THE ISOLATION OF A LYSINE-SENSITIVE ASPARTATE KINASE FROM PEA LEAVES AND ITS INVOLVEMENT IN HOMOSERINE BIOSYNTHESIS IN ISOLATED CHLOROPLASTS

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1. Introduction

Homoserine is an intermediate in the biosynthesis of the essential amino acids Thr and Met in microorganisms [1] and higher plants [2]. Pea seedlings synthesise massive amounts of Hse, mainly in the roots [3–5], although more recent studies suggest that Hse metabolism also takes place in the leaf [6].

Aspartate kinase (EC 2.7.2.4) catalyses the first reaction in Hse biosynthesis, and is subject to complex feedback regulation by Lys, Thr and Met in microorganisms [7]. In various plants aspartate kinase is also inhibited by the same end products (reviewed [8]), but in pea seedlings the enzyme has only been shown to be inhibited by Thr [9].

This paper reports the light-dependent synthesis of Hse from Asp by isolated pea chloroplasts. The enzyme aspartate kinase has been isolated from pea leaves and chloroplasts and found to be sensitive to both Lys and Thr. We believe this is the first report of a Lys-sensitive aspartate kinase in legume plants.

2. Materials and methods

2.1. *Plant materials*

Plants of *Pisum sativum*, c.v. Feltham First were grown as in [10]. Young shoots (9–11 days old) were harvested after 12 h in the dark, and 30 min illumination.

2.2. *Isolation and characterisation of chloroplasts*

Shoots were cut into very small pieces and placed directly into ice-cold isolation medium (330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 50 mM *N*-tris (hydroxymethyl) methylglycine buffer (pH 8.4) and 4 mM 2-mercaptoethanol) with a tissue : medium ratio of 1:4. The plants were homogenised in a Polytron (PT 1000) for 2–3 s at setting 5, and filtered through two layers of muslin and two layers of 'Miracloth'. Chloroplasts were pelleted by centrifugation at 2000 × *g* for 50 s. The intactness of the chloroplast preparation was determined by measuring potassium ferricyanide-dependent O_2 evolution [11]. CO_2-dependent O_2 evolution was measured as in [10] and chlorophyll assayed by the method in [12].

2.3. *Analysis of the products of Asp metabolism*

Incubations were carried out in 0.4 ml, in a shaking water bath at 20°C illuminated by a 1 KW lamp at 30 cm, the reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid. After centrifugation to remove precipitated protein, the supernatant was washed twice with ether. The pH was adjusted to ~2 and samples purified by cation exchange chromatography [13]. After concentration by rotary evaporation, samples were analysed by two-dimensional thin-layer chromatography [13]. Plates were developed twice in the first dimension, initially in methylethyl ketone:acetone:pyridine:water:formic

Abbreviations: Lys, lysine; Thr, threonine; Asp, aspartate; Hse, homoserine; Met, methionine; Cys, cysteine; EPPS, 4-(2-hydroxyethyl)-1-piperazine propane sulphonic acid; Chl, chlorophyll

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acid (35:35:15:15:2) and then in propan-2-ol:formic acid:water (20:1:5). In the second dimension, plates were run in butan-1-ol:acetone: diethylamine: triethylamine:water (10:10:1:1:5). Hse was identified by co-chromatography with a commercial standard. Radioactivity was determined by a modification of the methods in [14].

2.4. *Isolation and assay of aspartate kinase*

Pea shoots were homogenised in buffer (50 mM potassium phosphate (pH 7.4), 1.0 mM EDTA, 12.5%, v/v, glycerol and 25 mM 2-mercaptoethanol) at 4°C in a 'Polytron' (setting 5), for three bursts of 30 s. The brei was squeezed through four layers of muslin and centrifuged at 15 000 × *g* for 20 min. The supernatant was applied directly to a DEAE-cellulose column equilibrated in the extraction buffer, and the column thoroughly washed until the eluent was clear. The enzyme was eluted from the column with 0.4 M KCl, and all the fractions containing protein (assayed by the method in [15]) pooled and used as the source of aspartate kinase.

Aspartate kinase was assayed by the hydroxamate method [16]. The assay mixture contained 75 mM L-aspartate, 15 mM $MgCl_2$, 30 mM ATP and 225 mM hydroxylamine hydrochloride at pH 7.4; and 1–2 mg protein. Enzyme activity was linear up to a rate of 1 μmol/h at 30°C. All rates quoted were aspartate-dependent, and the product was confirmed by the radioactive assay in [9]. No asparagine synthetase activity [17] could be detected in the extracts.

2.5. *Chemicals*

Radioactively-labelled L-[U-^{14}C]aspartic acid (354 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. All amino acids were obtained from Sigma Chemical Co.

