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Direct identification and quantification of the cofactor in glutamate semialdehyde aminotransferase from pea leaves

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Glutamate semialdehyde aminotransferase, a key enzyme in the synthetic pathway leading to chlorophyll was purified from pea (*Pisum sativum*) leaves. Although the preparation contained a single contaminant the enzyme could be unambiguously identified as a dimer of subunit molar mass 45 kDa having an absorption spectrum consistent with the presence of pyridoxamine phosphate as cofactor. The cofactor was released by treatment with strong phosphate at low pH and was identified and quantified fluorimetrically. The specific activity of the enzyme (1.4 μmol·min^{-1}·mg^{-1}; 23 nkatal·mg^{-1}) is very much higher than previously reported.

Glutamate semialdehyde; Aminotransferase; Glutamate semialdehyde aminotransferase; Pyridoxamine phosphate

1. INTRODUCTION

The early stages of the pathway leading to the formation of tetrapyrrole for chlorophyll synthesis in plants differ quite markedly from those in animals. In both cases aminolevulinate is an early intermediate. However, in animals, this compound is formed by a condensation of succinyl coenzyme A with glycine [1] whereas in plants it arises from glutamate by steps which involve reduction of glutamyl tRNA and isomerisation of the resulting glutamate semialdehyde [2]. Using barley as source, the enzyme catalysing this isomerisation, glutamate semialdehyde aminotransferase, has been purified from electrophoretic gels in amounts sufficient to allow microsequencing and thereby elucidation of the sequence of the relevant gene [3,4]. Several lines of indirect evidence indicate that the enzyme contains pyridoxal phosphate or pyridoxamine phosphate and that the mechanism involves the interconversion of the cofactor between these two forms [5,6]. However, the presence of the cofactor has not so far been demonstrated directly. This may be because the enzyme has not been purified in sufficient quantity or possibly because of loss of cofactor during purification. In this paper we describe a purification of the enzyme from a new source namely greening pea leaves which, although yielding a preparation still containing one major contaminating protein, provides sufficient of the enzyme to identify and quantify the coenzyme by absorption and fluorescence spectroscopy.

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2. MATERIALS AND METHODS

2.1. *Purification*

The early stages of the preparation follow those published elsewhere [7] for the purification of the same enzyme from barley. Chloroplast stroma was prepared as a 50% extract in 0.1 M tricine, pH 7.9, containing 0.3 M glycerol and 1 mM dithiothreitol (buffer A) from peas grown in the dark for 10 days and exposed to light for 8 h. The extract was treated with ammonium sulphate to provide the fraction precipitating between 45% and 75% saturation. This was redissolved in 10 ml of buffer A and fractionated on a column of Sepharose 6B (5 × 49 cm) equilibrated with the same buffer solution. Active fractions, eluting between 450 ml and 520 ml, were concentrated to 10 ml using an Amicon PM30 filter, passed through a column (2.5 × 12 cm) of Blue Sepharose from which all non-binding protein was collected and applied to a Q-Sepharose Fast flow column (1 × 14 cm). This column was washed with 100 ml of buffer A before eluting with a linear gradient of 0 to 0.5 M NaCl in the same buffer. Active fractions eluting at about 0.2 M NaCl were concentrated to 0.5 ml, dialysed against buffer A and passed through a column of Red Sepharose collecting all non-binding protein. After concentration to 1 ml the preparation was applied to a 1 ml Mono-Q FPLC column, washed with 5 ml of buffer A and eluted with a 20 ml linear gradient of NaCl. The enzyme, eluting at 0.18 ml, was concentrated to 0.2 ml and fractionated on a 24 ml Superose 12 FPLC column (Fig. 1).

2.2. *Preparation of glutamate semialdehyde*

Glutamate semialdehyde, was prepared by ozonolysis of 4-amino-hex-5-enoate (obtained as a gift from Merrell Dow Pharmaceuticals, Egham, Surrey) using the method of Gough et al. [8].

2.3. *Enzyme assay*

Glutamate semialdehyde aminotransferase was assayed using glutamate semialdehyde (50 μM) in 0.1 M tricine, pH 7.9, containing 0.3 M glycerol and 1 mM dithiothreitol. The reaction was stopped after 3 min by adding 50 μl of 35% perchloric acid. After centrifugation, the supernatant was treated with 85 μl of 6 M sodium acetate, pH 4.7, and 30 μl of ethylacetoacetate and kept at 100°C for 20 min. The pyrrole formed was quantified with Erlich's reagent using ϵ_{553} = 11600 l·mol^{-1}·cm^{-1}.

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3. RESULTS AND DISCUSSION

Fig. 1 compares enzyme activity, protein concentration and SDS gel analyses of fractions separated by gel filtration on Superose 12 FPLC during the final stage of the preparation. Prior calibration of the Superose 12 column used with standard proteins showed that all of the material was of molar mass approximately 100 kDa. Analysis of the separate fractions by SDS gel electrophoresis showed the presence of two proteins of very different subunit molar mass. Despite the overlap occurring between the two proteins present in all fractions, enzyme activity is clearly seen to rise and fall simultaneously with the faster running electrophoretic band. Analysis of the SDS gel by scanning the Coomassie Blue stained bands showed that 63% of the fractions with highest specific enzyme activity consisted of the protein of subunit molar mass 45 kDa.

