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CYSTEINE DESULPHYDRASE ACTIVITY IN HIGHER PLANTS: EVIDENCE FOR THE ACTION OF L- AND D-CYSTEINE SPECIFIC ENZYMES

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Key Word Index—*Cucurbita pepo*; Cucurbitaceae; *Nicotiana tabacum*; Solanaceae; cysteine catabolism; cysteine desulphydrase; D-cysteine; hydrogen sulphide; L-cysteine; subcellular localization; sulphur metabolism.

Abstract—Emission of hydrogen sulphide in response to D-cysteine by leaf discs of cucurbit plants or cultured tobacco cells was considerably smaller than in response to L-cysteine. Whereas hydrogen sulphide emission from L-cysteine was inhibited by 100 μM aminooxyacetic acid (AOA), emission from D-cysteine was unaffected. These results from *in vivo* studies were found to be inconsistant with the L- and D-cysteine desulphydrase activities measured in crude homogenates. *In vitro*, D-cysteine desulphydrase activity was more than one order of magnitude higher than L-cysteine desulphydrase activity; L-cysteine desulphydrase was inhibited by 100 μM AOA to a smaller, D-cysteine desulphydrase to a higher extent than *in vivo*. Cystine lyase activity, which may interfere in the cysteine desulphydrase assay, was not found. In cucurbit leaves, the differences between *in vivo* and *in vitro* experiments can partially be explained by differences in the influx of L- and D-cysteine into the leaf discs. Influx of L-cysteine proceeded at a rate about four times higher than the influx of D-cysteine; it was inhibited by 100 μM AOA, whereas influx of D-cysteine was unaffected. Subcellular distribution of L- and D-cysteine desulphydrase was analysed in cultured tobacco cells. Both enzyme activities were found to be soluble. The D-cysteine activity was predominantly localized in the cytoplasm whereas L-cysteine activities were also found in chloroplasts and mitochondria. The L-cysteine desulphydrase in the cytoplasmic fraction may entirely be due to broken chloroplasts and mitochondria. Inhibitor studies with ammonium, pyruvate, AOA and O-acetylserine revealed considerable differences between L- and D-cysteine desulphydrase activity and between L-cysteine desulphydrase activity in chloroplasts and mitochondria. Therefore, the present data suggest that degradation of L- and D-cysteine are catalysed by different enzymes in different compartments of the cell.

INTRODUCTION

Enzymatic desulphydration is a major path for the degradation of sulphur-containing amino acids in living cells. Methionine is decomposed by soil microorganisms [1–3] and by leaf tissue of cucurbit plants [4] to methanethiol and its oxidation product dimethyl disulphide; methionine lyase activity (EC 4.4.1.11) catalysing the liberation of methanethiol from methionine by an α,γ-elimination reaction (I) is found in bacteria [5–9] and several fungi [10, 11]. Desulphydration of cysteine and the presence of enzymes synthesizing hydrogen sulphide from cysteine by an α,β-elimination reaction (II; cysteine desulphydrase, EC 4.4.1.1) have been reported in numerous bacterial [12–16] and higher plant cells [*cf.* 17–19]. Recently, a cystine lyase degrading cystine, cysteine, *O*-acetylserine and several *S*-substituted cysteines by an α,β-elimination reaction (III) has been partially purified from cabbage [20]. From this finding it was concluded that cystine lyase, cysteine desulphydrase and *O*-acetylserine lyase are all activities of a single enzyme catalysing α,β-elimination reactions [20]. In the present investigation evidence is presented that in cucurbit and in tobacco cells cysteine is not degraded by a cystine lyase, but by different enzymes with L- or D-cysteine desulphydrase activity.

I $MeSCH_2CH_2CHNH_2COOH + H_2O \rightarrow MeSH + MeCH_2COCOOH + NH_3$

II $HSCH_2CHNH_2COOH + H_2O \rightarrow H_2S + MeCOCOOH + NH_3$

III $RCH_2CHNH_2COOH + H_2O \rightarrow RH + MeCOCOOH + NH_2$

R: –SH, –S–Cys, –OCOMe.

