IMMOBILIZATION OF YEAST PYRIDOXAMINE (PYRIDOXINE) 5'-PHOSPHATE OXIDASE BY ORGANOMERCURIAL AGAROSE

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1. Introduction

Pyridoxamine (pyridoxine) 5'-phosphate oxidase (EC 1.4.3.5) catalyzes the formation of pyridoxal 5'-phosphate (PLP) from pyridoxamine 5'-phosphate (PMP) or pyridoxine 5'-phosphate (PNP) [1–4]. The enzyme has been highly purified from rabbit liver [4,5] and one bacterium [3].

Baker's yeast contains a high amount of this enzyme activity [6], however, trials for the purification have not been done since Pogell reported the existence of the enzyme in 1958 [1]. Recently, we have reported the results on the partial purification of the enzyme from yeast [6]. In the advances of the purification, we have now found that the enzyme covalently attached to an organomercurial agarose, Affi-Gel 501, is active in situ, and the enzyme column can be applicable to not only kinetic studies but also the production of PLP from PMP or PNP in relatively high yield.

The preparation and some properties of the immobilized enzyme are described here.

2. Materials and methods

2.1. *Materials*

Canned dry baker's yeast was a generous gift from Oriental Yeast Co. (Tokyo). PLP, PMP, and PMSF were purchased from Sigma. PNP was prepared by reduction of PLP with $NaBH_4$ [4]. Affi-Gel 501 was obtained from Bio-Rad. All other reagents were of analytical grade.

Abbreviations: PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; PNP, pyridoxine 5'-phosphate; PMSF, phenylmethylsulfonyl fluoride; KP*i*, potassium phosphate buffer

2.2. *Measurement of activity*

All experiments were carried out in the dark.

The measurement of pyridoxamine (pyridoxine) 5'-phosphate oxidase activity in soluble and immobilized state, unless otherwise noted, was standardized as follows: 285 μM substrate (PMP or PNP) with enzyme preparation was assayed for 30 min at 37°C in 0.2 M Tris–HCl buffer (pH 8.0) in the presence of 50 μg bovine serum albumin, as in [2].

An $\epsilon_{410} = 23 \times 10^3$ was used for the phenylhydrazone derivative of PLP. One enzyme unit catalyzes the formation of 1 nmol PLP/h under the conditions used. Specific activity is given in units/mg protein.

Protein was determined by the biuret method [7] or by the Lowry method [8] using bovine serum albumin as standard.

2.3. *Partial purification of enzyme*

Canned dry baker's yeast (500 g) was milled for 48 h at low temperature (~ 10°C) in Ball mill with 500 g sea sand (40–80 mesh), and the enzyme was extracted with 3 l 0.02 M KP*i*/10 μM PMSF (pH 7.0) for 3 h at 5°C. The suspension was centrifuged for 30 min at 18 000 × *g* and the precipitate discarded. The supernatant was adjusted to pH 5.0 with 2 N acetic acid, and the precipitate removed by centrifugation. The supernatant was readjusted to pH 7.0 with 10% KOH. The solution was fractionated by solid $(NH_4)_2SO_4$ from 0.45–0.8 saturation, and the precipitate dissolved in 0.02 M KP*i*/10 μM PMSF

(pH 8.0). After dialysis against the same buffer, the solution was applied to a DEAE–Sephadex A-50 column (5 × 20 cm), equilibrated with the same buffer, and material eluted with a linear gradient established from 0.02–0.2 M KP*i* (pH 8.0) totalling 2 l. Fractions containing high activity were pooled and brought to 0.8 saturation with solid $(NH_4)_2SO_4$. The precipitate was collected and dissolved in 0.02 M KP*i*/10 μM PMSF (pH 7.0). Absolute EtOH (0.5 vol. at 0°C) was added and the precipitate centrifuged immediately. The supernatant was dialyzed completely against several changes of 0.02 M KP*i*/10 μM PMSF/1 mM EDTA (pH 7.0) and insoluble material removed by centrifugation. After condensation by adding solid $(NH_4)_2SO_4$ to 0.8 saturation, the precipitate was dissolved in a min. vol. of 0.02 M KP*i*/10 μM PMSF (pH 7.0) and the resulting solution chromatographed on a Sephadex G-100 column (2.5 × 94 cm). Fractions having spec. act. > 1200 units/A_{280} were pooled, lyophilized, and dialyzed against 0.02 M KP*i*/10 μM PMSF (pH 7.0).

