EVIDENCE FOR A PYRIDOXINE (PYRIDOXAMINE) 5'-PHOSPHATE OXIDASE FROM WHEAT SEEDLINGS

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1. Introduction

In spite of its great importance in plant biochemistry, the biosynthetic pathway of pyridoxal 5'-phosphate (PLP) in the higher plant remained obscure. Very little information is available concerning the metabolic transformations of vitamin B_6 in the higher plant [1].

We have now detected an enzyme catalyzing the final oxidation step of the conversion of PNP (or PMP) into PLP from day 6–7 wheat seedlings. PLP production by a partially purified enzyme sample, PNP (or PMP) oxidase (EC 1.4.3.5), is shown here.

2. Materials and methods

2.1. *Materials*

Wheat seeds were purchased from a local market. PLP, PMP, pyridoxamine and pyridoxine were purchased from Sigma. PNP was prepared by reduction of PLP with $NaBH_4$ [2]. Apotryptophanase from *E. coli* (Type II) was also purchased from Sigma.

Abbreviations: PLP, pyridoxal 5'-phosphate; PNP, pyridoxine 5'-phosphate; PMP, pyridoxamine 5'-phosphate; PMSF, phenylmethylsulfonyl fluoride

2.2. *Measurement of activity*

PNP (or PMP) oxidase activity was measured by incubating the substrate with the enzyme preparation for 5 h or 10 h at 37°C in 0.2 M Tris–HCl buffer (pH 8.0), as in [3]. PMP (285 μM) was routinely used as substrate.

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 [4] using bovine serum albumin as standard.

3. Results

3.1. *Preparation of acetone powder from wheat seedlings.*

Wheat seeds soaked in water for 5 h were germinated on wet cotton for 6 days at room temperature (<25°C) in the dark. The day 6 seedlings were illuminated overnight under a fluorescent lamp (40 W). On day 7 of germination, the seedlings were harvested, the cotyledons were separated from the root and the endosperm, and were thoroughly washed with water. The cotyledons were homogenized in a Waring Blender in acetone chilled with dry ice. The homogenate was

filtered on a Büchner funnel, residual material was washed with acetone, and with 2–3 vol. ether. The resulting cake was spread over a sheet of filter paper and allowed to dry.

3.2. *Extraction of the enzyme*

From the powdered cotyledon, proteins were extracted with 5 vol. 0.02 M potassium phosphate buffer (pH 7.0) containing 10 μM PMSF at 5°C. After centrifugation, the supernatant was adjusted to pH 5.0 with 2 N acetic acid, and the resulting precipitate was discarded. To the clear supernatant, solid ammonium sulfate was added to 80% saturation, and the solution was allowed to stand for 1 h in a cold room. The protein precipitate was collected by centrifugation, dissolved in a small volume of 0.02 M potassium phosphate buffer (pH 7.0), then dialyzed overnight against 2 changes of the same buffer.

3.3. *DEAE–Sephadex A-50 column chromatography*

The dialyzed sample was applied to a DEAE–Sephadex A-50 column (2.5 X 23 cm), which was equilibrated with 0.02 M potassium phosphate buffer (pH 8.0). The column was eluted with a linear gradient established from 0.02–0.2 M potassium phosphate buffer totaling one liter. Fractions of high specific activity were pooled (fig.1) and lyophilized to dryness.

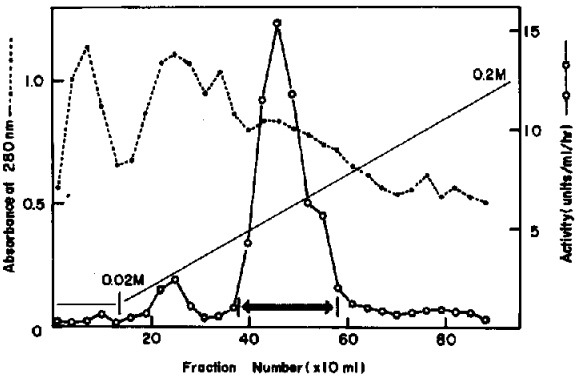

Fig.1. DEAE–Sephadex A-50 column chromatography of plant PNP oxidase. About 700 mg protein was applied to a column. The enzyme activity was assayed by incubating 0.4 ml aliquots of the eluate for 10 h with the substrate in the dark. Other conditions for chromatography and analysis were as in section 3.

