obtained after extraction with Tris-HCl buffer; Pellet, precipitate fraction obtained after 1% Triton X–100 extraction.

treatment with Triton X–100, 80% of the activity was recovered in the supernatant fraction obtained by centrifugation at 14,000×*g* for 1 h.

NADP degradative enzyme (NADP pyrophosphatase) was adsorbed to phosphocellulose at pH 7.4[16]. This fact suggests that the enzyme protein is basic. When the enzyme extracted by Triton X–100 as described above was applied on an electrofocusing column, two peaks of enzyme activity were observed at pH 4.6 and pH 4.9, respectively (see Fig. 3). The enzyme supplemented with $CoCl_2$ was also analyzed, and two peaks were observed at pH 4.6 and pH 5.3 (see Fig. 4). It seemed that a peak at pH 4.9 was shifted to pH 5.3 by supplement with $CoCl_2$. These results suggest that the enzyme solution extracted by 1% Triton X–100 may contain two enzymes concerning the degradation of NADP.

The enzyme extracted from the ethanol-ether powder by 1% Triton X–100 had no activity unless supplemenet with $CoCl_2$, and the electro-

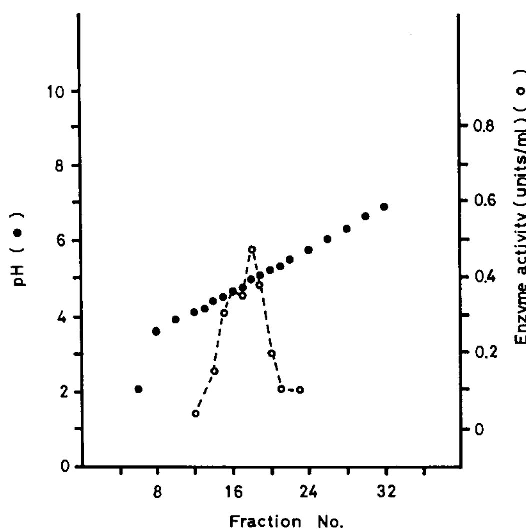

Fig. 3. Elution profile of electrofocusing column chromatography.

The enzyme extracted from the ethanol-ether powder (40 mg) by Triton X–100 was applied without supplement of $CoCl_2$. Electrofocusing was carried out under stabilization by sucrose density gradient from 0 to 50% at 600 V for 48 h at 10 C in a final volume of 110 mℓ. Range of pH was chosen between 3.0 and 8.0. The activity of each fraction was assayed in the reaction mixture containing 0.1 μ mole of $NADP^+$, 170 μ moles of Tris-HCl buffer (pH 7.0), 1.0 μ mole of $CoCl_2$ and 0.2 mℓ of enzyme solution in a final volume of 3.0 mℓ. Symbols: ●, pH; ○, enzyme activity.

focusing pattern was altered by supplement with $CoCl_2$. So, the adsorbability of the enzyme to phosphocellulose was examined with or without addition of $CoCl_2$. The enzyme which was not supplied with $CoCl_2$ could not be adsorbed to phosphocellulose equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58. On the other hand, the enzyme supplied with 10 mM $CoCl_2$ and dialyzed against 20 mM Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58 could be adsorbed to phosphocellulose under the same condition. These results are shown in Fig. 5 and Fig. 6.

Purification of the Enzyme by a Simple Procedure.