Absorption spectra of all of the fractions from the gel filtration were determined. Fig. 2 shows the spectrum of the fraction with maximal activity (fraction 22). The clear absorbance in the region of 330–350 nm was not present in the enzymically inactive fractions eluting early in the separation but coincided quantitatively with fractions containing enzyme activity. This observation provides positive support for earlier suggestions [6] that pyridoxamine phosphate is a cofactor in the enzyme. However, an absorbance at this wavelength is insufficient in itself to make a definite identification. Pyridoxamine phosphate has a characteristic fluorescence which in other vitamin B_6-dependent enzymes is strongly quenched by its combination with enzyme protein. Separation from the apo-protein relieves this quenching and the characteristic excitation and emission maxima of 330 nm and 390 nm are then detectable. To separate the chromophore the enzyme

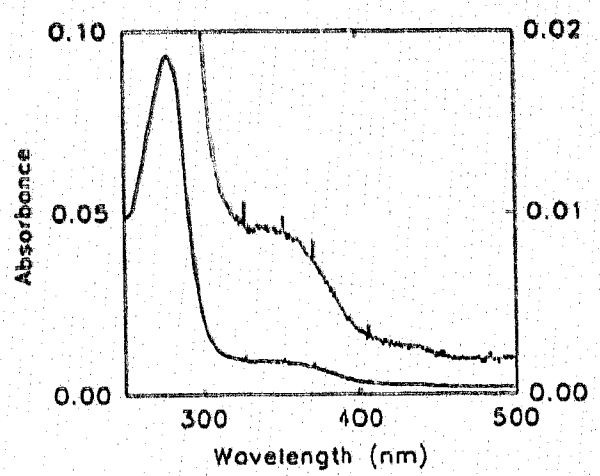

Fig. 2. Absorption spectrum of fraction 22 obtained at the final stage of purification (gel filtration) shown in Fig. 1. The same spectrum is shown but with different absorbance scales.

preparation (2 ml, 5 μg/ml) was maintained in the presence of 0.2 M NaH_2PO_4 (pH 5.5) for 45 min at 20°C. Increase in fluorescence was monitored at constant excitation and emission wavelengths of 330 nm and 390 nm and was seen to follow an apparently first-order process with a half-time of 1.6 min. The sample was separated into high and low molecular weight fractions by forced dialysis through a Centricon PM 30 filter. The emission spectrum of the resulting low molecular weight fraction (Fig. 3) is the same as that of authentic pyridoxamine phosphate.

The amount of pyridoxamine phosphate released was determined from its fluorescence by using a standard solution of the authentic compound. The amount of PMP released (62.8 pmol) from the 6.3 μg of glutamate semialdehyde aminotransferase protein present in the

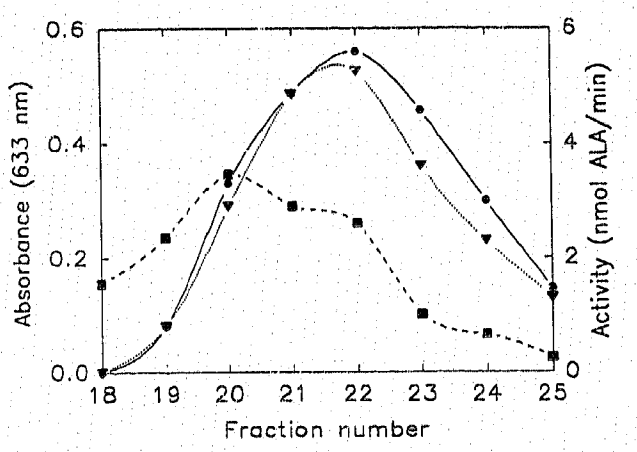

Fig. 1. Fractionation of glutamate semialdehyde aminotransferase by FPLC gel filtration on Superose. Enzyme activity present in each fraction (●) is compared with the amounts of the 45 kDa band (▼) and of the 100 kDa band (■) present in each fraction as determined by scanning each lane of the SDS-polyacrylamide gel.

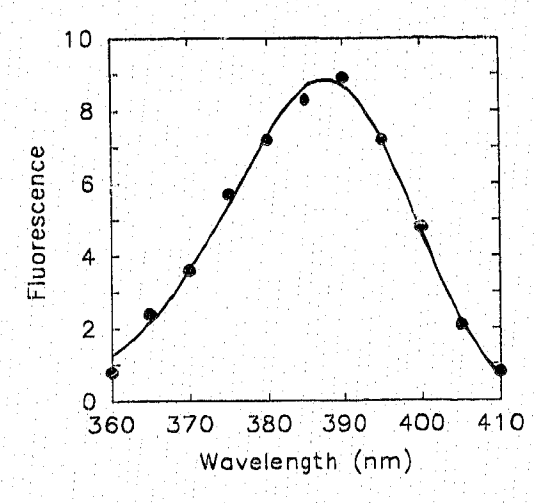

Fig. 3. Fluorescence emission spectrum of low molecular weight material separated from glutamate semialdehyde aminotransferase by forced dialysis. The continuous line through the points is that obtained by best fitting to a log normal expression [13].

sample (molar mass = 45 kDa) corresponds to 44% of the theoretical maximum if all of the coenzyme present was PMP. Possible reasons for the fact that this figure is lower than expected for perfect stoichiometry are that release of the coenzyme was incomplete or that some of the coenzyme was present in an inactive form that absorbs near 340 nm and that has been observed in other vitamin B_6-dependent enzymes [9–11].

The specific enzyme activity of the material used for these analyses was 900 $nmol \cdot min^{-1} \cdot mg^{-1}$ (15 $nkatal \cdot mg^{-1}$). Because the sample was only 63% pure, we estimate that the pure enzyme must have specific activity of approximately 1400 $nmol \cdot min^{-1} \cdot mg^{-1}$ (23 $nkatal \cdot mg^{-1}$). This value is at least 7-fold higher than other values reported for the specific enzyme activity of this enzyme (e.g. [12]).

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