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CHLOROPLAST CYSTEINE SYNTHASES OF *TRIFOLIUM REPENS* AND *PISUM SATIVUM*

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Key Word Index—*Trifolium repens*; white clover; *Pisum sativum*; peas; Leguminosae; cysteine synthase (*O*-acetylserine sulphydrylase); purification and properties; chloroplast localization; sulphide assimilation.

Abstract—About 68–86% of the cysteine synthase activity in leaf tissue of white clover (*Trifolium repens*) and peas (*Pisum sativum* cultivar Massey Gem) was associated with chloroplasts. The enzymes from white clover and peas were purified *ca* 66 and 12-fold respectively. For clover, the K_m values determined by colorimetric and S^{2-} ion electrode methods were: S^{2-} 0.51 and 0.13 mM; *O*-acetylserine (*O*AS), 3.5 and 2.0 mM respectively. The analogous values for the pea enzyme were: S^{2-}, 0.24 and 0.06 mM; *O*AS, 3.1 and 0.24 mM. Both enzymes were inhibited by cystathionine and cysteine. Pretreatment with cysteine inactivated the enzyme, but addition of pyridoxal phosphate caused partial reactivation. Isolated pea chloroplasts (70–75% intact) catalysed *O*AS-dependent assimilation of sulphide at a mean rate of 88 μmol/mg Chl/hr. About 85% of the *O*AS-dependent sulphide assimilated was recovered as cysteine. The rates were unaffected by light and 2 μM DCMU. Sonicating the chloroplasts enhanced the rate by 1.3–2 fold. Cysteine synthase activity was associated with the chloroplast stroma. Similar results were obtained for clover chloroplasts except that both the intactness and the rates were lower.

INTRODUCTION

Cysteine synthase (*O*AS sulphydrylase, EC 4.2.99.8) catalyses the assimilation of inorganic sulphide according to the reaction:

$$O\text{AS} + S^{2-} \longrightarrow \text{Cysteine} + \text{Acetate}$$

The enzyme has been purified from several plant species and some properties studied [1–5]. The subcellular distribution of the enzyme differs between plant species. In wheat leaves, kidney-bean seedlings and rape leaves the enzyme is associated with the soluble fraction [3, 4, 6]. In spinach-leaf, however, cysteine synthase is associated with chloroplasts [7]; chloroplasts are also known to be the main site of SO_4^{2-}, SO_3^{2-} and S^{2-} assimilation into cysteine in this species [8, 9]. With the exception of the pioneering work on the spinach enzyme by Giovanelli and Mudd [5, 10], all subsequent detailed investigations of cysteine synthases have been conducted with enzymes purified from species in which the enzyme is associated with the soluble fraction (cytoplasm) [1–4]. In this paper we report that the cysteine synthase activity of the leaves of *Trifolium repens* (white clover) and *Pisum sativum* (peas) is associated with chloroplasts and some properties of purified clover and pea enzyme are described.

RESULTS

Subcellular localization

The P1 and S1 fractions prepared by extracting pea and clover leaf tissue in iso-osmotic medium and fractionating by differential centrifugation contained *ca* equal amounts of cysteine synthase activity when assayed in iso-osmotic medium by method 1 (Table 1). However, when washed chloroplasts (P2) were sonicated, the total amount of activity *ca* doubled (sum S3 plus P3). The bulk of the activity associated with chloroplasts was recovered in the stroma (S3). The total activity associated with chloroplasts by this method was 68% for clover and 76% for pea (Table 1). In a second approach the enzyme activity of chloroplast stroma (expressed per mg Chl of the chloroplasts from which it was prepared) was compared with the activity of crude extracts prepared in non-iso-osmotic medium; the activity of crude extracts, expressed per total Chl in the leaf tissue, was used as an index of total enzyme activity. The sp. act. of clover and pea stroma respectively were 86 and 78% of the values for crude extracts (cf. chloroplast intactness 56 and 80% respectively) (Table 2).

Table 1. Subcellular distribution of cysteine synthase in white clover and peas

Treatment	Fraction	Cysteine synthase (units/g fr. wt) Clover	Peas
Extraction	P1 unwashed chloroplasts	2.68	3 56
	S1 crude supernatant	4.07 (29 5)	2 76 (23 7)
Wash P1	P2 washed chloroplasts	3.74	4 16
	S2 chloroplast washings	0.43 (3.0)	0 12 (1.0)
Sonicate P2	P3 chloroplast grana	1 59 (11.5)	0.21 (1.7)
	S3 chloroplast stroma	7.72 (56 0)	9.08 (74.6)

Chloroplasts were prepared by method A and enzyme activity measured at 35° by method 1. Values in parentheses denote the percentage distribution.