Preparation of the ethanol-ether powder. The washed cells (about 32 g) of *P. vulgaris* were suspended in 160 m*l* of 83 mM phosphate buffer (pH 7.4). Each 40 mℓ of the suspension was exposed to sonic vibration for 20 min at interval of 3 min in an ice-bath. The suspension of the disrupted cells was centrifuged at 7,000 rpm for 7 min. The precipitate fraction was resuspended in 20 mℓ of 83 mM phosphate buffer and exposed

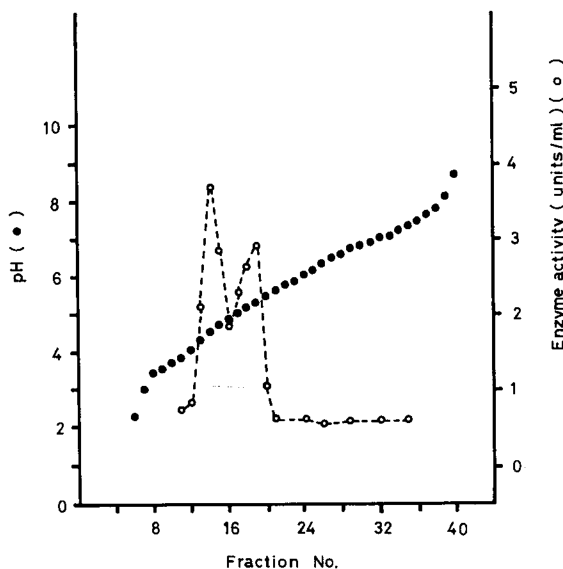

Fig. 4. Elution profile of electrofocusing column chromatography.

The enzyme extracted from the ethanol-ether power (500 mg) by Triton X-100 and supplemented with 10 mM $CoCl_2$ was dialyzed against deionized water for 12 h at 4 C, then applied to an electrofocusing column under the same condition described in the legend to Fig. 3. Symbols: ●, pH; ○, enzyme activity.

to sonic vibration as above. The supernatant fraction and the redisrupted suspension were combined and dialyzed against 10 mM Tris-HCl buffer (pH 7.4) overnight, then centrifuged at 13,000 × *g* for 1.5 h. The precipitated materials were suspended in 100 mℓ of 100 mM Tris-HCl buffer (pH 7.4) containing 50 mM EDTA, then dialyzed against 10 mM Tris-HCl buffer (pH 7.4) overnight. The dialyzed suspension was centrifuged at 13,000 × *g* for 1.5 h. The precipitate was suspended in 20 mℓ of 10 mM Tris-HCl buffer (pH 7.4) in an ice-bath, then dropped into 250 mℓ of ethanol-ether (3 : 1) chilled at −20 C with stirring. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min at 0 C and suspended in 100 mℓ of ethanol-ether, then the insoluble materials were harvested by centrifugation. The resultant precipitate was dried by evaporation for several hours, homogenized and stored in a desiccator at 4 C. From 32 g

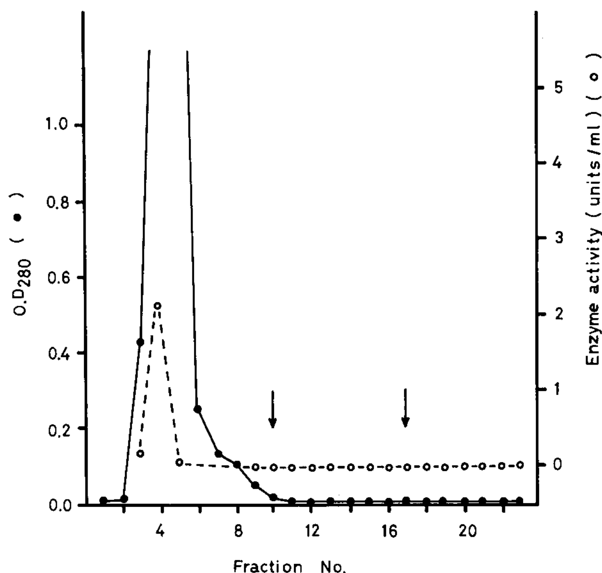

Fig. 5. Adsorbability of the enzyme to phosphocellulose.