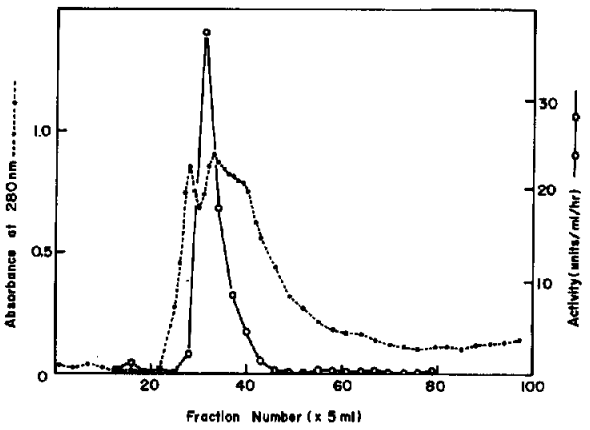

Fig.2. Sephadex G-100 column chromatography of plant PNP oxidase. About 80 mg protein dissolved in 10 ml 0.02 M potassium phosphate buffer (pH 7.0) was applied on a column. The enzyme activity was assayed by incubating 0.3 ml aliquots with the substrate for 5 h in the dark. Other conditions for chromatography and analysis were as in section 3.

3.4. *Sephadex G-100 column chromatography*

The resulting solid material was dissolved in a minimum volume of 0.02 M potassium phosphate buffer (pH 7.0), and the solution, after centrifugation, if necessary, was applied to a Sephadex G-100 column (2.6 X 91 cm) equilibrated with the same buffer. The column was eluted with the buffer, 3 fractions of high specific activity were combined (see fig.2), and used as a partially-purified enzyme sample.

3.5 *Principal properties of the enzyme*

The specific activity of the combined sample was about 71 units/mg protein for PNP and a ratio of PNP oxidase to PMP oxidase activity was about 1.6 at a substrate concentration of 34.3 μM K_m values for PNP and PMP were calculated to be 17 μM and 5 μM, respectively, from Lineweaver-Burk plots. Pyridoxine or pyridoxamine does not serve as substrate in these conditions.

The reaction product of the enzyme was identified as PLP by the apotryptophanase method in [5]. Under anaerobic conditions, bubbled with N_2 gas and sealed tightly, neither PNP nor PMP was converted to PLP.

4. Discussion

Higher plants produce relatively low amounts of PLP. Perhaps, this explains why no reports are availabe on the enzymes involved in the synthesis of PLP in plants. A further difficulty is the interference of plant pigments abundant in the leaf, with the enzyme assay. These pigments turn brown in the incubation mixture during the incubation, or upon the addition of phenylhydrazine supposed to react only with PLP formed. To minimize the browning of the reaction medium, acetone powder was prepared and thus the PLP formed could be detected.

At the early stage of purification, the production of PLP could not be demonstrated unequivocally. After the purification step on DEAE-Sephadex A-50 the production of PLP was clearly demonstrated. The enzyme was shown to be relatively stable at neutral pH. It seems important that the enzyme eluted near the void volume of the Sephadex G-100 column. This suggests that the enzyme is different from that in rabbit liver [2] and baker's yeast [6], especially as to its molecular feature in solution.

The catalytic properties of the plant enzyme resemble those of the enzyme preparations obtained from rabbit liver [2] and baker's yeast [6], except for the relative substrate specificity for PNP and PMP.

References

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