Table 2. Cysteine synthase activity associated with crude extracts and the stroma from isolated chloroplasts of peas and white clover

Source	Activity of crude extracts (units/ mg protein)	Activity of crude extracts (units/ mg Chl)	Chloroplast intactness (%)	Activity of chloroplast stroma (units/ mg protein)	Activity of chloroplast stroma (units/ mg Chl)
White clover	0.54	6.1	55.7	0.70	5.3
Peas	0.78	7 7	79 8	0.80	6.0

Chloroplasts were prepared by method B and enzyme activity measured at 35° by method 1. Activities per mg Chl are expressed relative to the Chl content of the material from which the appropriate extracts were prepared (stroma from chloroplasts, crude extracts from fresh tissue)

S^{2-} assimilation by intact and sonicated chloroplasts

Pea chloroplasts (70–75 % intact) catalysed S^{2-} consumption at initial rates of 30–50 μmol/mg Chl/hr as measured by method 2. Addition of *O*AS typically caused a 3–4 fold increase in the rate of S^{2-} consumption which remained constant for *ca* 3 min, after which the rate decreased (Fig. 1a). The *O*AS-dependent assimilation of S^{2-} was accompanied by *O*AS-dependent synthesis of cysteine as measured by method 1; for intact chloroplasts, *ca* 85 % of the S^{2-} assimilated was recovered in cysteine (Table 3). Similar results were obtained with sonicated chloroplasts (grana not removed) and chloroplast stroma (grana removed) except that both the rates of *O*AS-dependent cysteine synthesis and *O*AS-dependent S^{2-} assimilation were invariably 1.3–2 fold greater than for intact chloroplasts (Table 3) and the initial rates of *O*AS-dependent S^{2-} assimilation remained constant for a shorter period (Figs 1b and 1c). The rates for S^{2-} assimilation were unaffected by light and 2 μM DCMU (Fig. 1). The effect of concentration of S^{2-} and *O*AS on the rate of cysteine synthase associated with pea chloroplast stroma were studied by methods 1 and 2 (results not shown). The K_m values, calculated from double reciprocal plots of the data for methods 1 and 2 respectively were 2.6 and 0.74mM for *O*AS and 0 35

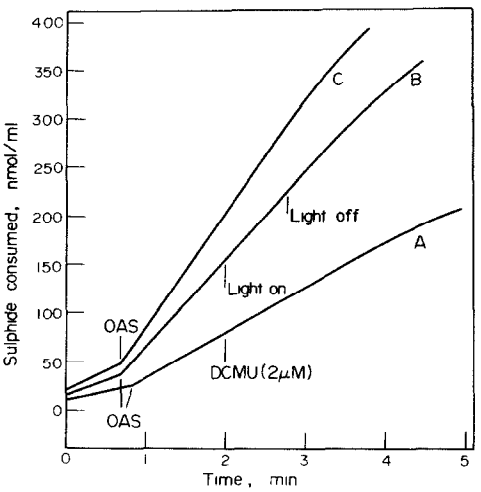

Fig. 1. Assimilation of sulphide by (a) intact pea chloroplasts, (b) sonicated chloroplasts and (c) stroma from sonicated chloroplasts. The two derived fractions (b and c) were prepared from the chloroplasts shown in (a) (74 % intact). Activity was monitored at 28° by method 2, suitably modified for working with chloroplasts, and additions made as shown Incubation mixtures contained 33.5 μg Chl/ml (a and b) or stromal protein derived from an equivalent amount Reaction vol, 1 ml

Table 3. *O*AS-dependent S^{2-} assimilation and *O*AS-dependent cysteine synthesis catalysed by intact and sonicated chloroplasts and chloroplast stroma from peas and clover

Source material	Chloroplast treatment	Endogenous consumption of S^{2-} (μmol/mg Chl/hr)	*O*AS-dependent S^{2-} assimilation (μmol/mg Chl/hr)	*O*AS-dependent cysteine synthesis (μmol/mg Chl/hr)	*O*AS-dep. S^{2-} assim. / *O*AS-dep. cysteine syn (%)
1 Pea chloroplasts (74 % intact)	A 'intact'	47 1	91.6	80.1	87.4
	B 'intact'	35.5	74.8	60.9	81.4
	C 'intact'	29 8	96.1 (87.5)	83.3 (74 8)	86.7 (84 9)
	D sonicated	55.4	193	138	71.6
	E sonicated	38 0	150	95.8	63.9
	F sonicated	35 4	142 (161.5)	104 (112.6)	73 2 (69.6)
	G sonicated, grana removed	73.0	176	129	73 5
	H sonicated, grana removed	64.7	147 (161.5)	110 (119.5)	74.4 (73 9)
2. Clover chloroplasts (56 % intact)	A 'intact'	11.1	52.6	n.d.	
	B sonicated	22.8	95.3	n.d.	
3. Clover chloroplasts (15 % intact)	A 'intact'	6.9	25.3	n.d.	
	B sonicated	26.0	30.1	n.d.	