The enzyme extracted from the etanol-ether powder (20 mg) by Triton X-100 was applied to a phosphocellulose column (15×100 mm) equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58. Elution was carried out stepwise with 50 mℓ of 20 mM Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58 and with 40 mℓ of the same buffer containing 0.25 M NaCl and 0.5 M NaCl respectively at 4 C. The activity of the enzyme was measured in the reaction mixture containing 0.1 μ mole of $NADP^+$, 170 μ moles of Tris-HCl buffer (pH 7.0), 1.0 μ mole of $CoCl_2$ and 0.1 mℓ of the enzyme solution in a final volume of 3.0 mℓ. Symbols: ●, $O.D_{280}$; ○, enzyme activity.

of the washed cells, 2.15 g of the powder was obtained. The powder was stable for three months at least at room temperature.

Extraction of the enzyme. The prowder (500 mg) was suspended in 120 m*l* of 100 mM Tris-HCl buffer (pH 7.4) containing 50 mM EDTA and dialyzed against 10 mM Tris-HCl buffer (pH 7.4) overnight, then centrifuged at 14,000 × *g* for 1 h. The precipitate was suspended in 100 mℓ of 10 mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100 and allowed to stand with stirring overnight. The suspension was centrifuged at 14,000 × *g* for 1 h. The supernatant fraction was supplemented with 3 mℓ of 100 mM $CoCl_2$ solution neutralized with 100 mM trisaminomethane and concentrated to about 30 mℓ with polyethylene glycol No. 6,000, then exposed to sonic vibration for 5 min in an ice-bath. The concentrated solution was centrifuged at 100,000 × *g* for 1 h. Finally, 23.8 mℓ of the clear solution was obtained.

Phosphocellulose column chromatography. The enzyme solution (23.8

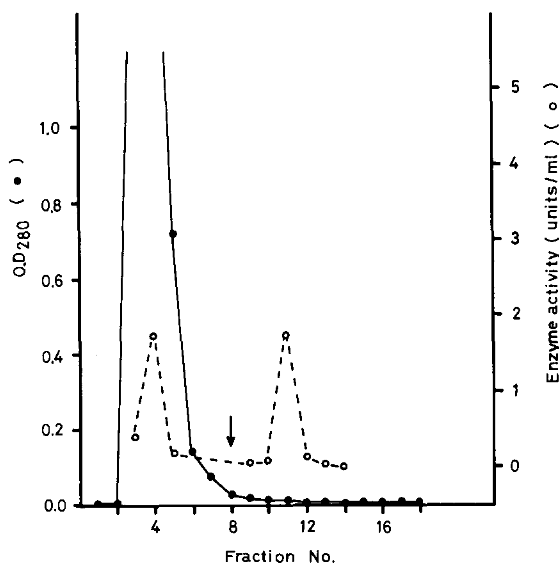

Fig. 6. Adsorbability of the enzyme to phosphocellulose.

The enzyme extracted from the ethanol-ether powder (40 mg) by Triton X-100 and supplemented with 10 mM $CoCl_2$ was dialyzed against 20 mM Tris-HCl buffer (pH 7.4) overnight at 4 C and applied to a phosphocellulose column as described in the legend to Fig. 5. Elution was carried out stepwise with 40 mℓ of 20 mM Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58 and with the same buffer containing 0.5 M NaCl at 4 C. Enzyme activity was assayed as described in the legend to Fig. 5. Symbols: ●, $O.D_{280}$; ○, enzyme activity.

mℓ) obtained by centrifugation at 100,000×*g* was applied to a phosphocellulose colum (25×100 mm) bufferized with 10 mM Tris-HCl buffer (pH 7.4) containing 0.2% Brij-58. Then the column was washed with 50 m*l* of the same buffer. After washing, the enzyme was eluted with the same buffer containing 0.2 M NaCl. The elution profile is shown in Fig. 7. The fractions eluted with 0.2 M NaCl were combined, then enzyme activity and the concentration of protein were determined. The yields of enzyme activity are summarized in Table 3. The specific activity of some frac-

