CRYSTALLINE ACID PHOSPHATASE HAVING PYRIDOXINE-PHOSPHORYLATING ACTIVITY

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Recent work from our laboratory reported that pyridoxine-5'-phosphate was synthesized from pyridoxine and various organic phosphates via transphosphorylation in the presence of cells or cell extract of several microorganisms (Ogata et al., 1964; Ogata et al., 1966). It has already been demonstrated that many transphosphorylations were catalyzed by nonspecific and specific phosphatases (Atkinson and Morton, 1960). As yet, only brief allusions to the detailed mechanism of the reaction have been discussed, since few of these phosphatases have been obtained in a pure state. In the course of further investigation on the mechanisms of transphosphorylation, we obtained from the cell extract of Escherichia freundii K-1 the crystalline protein which catalyzed both the hydrolysis of the phosphoryl substrate and the phosphorylation of pyridoxine. The present communication will report the purification and crystallization of this phosphatase.

The cells of Escherichia freundii K-1 were disrupted by ultrasonic oscillations, and then the cell-free extract containing about 340 g protein was obtained. The extract was heated for 10 min at 60°C in a water bath. After centrifugation, the supernatant solution was fractionated with

ammonium sulfate (35-65 % saturation). Further treatment was followed by the addition of one tenth amount of neutralized protamine sulfate of protein content and the precipitate formed was centrifuged off. The supernatant solution was applied to a DEAE-cellulose column which had been equilibrated with 0.01 M phosphate buffer, pH 7.0. The active fractions eluted with the same buffer were combined and concentrated by the addition of ammonium sulfate (80 % saturation). The preparation was fractionated with ammonium sulfate (45-55 % saturation), then applied to a hydroxylapatite column equilibrated with 0.01 M phosphate buffer, pH 6.8. After the column was washed with 0.03 M of the same buffer, the enzyme eluted with 0.1 M of the same buffer containing 0.1 M sodium chloride was combined and concentrated by the addition of ammonium sulfate (80 % saturation). It was then applied to a CM-sephadex column equilibrated with 0.01 M phosphate buffer, pH 6.6. The purified enzyme fractions were

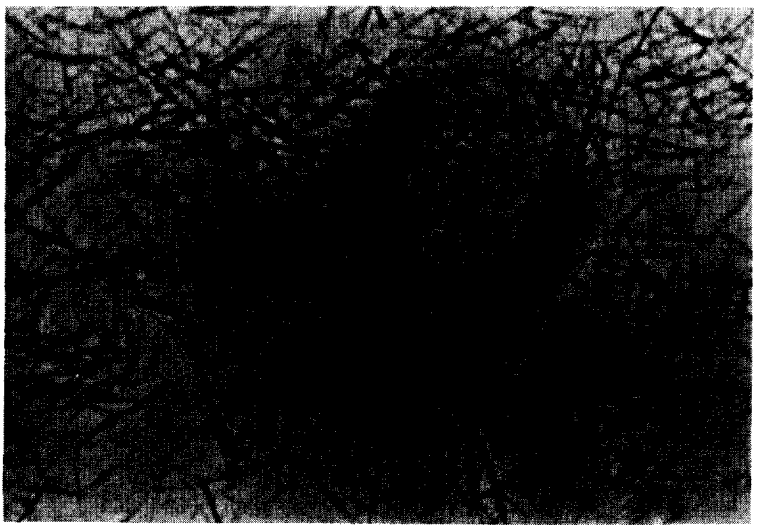

Fig. 1. Microphotograph of Crystalline Acid Phosphatase of E. freundii K-1 (Magnified 600-fold)

obtained by elution with 0.03 M of the same buffer. The active fractions which had approximately 3000-fold specific activity were combined and precipitated by the addition of ammonium sulfate (80% saturation). The precipitate was dissolved in a small volume of 0.03 M phosphate buffer, pH 7.0. The gradual addition of solid ammonium sulfate to the solution made the crystalline enzyme obtainable. Crystals appeared as highly refractive needles of a white color (Fig. 1). This crystals appeared homogeneous upon ultracentrifugation and had a Svedverg constant of 7.5 S (20°C, about 0.7 % protein concentration). A summary of the purification procedure is presented in Table I.

Table I. Purification of Acid Phosphatase Having Pyridoxine-Phosphorylating Activity

The enzyme assays were performed by measurements of p-nitrophenol liberation (Omori, 1937) and pyridoxine-5'-phosphate formation (Ogata et al., 1966) in a reaction mixture containing enzyme, 10 µmoles of pyridoxine hydrochloride, 50 µmoles of sodium p-nitrophenyl phosphate and 200 µmoles of Tris-maleate buffer, pH 6.0, in a total volume of 3 ml. Specific activity was expressed in µmoles of p-nitrophenol per mg of protein per 30 min. The protein was determined by the method of Lowry et al. (1951).

Step	Fraction	Total protein	Total activity	Specific activity	Ratio p-nitrophenol/ pyridoxine-P
I	Cell-free extract	343,300	308,970	0.9	33
II	Heat treatment	58,200	302,640	5.2	35
III	Ammonium sulfate	27,500	299,750	10.9	35
IV	Protamine treatment	18,200	305,760	16.8	35
V	DEAE-cellulose	4,000	228,400	57.1	32
VI	Ammonium sulfate	1,420	116,080	81.7	33
VII	Hydroxylapatite	113	133,815	1184.2	32
VIII	CM-sephadex	18	37,803	2716.1	31
IX	Crystallization	12	33,602	2800.2	32

Although the purification of phosphatase has been tried by many workers, the enzyme has never been crystallized, until recent work on the crystallization of alkaline phosphatases of Escherichia coli (Malamy and Horecker, 1964) and Bacillus subtilis (Takeda and Tsugita, 1967). The crystalline preparation presented herein, having pyridoxine-phosphorylating activity, is an acid phosphatase which showed the optimum pH of 6.0 with p-nitrophenyl phosphate as phosphoryl substrate. The enzyme also hydrolyzed other various phosphates i.e., sugar phosphates, nucleotides, phenyl phosphate and acetyl phosphate, and accompanied the transphosphorylation of pyridoxine under similar pH ranges. Metal ion did not stimulate the catalytic action of the enzyme, although alkaline phosphatase contains metal ion as a functional component (Plocke et al., 1962).

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