Chloroplasts were prepared by method B Reaction mixtures were prepared as for method 2 and activity measured at 28° by method 2. In addition, samples were removed after 5 min and activity estimated by method 1. Cysteine synthesis did not occur in the absence of *O*AS. Reaction mixtures for experiments 1, 2 and 3 contained 33.5, 60.0 and 24.3 μg Chl/ml incubation mixture respectively. For experiments 1G and 1H protein derived from chloroplasts containing 33.5 μg Chl/ml was used. All experiments were conducted in the dark. Abbreviation: n.d., not determined. Mean values are shown in parentheses

and 0.12 mM for S^{2-}. Neither intact nor sonicated pea chloroplasts catalysed serine-dependent assimilation of S^{2-} or cysteine synthesis either in the light or in the dark. Preliminary attempts to demonstrate light-dependent consumption of S^{2-} in the presence of coenzyme A, acetate and serine (with and without ADP, PP_i and $MgCl_2$) in intact and sonicated chloroplasts were unsuccessful.

The rates of *O*AS-dependent S^{2-} assimilation for two clover chloroplast preparations are shown in Table 3. The results are qualitatively similar to those described for pea chloroplasts, but the rates appear to be correlated with the intactness of the chloroplast preparations. In general, most clover preparations were only 15–50% intact and exhibited *O*AS-dependent rates for S^{2-} assimilation and cysteine synthesis of 20–45 μmol/mg Chl/hr.

Enzyme purification

Enzyme activity of crude clover extracts was proportional to protein concentration up to *ca* 30 μg protein per assay (method 1); the sp. act. decreased at higher concentrations of protein. Accordingly, not more than 30 μg of protein was used to estimate activity in crude clover extracts by method 1.

A typical purification of clover enzyme is shown in Table 4. With the exception of the gel filtration treatment on Sepharose, each step effected a 3–5 fold increase in sp. act. resulting in an overall purification of 66-fold. Enzyme activity tended to elute at lower concentrations of KCl than the bulk of the protein during ion exchange chromatography on DEAE-cellulose. Various modifications to the conditions for binding and eluting the enzyme during this treatment failed to effect improved separation of enzyme from protein. Fractionation with acetone and protamine sulphate in the initial stages of the purification procedure were unsatisfactory. *O*AS-lyase, homocysteine-forming sulphydrylase and serine sulphyrylase were not detected in either crude extracts or the purified cysteine synthase preparation.

Purification of pea shoot cysteine synthase was not examined in detail. Per g fr. wt, pea shoots frequently contained more activity and less protein than clover leaflets. Thus, although the pea enzyme was purified only 12-fold, the sp. act. (8.4 units/mg protein) was greater than that of some of the more highly purified clover preparations (Table 4).

Stability of purified clover cysteine synthase

The clover enzyme was unstable during all stages of purification, being least stable when purified. The activity of purified enzyme in 50 mM K-P_i buffer decreased by 80–85% after one week at 2°. Storage of the enzyme in *O*AS enhanced the loss of activity (90% loss after 1 week); addition of 0.1 mM pyridoxal phosphate (PyrP) to enzyme stored with *O*AS did not restore activity. Varying the enzyme concentration during storage and adding 5% (w/v) bovine serum albumin (BSA) did not affect the loss of activity. However, the rate of loss of activity was 35% less at −15° and was retarded a further 5% by addition of 5 mM dithiothreitol (DTT). Addition of 5% (w/v) polyethylene glycol (PEG) lessened the loss of activity at 2° (52% loss), but not at −15°. Accordingly, purified cysteine synthase was routinely stored at −15° in 5 mM DTT.

In contrast to the relative cold lability, treatment of purified enzyme at 50° for 20 min and 60° for 5 min caused 0 and 5% loss of activity respectively.

Table 4. Typical purification of cysteine synthase from leaf tissue of white clover

Treatment	Total Protein (mg)	Specific activity (units/mg protein)	Recovery (%)	Relative purification
Dialysed crude extract	3400	0.029	100 0	1
Heat (55° for 2 min)	630	0.153	97 1	5 3
Ammonium sulphate (35–55% satd)	144	0.523	76 8	18.1
DEAE-cellulose	22.6	1 38	31.8	47 8
Sepharose 2B	6 51	1.91	20.2	65.9

Enzyme activity was measured at 35° by method 1.

