RESOLUTION OF ASPARTATE AMINOTRANSFERASE FROM A HIGHER PLANT SOURCE*

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Numerous detailed reports regarding aspartate aminotransferase (EC 2.6.1.1) from animal tissues have demonstrated a requirement for pyridoxal-5-phosphate which acts as a coenzyme. Evidence for this has been largely based on studies with the purified and resolved enzyme (e.g. O'Kane and Gunsalus, 1947; Cammarata and Cohen, 1951; Meister et al., 1954). Although aspartate aminotransferase is known to occur in higher plants (Smith and Williams, 1951; Cook, 1957; Cruickshank and Isherwood, 1958; Patwardhan, 1960; Ellis and Davies, 1961; Fasella et al., 1966), complete resolution of the holoenzyme has not been reported. However considerable indirect evidence suggests that the plant enzyme is pyridoxal phosphate dependent (Cruickshank and Isherwood, 1958; Davies and Ellis, 1961). In this communication the resolution of aspartate aminotransferase from germinating pea cotyledons and its absolute requirement for pyridoxal and pyridoxamine phosphates is demonstrated.

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MATERIALS AND METHODS

Pyridoxal and pyridoxamine 5-phosphates were purchased from Nutritional Biochemicals Corporation. Phosphoric acid-^{32}P in hydrochloric acid was supplied by Atomic Energy of Canada Ltd. Pyridoxal-5-phosphate labelled with ^{32}P was prepared by the method of Sober and Peterson (1954). Radioactivity was determined in a Nuclear Chicago gas flow counter.

Pea seeds (Pisum sativum L. variety Homesteader) were soaked in distilled water for 24 hr at room temperature. They were then germinated in vermiculite at 28° for two days in darkness. For extraction of aminotransferase activity 300 g of the cotyledons were ground in 500 ml of 0.05 M Tris-HCl (pH 7.8) at 2°. The homogenate was centrifuged at 10,000 xg for 20 min and the supernatant brought to 40% saturation with $(NH_4)_2SO_4$. After removal of the precipitated protein by centrifugation, more saturated $(NH_4)_2SO_4$ solution was added to 60% saturation. The protein precipitated was then collected by centrifugation and dissolved in 50 ml of 0.05 M Tris-HCl (pH 7.8). Further fraction of the enzyme was achieved by adding 50 ml of a solution containing 50% $(NH_4)_2SO_4$ in 0.05 M Tris-HCl (pH 7.0). The resulting precipitate was collected by centrifugation, dissolved in approximately 30 ml of 0.005 M Tris-HCl (pH 7.8) and dialyzed for 15 hr against the same buffer.

A sample of the dialyzed extract (300 mg protein) was then applied to a column of DEAE cellulose (2 x 20 cm) which had previously been equilibriated with 0.005 M Tris-HCl (pH 7.8). The enzyme was washed onto the column using 200 ml of this buffer and then eluted using a gradient of Tris-HCl (pH 7.8) up to 0.2 M. Fractions of 5 ml were collected every 2.5 min. Fractions 76-90 which contained the highest specific enzyme

activity were pooled for isolation of the apoenzyme. Resolution of the holoenzyme was achieved using the method of Banks and Vernon (1961) with the exception that the time of incubation at 60° was reduced to 30 min.

Aspartate aminotransferase activity was assayed at 280 mμ (Green et al., 1945). One unit of enzyme activity is defined as the amount of enzyme producing 1 μmole of oxaloacetate per min.

RESULTS AND DISCUSSION

Partial and complete resolution of the enzyme is shown by the data in Table I.

Table I

Effect of Pyridoxal and Pyridoxamine Phosphates on Aspartate Aminotransferase Activity

Treatment	Specific Enzyme Activity (μmoles OAA/min/mg protein) Holoenzyme	Apoenzyme
No coenzyme added	0.46	0
Pyridoxal-5-phosphate	0.51	0.81
Pyridoxamine-5-phosphate	0.51	0.81

Assay conditions: 20 μmoles L-aspartate, 100 μmoles Tris-HCl, 50 μg pyridoxal or pyridoxamine phosphate as indicated and enzyme (0.37 mg protein, holoenzyme or 0.14 mg protein, apoenzyme) were incubated at 37° for 5 min. Reaction was initiated by addition of 10 μmoles α-ketoglutarate. Final volume 3 ml. Final pH 8.0.