3. Results

3.1. *Preparation of immobilized enzyme*

Commercially available organomercurial agarose, Affi-Gel 501 (capacity 1.5 μmol/ml), was packed in a 1.5 i.d. × ~18 cm glass column and the column washed with the following solutions in the order: 250 ml 0.05 M sodium acetate buffer (pH 4.8); 200 ml 0.05 M sodium acetate buffer/10 mM $HgCl_2$/20 mM EDTA (pH 4.8); 500 ml 0.1 M KP*i*/0.2 M KCl/1 mM EDTA (pH 6.0), as in [9]. The column was then equilibrated with 0.02 M KP*i* (pH 7.0). About 18 mg protein of dialysate (spec. act. 1440) was applied to the column and allowed to react with relatively slow flow speed at 5°C. The column was washed exhaustively with 0.2 M KP*i* (pH 7.0). The leakage of the enzyme activity was negligible during the washing, although ~ 50% total protein applied was eluted through. The column was faint yellow.

3.2. *Activity by continuous flow of substrate*

To the immobilized enzyme column placed at room temperature (~ 25°C), each 200 ml substrate (285 μM) in 0.2 M Tris–HCl buffer (pH 8.0) was continuously applied with various flow rates and co-existence of the compound which might be expected to affect the enzyme reaction. The production of PLP was estimated by comparing the standard PLP in 0.2 M Tris–HCl buffer (pH 8.0) at 413 nm. Typical conversion ratios are summarized in table 1.

Table 1
Conversion ratio of substrate under various conditions[a]

System (substrate 285 μM)	Flow rate (ml/min)	Av. conversion ratio of 200 ml (%)
PMP	0.91	22.4
PMP	0.10	53.1
PNP	0.91	9.2
PNP	0.10	34.5
PMP + PLP (143 μM)	0.91	8.1
PMP + H_2O_2 (400 μM)	0.67	44.9[b]
PMP	0.67	16.7
PMP + Pi (0.2 M)	0.67	16.7

[a] Experiments were performed in a 1.5 i.d. × 18 cm column using continuous flow method at 25°C, by alternating 200 ml 0.2 M Tris–HCl buffer (pH 8.0), and 200 ml same buffer containing the substrate and/or the compound indicated

[b] Bubbles evolved in the column, the top turned grey, and the enzyme denatured, herewith

3.3. K_m *values for PMP and PNP*

To determine the K_m value for PMP or PNP, an immobilized sample was extruded from the column and an aliquot well suspended in 0.2 M Tris–HCl buffer (pH 8.0) was assayed with various concentrations of substrate under the standard conditions. K_m values for both substrates, calculated according to the least squares method [10], are shown in table 2 in comparison with the data obtained from the most purified soluble preparation (spec. act. 26 000, unpublished data).

3.4. *Other properties of immobilized enzyme*

Optimum temperature determined under the standard conditions was significantly higher than that of the purified soluble enzyme as shown in fig.1. However, optimum pH of the immobilized enzyme when PNP was used as substrate was slightly lower (see table 2). The enzyme column stored in 0.2 M KP*i* (pH 7.0) at room temperature (~ 25°C) remained stable, for > 1 week, and at low temperature (~ 5°C)

Table 2
Comparison of some physical and catalytical properties of immobilized and soluble PMP oxidase

Properties	Enzyme	
	Immobilized	Soluble[a]
K_m[b] (μM)		
PMP	10	18
PNP	1.2	2.7
Relative act. (PNP/PMP oxidase)	0.34	0.28
Optimum pH[c]		
PMP	9.0	9.0
PNP	7.5	8.0
Optimum temp. (PMP and PNP)	55°C	45°C
Half-life, 25°C[d]	4 days	<1 day
PLP inhibition	+	+