Properties of clover and pea cysteine synthases

The initial rates (0 to 4 min) catalysed by clover enzyme increased with temperature up to 45°. However, whereas the rates at 25 and 35° were constant for at least 20 min the rates at 35 and 45° decreased after *ca* 5 and 10 min respectively. Accordingly, standard assays were performed for 15 min at 35°. For purified enzyme, activity was proportional to enzyme concentration up to at least 30 μg protein per assay. The optimum pH in K-P_i buffer was 7.8. The optimum pH in Tris–HCl buffer was *ca* 7.6, but this buffer, relative to K-P_i, was *ca* 40% inhibitory.

The activity of purified clover enzyme was not enhanced by 0.01–1 mM PyrP either before or after gel filtration on Sephadex G-25. Similarly, no effect with PyrP could be demonstrated when the enzyme was preincubated with hydroxylamine as described by Smith and Thompson [1]. Dialysis of purified enzyme in the presence of 50 mM L-cysteine followed by dialysis to remove excess cysteine [11] inactivated the enzyme. Preincubation of inactivated enzyme with 0.1 mM PyrP for 10 min restored activity to 23% of the original activity (Table 5). The optimum concentration of PyrP was *ca* 0.1–0.25 mM (Fig. 2).

The effect of concentration of S^{2-} and *O*AS were measured by both the colorimetric and S^{2-} ion electrode techniques (methods 1 and 2 respectively) for the clover (Fig. 3) and pea (data not shown) enzymes. The K_m values, determined from double reciprocal plots of the data

Table 5. Inactivation of purified clover cysteine synthase by cysteine and reactivation by PyrP

Pretreatment	Additions to incubation	Activity (units/mg protein)
Gel filtration	none	0.561
	PyrP (0.1 mM)	0.570
Gel filtration and dialysis against cysteine	none	0.040
	PyrP (0.1 mM)	0.129

Purified enzyme was passed through Sephadex G-25 (gel filtration). A sample of the enzyme was preincubated with 10 mM L-cysteine at 2° for 90 min. Protein was recovered by precipitation with $(NH_4)_2SO_4$ (0.561 g/ml), redissolved in buffer and dialysed against medium containing 10 mM K-Pi buffer pH 7, 1 mM Na_2EDTA and 50 mM L-cysteine for 12 hr followed by 12 hr dialysis against the same medium without cysteine. PyrP was added to the enzyme where indicated 10 min prior to initiating the assay.

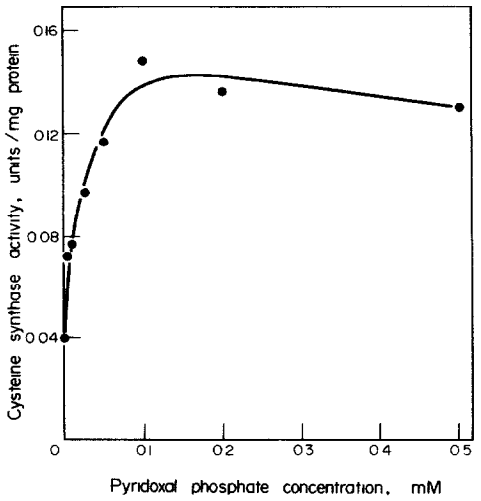

Fig. 2. Effect of concentration of PyrP on the reactivation of purified clover cysteine synthase inactivated with L-cysteine (see Table 5). Enzyme activity was measured by method 2.

are summarized in Table 6. Purified clover enzyme did not catalyse the formation of a product which reacted with the Gaitonde reagent 2 (method 1) when *O*AS was replaced with L-serine, L-homoserine, *N*-acetyl-L-serine, *O*-phospho-L-serine, *O*-acetyl-L-homoserine or *O*-succinyl-L-homoserine (each 10 mM). When clover enzyme was incubated with *O*AS-[^{14}C] and S^{2-} (method 3) and samples subjected to TLC in solvent II, a labelled compound indistinguishable from cysteine was detected. The reaction product was confirmed as cysteine by electrophoresis. When one of the thiols of ethane, 1-propane, 2-propane and 1-butane were supplied to method-3 assays instead of S^{2-}, the enzyme catalysed the synthesis of the ethyl, 1-propyl, 2-propyl and 1-butyl *S*-substituted derivatives of cysteine respectively as determined from analysis of reaction mixtures by TLC in solvent I.