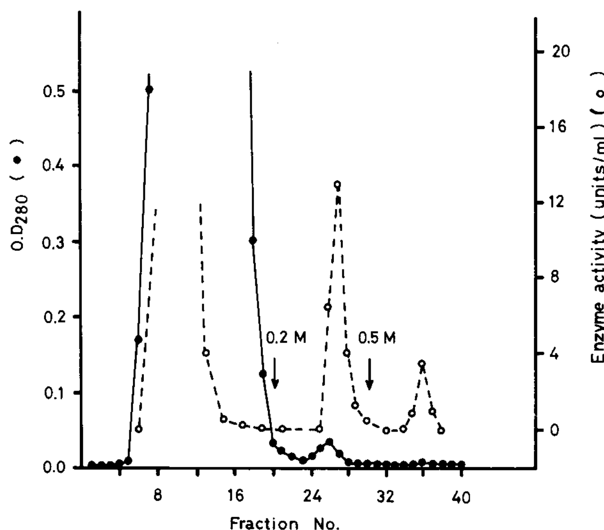

Fig. 7. Elution profile of phosphocellulose column chromatography. Symbols: ●, O.D_{280}; ○, enzyme activity.

TABLE 3. Purification of NADP degradative enzyme from the ethanol-ether powder.

Fraction	Total volume (mℓ)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Crude extracts	104.0	—	1,040	—	100
Supernatant after ultracentrifugation	23.8	—	980	—	94
Phosphocellulose	24.0	0.95	210	221	20.2

The reaction mixture contained 0.2 μmole of $NADP^+$, 170 μmoles of Tris-HCl buffer (pH 7.0), 1.0 μmole of $CoCl_2$ and suitable amounts of the enzyme in a final volume of 3.0 mℓ. The reaction was carried out under the standard assay method described in Materials and Methods.

tions at the final step was about the same level as the EDTA-treated enzyme prepared by the complex procedure described previously[16]. All procedures were carried out at 4 C unless otherwise stated.

Discussion

It seems that many pyrophosphatases from various organisms are membrane-bound enzymes. So, some devices for solubilization have been designed by the use of some detergents, organic solvents, lipase and the treatment with alkalin pH. In some cases, the purification was not successful because of the impossibility of solubilization[4~6]. By treatment with Triton X–100 alone pyrophosphatases could be extracted from membranes and particles of *E. coli*[2], *Micrococcus lysodeikticus*[7]. From rat liver the enzymes were solubilized by butanol[9,10], Triton X–100[3] or by treatment with lipase in 0.2% Triton X–100[12]. From rat cerebellum[8] and hepatocytes[1], the enzymes were extracted by 0.1% Triton X–100 and by 0.05% DOC at pH 10.7 respectively. The enzyme of bovine liver microsomes[18] was solubilized by treatment with NH_4OH at pH 10.4. As to the enzyme of *P. vulgaris*, Triton X–100 was available only after the treatment with ethanol-ehter (3 : 1). This suggests that the enzyme firmly binds to membranes or particles and forms the complex with some lipids which can be removed by treatment with ethanol-ether but can not be by Triton X–100 alone.

The solubilized enzyme had the isoelectric points at pH 4.6 and 4.9, and the supplement with $CoCl_2$ changed it from pH 4.9 to pH 5.3. The enzyme supplemented with $CoCl_2$ was adsorbed to phosphocellulose at pH 7.4. From these results, it seems that the enzyme activated by $CoCl_2$ can be adsorbed to phosphocellulose by affinity-binding rather than electrostatic binding.

The procedure of the enzyme preparation containing the treatment with ethanol-ether and solubilization by Triton X–100 provided two convenient merits ; one is that the ethanol-ether powder is very stable to be available at any time, and the other is the simplification of the purification procedure.

The physiological roles of our enzyme can only be speculated now. But the roles of pyrophosphatases in various organisms have been discussed by some investigators[19,20]. It was reported that pyrophosphatase activity and the levels of pyridine nucleotides were changeable with the environmental factors[21,22]. NADP degradative enzyme of *P. vulgaris* may also take part in the control of pyridine nucleotide-levels in the bacterial cells.

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