[a] Purified enzyme (spec. act. 26 000) was used
[b] Calculated according to the least squares method [10]
[c] Measured in 0.5 pH unit
[d] A 0.3 ml aliquot, of immobilized enzyme and 30 μl aliquot of soluble enzyme, were incubated at 25°C in 3.0 ml 0.2 M Tris–HCl buffer (pH 8.0); after the addition of 50 μg bovine serum albumin and 2 nmol FMN, residual activity was measured under standard assay conditions

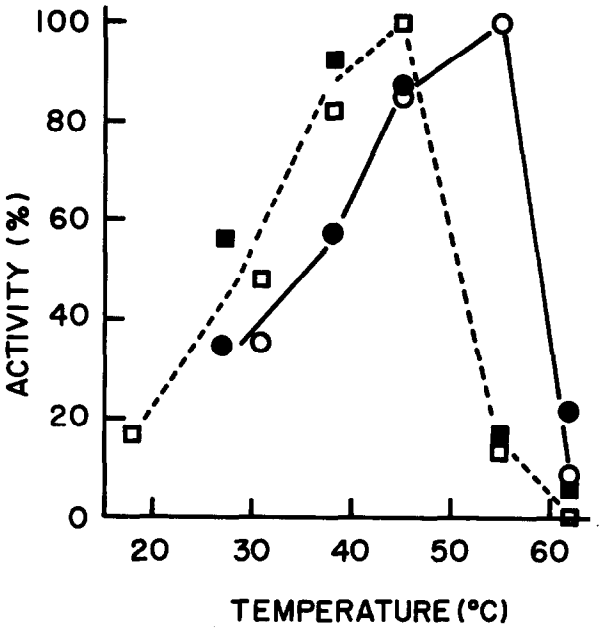

Fig.1. Effect of temperature on immobilized PMP oxidase. An aliquot (0.3 ml, 55 units) of immobilized PMP oxidase suspended in 0.2 M Tris–HCl buffer (pH 8.0) was assayed for 30 min at various temperatures under the standard conditions (———). For comparison, purified soluble enzyme (3 μg protein) was also examined (– – – –). Data are expressed as % activity of the highest value obtained. (○, □) PMP oxidase activity; (●, ■) PNP oxidase activity.

for > 1 month. The enzyme column, inactivated due to the long-term storage, however, was unable to regenerate the activity by the addition of its coenzyme, FMN (unpublished data).

4. Discussion

Pyridoxamine (pyridoxine) 5′-phosphate oxidase immobilized via mercaptide formation through sulfhydryl function(s) is expected to react in situ without affecting its activity, as it has been shown [5,11] that 2 free sulfhydryl groups at the enzyme surface were easily modified without loss of activity, though the enzyme was highly purified from rabbit liver.

Published data [2–4] show that one of the products, PLP, inhibits enzyme activity. This effect was confirmed, too, in the case of continuous flow reaction through the column. The great increase in the conversion ratio on PNP plus H_2O_2 (400 μM), another kind of product, is not so surprising, if mercurial cation at the supporter matrix can catalyze the formation of O_2 from H_2O_2 added. Probably, a higher conversion ratio in the continuous flow than in the usual reaction by the soluble enzyme (~ 40%) reflects the decomposition of H_2O_2 and thus the evolution of O_2.

The soluble enzyme is not so stable in solution. On the contrary, immobilization of the enzyme favours:
(i) Prolonged preservation even at room temperature;
(ii) Heat-stability up to 55°C.

It is also noteworthy that PNP was converted to PLP by continuous flow easier than by the usual reaction, and the immobilization process, in this case, was reversible in recovering the soluble enzyme whenever an appropriate concentration of reducing reagent, e.g., dithiothreitol, was applied to the column [5].

In conclusion, the preparation, and some physical and catalytic properties of immobilized pyridoxamine (pyridoxine) 5′-phosphate oxidase have been described. The immobilized enzyme has characteristics similar to

the soluble form with respect to optimum pH and relative specificity, but, smaller app. K_m values for both substrates, and is more heat stable.

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