Various metabolites affected the rate of cysteine synthase activity as measured by method 1 (Table 7). L-Cystathionine and L-cysteine strongly inhibited the enzyme activity; the effect of cysteine concentration is shown in Fig. 4. The following compounds had no significant effect on cysteine synthase activity: Na_2SO_4,

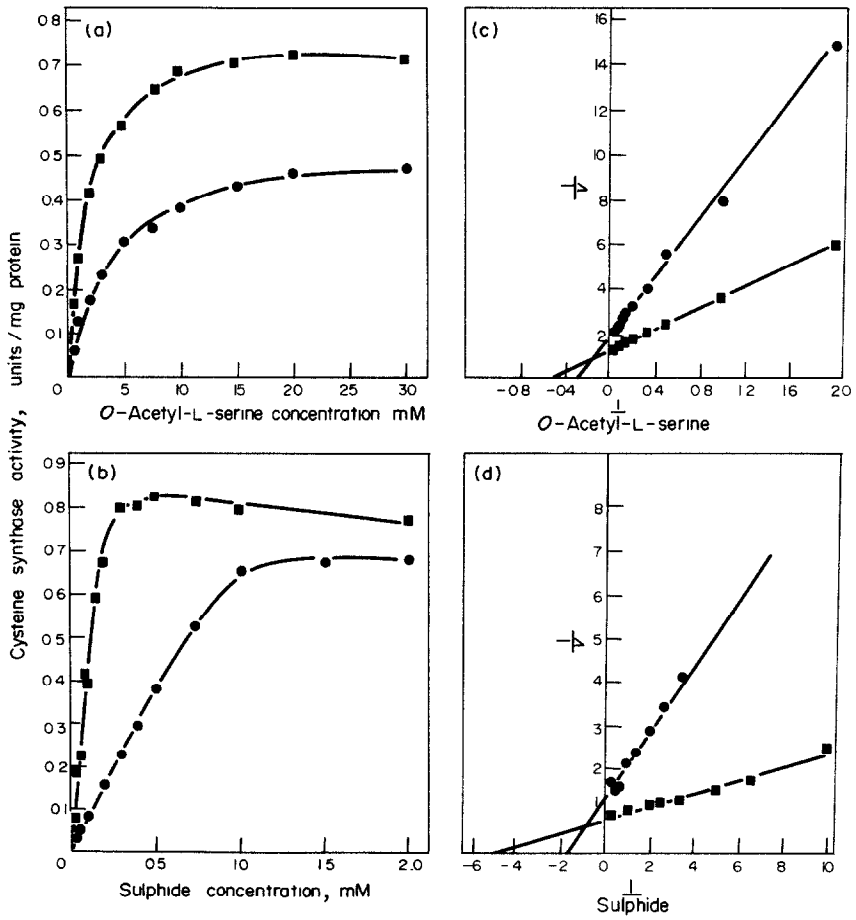

Fig. 3. Effect of concentration of (a) *O*AS and (b) S^{2-} on the activity of purified clover cysteine synthase as measured by (●) method 1 and (■) method 2. Double reciprocal plots of the data in (a) and (b) are shown in (c) and (d) respectively. The concentrations of S^{2-} in (a) and *O*AS in (b) was 1 and 20 mM respectively.

Table 6. K_m values for purified cysteine synthases from various sources

Source	Assay	K_m(OAS)	$K_m(S^{2-})$	Reference
Wheat leaf	colorimetric	0.37 μM	0.59 mM	[3]
Rape leaf	colorimetric	6.1 mM	—	[4]
Rape leaf	sulphide electrode	1.74 μM	0.43 mM	[2]
Radish root	colorimetric	2.17 mM	—	[12]
Salmonella	colorimetric	5.0 mM	<0.1 mM	[13]
	spectrophotometric	0.6 μM	—	
White clover leaf	colorimetric	3.5 mM	0.51 mM	this paper
	sulphide electrode	2.0 mM	0.13 mM	
Pea shoots	colorimetric	3.1 mM	0.24 mM	
	sulphide electrode	0.24 mM	0.06 mM	

Na_2SeO_4, Na_2SO_3, L-serine, *O*-acetyl-L-homoserine, *O*-succinyl-L-homoserine, *O*-phospho-L-serine (each 10 mM) and adenosine 5′-sulphatophosphate (5 mM). In addition, the following compounds caused less than 8% inhibition of activity: $MgCl_2$, EDTA, ATP and ADP (each 10 mM).

Table 7. Inhibition of clover cysteine synthase by various metabolites

Compound	Inhibition (%) Method 1	Method 2
L-Homoserine	n.d.*	16.1
L-Methionine	28.0	20.9
L-Homocysteine	12.6	8.9
L-Cystathionine	n.d.*	91.2
L-Cysteine	n.d.*	66.0
N-Acetyl-L-serine	17.4	20.6
3′-AMP	16.0	n.d.
5′-AMP	11.0	n.d.