3. Results and discussion

3.1. *Homoserine synthesis in chloroplasts*

All preparations used contained ⩾85% intact chloroplasts capable of CO_2-dependent O_2 evolution of more than 75 μmol/h/mg Chl. Such chloroplasts synthesised Hse from Asp in a light-dependent reaction (table 1). The requirement for light can readily be explained, as one molecule of ATP and two molecules of NADPH are consumed in the conversion of Asp to Hse. Thr at 2 mM inhibited Hse synthesis by 72.2%, as expected since Thr is known to inhibit both aspartate kinase and homoserine dehydrogenase from pea seedlings [9]. The inhibition of Hse formation by lysine suggests the presence of a Lys-sensitive enzyme in the pathway from Asp to Hse. Although there are several reports of Lys-sensitive aspartate kinase in cereals [8] we are not aware of any report of a Lys-sensitive aspartate kinase (or any other enzyme in the pathway) from legumes. The results however, support the observation [18] that a combination of Lys and Thr inhibited protein synthesis in pea chloroplasts. Since this inhibition of protein synthesis was overcome by supplying Met it may be concluded that Lys and Thr combine to prevent the formation of Met.

Table 1
Incorporation of [^{14}C]aspartate into homoserine by isolated pea chloroplasts

Treatment	Aspartate		Homoserine	
	cpm (×10^3) sample	% light control	cpm (×10^3) sample	% light control
Light	1538	100	110.3	100.0
Dark	1753	114	1.5	1.4
Light + Lys	1649	107	59.5	54.0
Light + Thr	1539	100	30.7	28.0
Light + Lys + Thr	1646	107	1.8	1.6

Final vol. 0.4 ml contained 104.5 μg Chl (85% intact chloroplasts), 2 μCi [^{14}C]aspartate, 80 μmol KCl, 20 μmol EPPS (pH 8.3) and 2.6 μmol $MgCl_2$. Lys and Thr were at 2 mM. All solutions were filter sterilised

3.2. *Properties of pea leaf aspartate kinase*

Initial attempts to isolate aspartate kinase from pea leaves were prevented by a component which formed a hydroxamate complex independent of Asp. The use of a DEAE-cellulose column led to a very active preparation with a high specific activity.

The effect of a number of potential feedback inhibitors on the enzyme is shown in table 2. The key point is that a large proportion of the activity is inhibited by Lys as predicted by the Hse labelling data shown in table 1. The additive nature of the Lys

Table 2
The effect of amino acids on aspartate kinase activity in 9 day old pea shoots

Amino acid[a]	% control activity[b]
Lys, 0.5 mM	58
Lys, 5 mM	34
Thr, 5 mM	76
Met, 5 mM	90
Lys, 0.5 mM + Met, 5 mM	30
Lys, 5 mM + Met, 5 mM	28
Thr, 5 mM + Met, 5 mM	64
Lys, 5 mM + Thr, 5 mM	13
Lys, 5 mM + Thr, 5 mM + Met, 5 mM	8
L-Cys, 5 mM	54
D-Cys, 5 mM	57

[a] L-form unless stated
[b] Rate calculated as 0.62 μmol/h/g fresh wt or 0.56 μmol Asp hydroxamate formed h/mg protein

and Thr inhibition suggests that two separate isoenzymes may be present as found in carrot tissue [19,20]. Further evidence in support of the suggestion that the inhibitions by Thr and Lys are due to two separate isoenzymes is that the two sensitivities change relative to each other according to the age of the plant. In 3-day old seedlings, the enzyme is almost solely Thr sensitive [9], a result we have been able to confirm (data not shown). Young rapidly growing shoots contain predominantly the Lys-sensitive enzyme (table 2), but old leaves and pods contain 70–80% Thr-sensitive activity. Young developing cotyledons during rapid protein synthesis also contain a predominantly (90%) Lys-sensitive enzyme. Similarly, in carrot tissue cultures (in which the two isoenzymes have been partially separated) the presence of a large proportion of the Lys-sensitive enzyme is correlated with times of rapid growth [19,20].

There is some suggestion from the results in table 2 of a synergistic action between Met and low concentrations of Lys (0.5 mM) as found for aspartate kinase from barley shoots [21]; the effect was not seen at high concentrations of Lys. Aspartate kinase was also inhibited by L-Cys, but D-Cys had a similar action suggesting that it was a non-specific effect; this conflicts with recent results obtained with a carrot cell enzyme preparation [22].

3.3. *Localisation of aspartate kinase*

The evidence that pea chloroplasts are able to synthesize Hse from Asp suggests that at least part of the total leaf aspartate kinase must be located within this organelle.

Differential centrifugation studies showed that chloroplasts which were 95% intact contained aspartate kinase activity of 2.18 μmol/h/mg Chl, compared to rates of 1.89 μmol/h/mg Chl obtained with crude total homogenates. Aspartate kinase activity co-sedimented with nitrite reductase activity, an enzyme totally located within the chloroplast [23] but did not co-sediment with either catalase (a peroxisome marker) or glutamate dehydrogenase and cytochrome oxidase (mitochondrial marker enzymes). The sensitivity of the chloroplast enzyme to Lys and Thr remained the same as that in the total extract.

We have demonstrated here that pea chloroplasts are capable of synthesising Hse from Asp, despite suggestions [6,24,25] that in pea seedlings Hse may be formed by other routes. Since Hse is a precursor of Met [2], regulation of its biosynthesis may also be expected to affect the rate of Met formation. Thus it may be expected that, at least during periods of rapid growth (e.g., in developing cotyledons), Met production will be subject to regulation by Lys and Thr as has been found in cereals [13]; as Met is the nutritionally-limiting amino acid in legume seeds, this may be considered an important finding.

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