Activity of the holoenzyme, after chromatography on DEAE cellulose, was increased by additions of pyridoxal phosphate. However the apoenzyme was found to display an absolute requirement for either pyridoxal-phosphate or pyridoxamine phosphate. The specific enzyme activity of the reconstituted enzyme was approxi-

mately 60 times greater than the initial homogenate. Reconstituting the apoenzyme with pyridoxal-5-phosphate-^{32}P followed by prolonged dialysis resulted in labelling of the protein and reactivation of enzyme activity. Thus indicating that the coenzyme is firmly bound in the reconstituted enzyme.

Several attempts to resolve the holoenzyme by the methods of Wada and Snell (1962); Schirch and Mason (1962); Scardi et al. (1963) and Torchinskii (1963) were unsuccessful. The criterion used for complete resolution being an absolute requirement for pyridoxal or pyridoxamine-5-phosphate in the transamination of aspartate. Failure to resolve the holoenzyme by these methods suggests that the coenzyme is more tightly bound in plant aspartate aminotransferase (Cruickshank and Isherwood, 1958) than occurs in the animal enzyme.

Activity of the apoenzyme as a function of coenzyme concentration is shown in Figures 1 and 2.

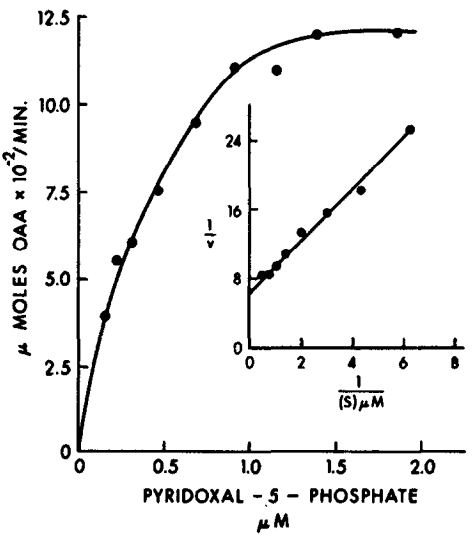

Figure 1. Effect of pyridoxal-5-phosphate concentration on aspartate aminotransferase activity. Assay conditions as in Table I.

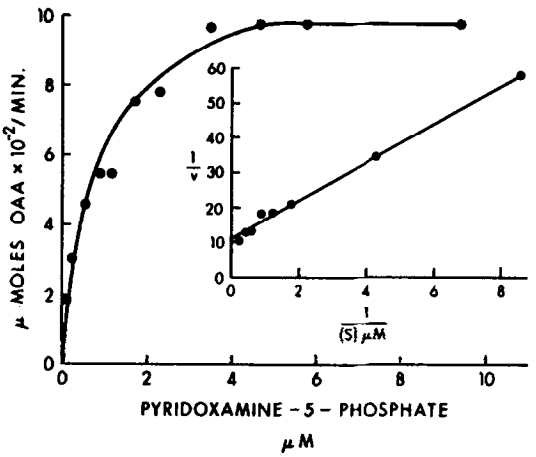

Figure 2. Effect of pyridoxamine-5-phosphate concentration on aspartate aminotransferase activity. Assay conditions as in Table I.

Under the experimental conditions used, the Michaelis constants for pyridoxal phosphate and pyridoxamine phosphate were 5.8×10^{-7} M and 5.3×10^{-7} M respectively. Concentrations of the coenzymes above saturation were not found to be inhibitory. Similarity in the Km values suggests that the plant enzyme can exist in two interconvertible forms as has been shown for the enzyme from pig hearts (Jenkins and Sizer, 1960).

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