All metabolites were tested at 10 mM. Incubation conditions for method 1 and method 2 assays were as described in Experimental, except that both assays contained 20 mM *O*AS and 1 mM S^{2-}. Asterisks denote that the compound tested interfered with the Gaitonde reagent used in method 1 assays. (n.d.: not determined).

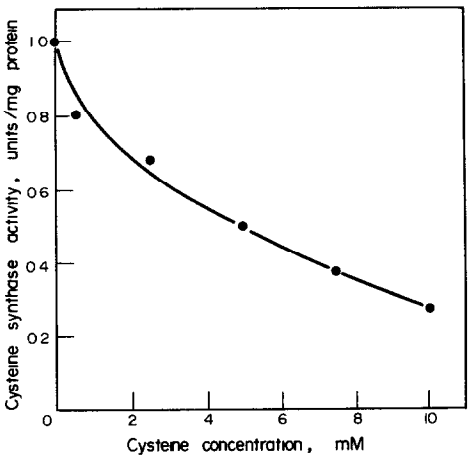

Fig. 4. Effect of concentration of cysteine on clover cysteine synthase activity. Enzyme activity was measured by method 2.

DISCUSSION

The subcellular fractionation study (Table 1) and the comparison of the activity of chloroplast stroma (corrected for Chl) with that of crude extracts (Table 2), indicate that at least 68–86% of the cysteine synthase activity of clover leaf and pea shoot tissues is associated with chloroplasts. We presume that the chloroplast membrane limits the rate of entry of *O*AS and/or sulphide since sonicating the chloroplasts invariably enhanced the rate of cysteine synthase activity (Fig. 1, Table 3). The association of the enzyme with the chloroplasts of clover and peas is consistent with that for spinach [7] and the incorporation of SO_4^{2-} and SO_3^{2-} into cysteine by chloroplasts of this species [8, 9], but is inconsistent with the localization of cysteine synthase in the cytosol of wheat, kidney bean and rape leaf tissue [3, 4, 6]. Perhaps one reason why negligible activity was detected in the chloroplasts of the latter species could be that the chloroplast membranes are impermeable to *O*AS and/or S^{2-} under the conditions employed. Alternatively, spinach, peas and, to a limited extent, clover, might yield chloroplast preparations with higher intactness, thereby causing greater retention of the stromal enzyme. However, we are unable to rule out the possibility that at least some cysteine synthase of clover and peas is associated with the cytosol (Tables 1 and 2) though at no stage during the purification of the clover enzyme from crude extracts (prepared in non-iso-osmotic medium) did we detect any consistent evidence for the existence of more than one enzyme. In one experiment (out of a total of 50) a slight tendency for cysteine synthase to resolve into two peaks during chromatography on DEAE-cellulose was noted. Subsequent investigation by gel electrophoresis showed no discernible differences.

The cysteine synthase of both intact and ruptured chloroplasts was unaffected by light and DCMU implying that the light reactions were not involved in S^{2-} assimilation. Since chloroplasts did not catalyse cysteine synthesis when *O*AS was replaced with L-serine (method 1) or serine-dependent consumption of S^{2-} (method 2), we conclude that the reaction catalysed by cysteine synthase acts as the major route for incorporation of S^{2-} into cysteine in clover and pea chloroplasts. Relative to the rates of carbon and nitrogen assimilation processes of chloroplasts [14, 15], the rates we observe at 28° for *O*AS-dependent S^{2-} assimilation by sonicated clover and pea chloroplasts and stroma (80–160 μmol/mg Chl/hr) are extremely high. This is in general agreement with the value of 123 μmol/mg Chl/hr reported for cysteine synthesis by spinach chloroplasts [7]. These rates,

however, are far in excess of those for SO_4^{2-} and SO_3^{2-} reduction by spinach chloroplasts (typically 0.5–3 μmol/mg Chl/hr [8, 16]) and suggest that the activity of cysteine synthase under the conditions employed in our experiments is not rate-limiting with respect to SO_4^{2-} and SO_3^{2-} assimilation.

The pH optimum, cold lability, heat stability, substrate specificity and the K_m (S^{2-}) we report for the purified clover enzyme are similar to those reported for the purified cytoplasmic enzymes from rape, kidney bean and wheat [1–4]. The K_m (*O*AS) values previously reported for the cysteine synthases from various sources using a range of methods differ greatly. In general, K_m (*O*AS) values estimated by the less sensitive colorimetric methods are *ca* 1000-fold greater than those determined by more sensitive techniques (Table 6), although the very low value for the wheat loaf enzyme [3] would appear to be well beyond the sensitivity of the colorimetric method employed. For the purified clover and pea enzymes and pea chloroplast stroma, however, we found that although the K_m (*O*AS) values determined with the S^{2-} ion electrode were consistently lower than those measured with the colorimetric method, both values were relatively high in comparison with the values obtained for enzymes from other sources using sensitive methods (Table 6). This then raises the question whether plants contain separate cysteine synthases with high and low affinity for *O*AS (possibly in different parts of the cell) and/or whether the affinity of plant enzymes is altered by a modifier. The proposal that chloroplasts possess separate mechanisms for the production of free and bound sulphite involving sulphite and thiosulphonate reductases respectively [17] might be relevant here; differences in the kinetics described in Table 6 might reflect the form of sulphide incorporated.

Clover cysteine synthase was not obtained in sufficiently pure form to establish that it contained PyrP. However, the partial restoration of activity by PyrP following inactivation by dialysis against cysteine (Table 5, Fig. 2) suggests that PyrP was required for the reaction and was present in purified enzyme.

Cystathionine, an intermediate in the synthesis of methionine from cysteine [18], inhibited cysteine synthase activity, but methionine itself caused little inhibition at 10 mM (Table 7). Cysteine also inhibited the reaction. These metabolites could, therefore, be important in regulating S^{2-} assimilation. However, the very high rates of cysteine synthase activity of chloroplasts relative to the rates of cysteine synthesis from SO_4^{2-} and SO_3^{2-} [8, 16] would suggest that if the enzyme is important in regulating cysteine synthase *in vivo* then the substrates and modifiers would need to be present at concentrations which would permit the enzyme to operate at *ca* 0.6–4 % of its maximum potential activity.

EXPERIMENTAL

Plant material. White clover (*Trifolium repens*) was raised in a glasshouse and the leaflets used immediately after harvest. Peas (*Pisum sativum* cv Massey Gem) were soaked in H_2O for 24 hr (1.6 ml/g dry seeds) and raised in shallow trays of a potting mixture (coarse sand–vermiculite–peat moss, 1.1.1 v/v) in a growth cabinet at 20° with a 12 hr photoperiod. The seeds/seedlings were watered regularly for 8 days and thereafter watered with 10 mM KNO_3. Pea shoots were harvested 12 to 15 days after imbibition.

Chemicals. *O*AS, *O*-acetyl-L-homoserine and L-cystathionine were purchased from Calbiochem. DTT, PyrP and *O*-succinyl-L-homoserine were obtained from Sigma. *O*-Acetyl-L-serine-[^{14}C] was prepared as per [19]. All other chemicals were purchased from commercial sources and were of the highest purity generally available.

Extraction and purification of clover cysteine synthase. Leaf tissue was extracted at 2° in a bottom-drive blendor using 0.2 g fr. wt per ml in 0.1 M K-P_i buffer, pH 7 containing 1 mM Na_2EDTA and 1 mM DTT. After squeezing through muslin, the supernatant soln was recovered by centrifuging at 20000 *g* for 15 min. The crude extract was treated at 55° for 2 min, rapidly cooled to 2° and insoluble material removed by centrifuging (20000 *g* for 15 min). $(NH_4)_2SO_4$ (0.209 g/ml of extract) was added and precipitated protein discarded. Further $(NH_4)_2SO_4$ (0.129 g/ml) was added and the precipitated protein was redissolved in medium 1 (50 mM K-P_i buffer pH 7 containing 1 mM Na_2EDTA and 1 mM DTT). The soln was dialysed against the same medium, applied to a DEAE-cellulose column (28 × 2 cm), equilibrated with medium 1, flushed with medium 1 (150 ml), and eluted with a linear gradient of K-P_i buffer (50–500 mM, pH 7) containing 1 mM Na_2EDTA and 1 mM DTT. Active fractions were passed through a Sepharose 2B column (80 × 3.5 cm) equilibrated with medium 1. Fractions containing cysteine synthase activity were combined, adjusted to 5 mM DTT and stored at −15°. This prepn, which was free from glutathione reductase activity, was used as the source of purified enzyme. Crude extracts were dialysed against medium 1 before estimating cysteine synthase activity.

Purification of pea-shoot cysteine synthase. The following method is an abridged version of [20] for the purification of glutathione reductase and is not specifically designed for the purification of cysteine synthase. Pea shoots were extracted with a bottom-drive blendor in 50 mM Tris–HCl buffer pH 8 (1 g fr wt/3 ml) and the supernatant soln recovered by centrifugation (17000 *g* for 45 min). $(NH_4)_2SO_4$ (0.313 g/ml) was added and pptd material discarded. Additional $(NH_4)_2SO_4$ (0.214 g/ml) was added and pptd protein was recovered and dissolved in 50 mM Tris HCl pH 8. The soln was fractionated with Me_2CO at −3° as per step 4 in ref [20]. Material precipitating between 34–48 % v/v Me_2CO was dissolved in 0.1 M Tris–HCl pH 8 and dialysed against the same buffer. The soln was applied to a DEAE-cellulose column equilibrated with 0.1 M Tris–HCl pH 8 and, after flushing, the column eluted with a linear gradient of KCl (0–300 mM) in the same buffer. Fractions containing cysteine synthase also contained glutathione reductase activity.

Preparation of chloroplasts. Method-A chloroplasts were prepared from clover leaflets and pea shoots according to ref. [21] and method-B chloroplasts as ref. [15]. Ruptured chloroplasts were prepared by sonicating for 1 min with a Rapidis 600 Sonicator (Ultrasonics Ltd., England) and chloroplast stroma was recovered by centrifuging sonicated chloroplasts at 12000 *g* for 20 min. Cysteine synthase activity of chloroplasts and derived fractions was measured by methods 1 and 2 except that the chloroplasts were incubated in the iso-osmotic media described in [21] and [15] for method A and B chloroplasts respectively. In addition K-P_i buffer and DTT were omitted from method 1 assays and K-P_i was omitted from method 2 incubation mixtures. Chloroplast intactness was determined as per [22] and Chl was measured in EtOH [23].

Assay of cysteine synthase. Three methods were used. Method 1 (colorimetric). Incubations were conducted at 35° in small capped test tubes. Reaction mixtures (1 ml) contained (in μmol) *O*AS (20), Na_2S (1), K-P_i buffer pH 7.8 (200), DTT (1) and enzyme. Reactions were started by addition of Na_2S and terminated after 15 min with 0.2 ml of 1.5 M TCA. Cysteine was estimated with reagent 2 as per [24]. The colorimetric method of [25] was unreliable. Cysteine synthase activity is expressed as μmol of cysteine synthesized/min (cysteine synthase units). Method 2. S^{2-} consumption was monitored with a S^{2-} ion electrode [2] (model no. 94-16A, Orion Research Inc., Cambridge, Mass.) Incubation mixtures (1 ml) contained (in μmol) *O*AS (10), Na_2S (1), K-P_i buffer pH 7.8 (200) and enzyme. Solns of

*O*AS and S^{2-} were prepared shortly before each expt. *O*AS was prepared in 100 mM K-Pi buffer pH 5 and S^{2-} in 100 mM K-P_i buffer pH 11.5 containing 10 mM Na_2EDTA. Reactions were initiated with enzyme and the EMF recorded for 1 min. S^{2-} assimilation was calculated from a standard curve constructed as in ref. [2] and activity was corrected for non-enzymic loss of S^{2-}. Activity is expressed as *O*AS-dependent assimilation of S^{2-} in μmol/min (cysteine synthase units). Method 3. Reaction mixtures (1 ml) were as described for method 1, except that 1 μmol *O*AS-[^{14}C] (1 μCi/μmol) was used. Incubations were conducted at 35° and terminated after 30 min with 0.2 ml 1.5 M TCA. Samples were removed for analysis by TLC and paper elctrophoresis (PE) and reaction products detected by autoradiography (TLC) or with a gas-flow strip detector (PE). Method 3 was also used to examine the synthesis of *S*-substituted cysteines by replacing S^{2-} with 5 μl of one of the thiols of ethane, 1-propane, 2-propane and 1-butane.

Protein. Protein in crude extracts, $(NH_4)_2SO_4$ fractions, chloroplasts and chloroplast fractions was measured (after precipitating with TCA and washing with Me_2CO) as in ref. [26]. More purified protein was measured as in ref. [27].

TLC and paper electrophoresis. TLC was performed on Si gel plates (20 × 20 cm) using the following solvents: I. MeCOEt–Py–H_2O–HOAc (70:15:15:2) [28]; II. $CHCl_3$–EtOH–HOAc–H_2O (25:16:5:4) [25]. Compounds were detected with ninhydrin spray, UV fluorescence quenching or autoradiography [28]. Electrophoresis was conducted on Whatman 3 MM paper for 3–4 hr at 45 V/cm in HCO_2H–HOAc buffer pH 2 [28]. Electrophoresis strips were monitored for radioactivity with a gas flow scanner.

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