RESULTS

Degradation of L- and D-cysteine by cucurbit and tobacco cells

Hydrogen sulphide emission by leaf discs from cucurbit plants and by photoheterotrophic tobacco suspension cultures was not only observed in response to L-cysteine, but also in response to D-cysteine (Table 1). The average rate of emission in response to D-cysteine calculated for leaf discs of cucurbit plants over a 3 hr period and for tobacco suspensions over a 22 hr period was 30%, and 18%, respectively, of the rate observed with L-cysteine. At 100 μm AOA, an inhibitor of pyridoxalphosphate-dependent enzymes [21], hydrogen sulphide emission from cells treated with L-cysteine was inhibited more than 90% in the case of *N. tabacum* and 75% in *C. pepo*; H_2S emission in response to D-cysteine was not significantly altered by this compound (Table 1). These observa-

Abbreviations: AOA, aminooxyacetic acid; OAS, *O*-acetylserine.

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Table 1. Hydrogen sulphide emission by cucurbit and tobacco cells *in vivo*

	C. pepo [nmol/hr/g fr. wt (%)]	*N. tabacum* [nmol/hr/g fr. wt (%)]
L-Cys	65.6 ± 7.4 (100 ± 11)	554.5 ± 79.1 (100 ± 14)
L-Cys + AOA	16.7 ± 3.1 (25 ± 5)	45.5 ± 2.7 (8 ± 0.5)
D-Cys	19.8 ± 2.6 (100 ± 13)	100.0 ± 16.4 (100 ± 16)
D-Cys + AOA	18.6 ± 4.9 (94 ± 25)	90.9 ± 3.6 (91 ± 4)

Ten leaf discs from 4–6-week-old cucurbit plants were exposed to 10 ml 10 mM L- or D-cysteine in 100 ml Erlenmeyer flasks; exponential phase tobacco cells were inoculated under sterile conditions into 5 ml culture medium in 25 ml Erlenmeyer flasks containing 0.8 mM L- or D-Cysteine. AOA was added at 100 μM final concentration. After 3 hr (cucurbit cells) or 22 hr (tobacco cells) of incubation hydrogen sulphide in the headspace of the flasks was determined with a flame photometric sulphur analyser.

Table 2. Cysteine desulphydrase activity in cucurbit and tobacco cells

	C. pepo [nmol/min/g fr. wt (%)]	*N. tabacum* [nmol/min/g fr. wt (%)]
L-Cys	0.55 ± 0.02 (100 ± 4)	0.13 ± 0.02 (100 ± 12)
L-Cys + AOA	0.23 ± 0.04 (42 ± 7)	0.06 ± 0.01 (46 ± 8)
D-Cys	8.98 ± 0.86 (100 ± 10)	5.76 ± 1.31 (100 ± 23)
D-Cys + AOA	4.76 ± 0.42 (53 ± 5)	1.75 ± 0.46 (30 ± 8)

12.5 g fr. wt leaves of 4–6-week-old cucurbit plants, exponential phase tobacco cells, were homogenized in 50 ml 0.1 M K-phosphate, pH 7.5. L- and D-Cysteine desulphydrase activity was determined in the presence or absence of AOA (100 μM final concentration) with a flame photometric sulphur analyser.

tions on hydrogen sulphide emission in response to L-cysteine and D-cysteine with intact cells are only partially consistant with cysteine desulphydrase activities determined *in vitro* (Table 2). In crude homogenates of cucurbit and tobacco cells both L- and D-cysteine-specific desulphydrase activities were found. D-Cysteine desulphydrase activity was, however, more than one order of magnitude higher than L-cysteine desulphydrase activity. Desulphydration of L-cysteine was inhibited *in vitro* by 100 μm AOA to a much smaller extent than in intact cells, desulphydration of D-cysteine to a greater extent. Degradation of cystine was not observed in the homogenates, as based on the generation of pyruvate in response to this sulphur source [20]. Hence, cystine lyase activity that may interfere in the cysteine desulphydrase assay, was not present in the homogenates.

The differences between the results of *in vivo* and *in vitro* experiments may partially be explained by differences in the influx of L- and D-cysteine into the cells. The net influx of cysteine into leaf discs of cucurbit plants was calculated from the decline in the cysteine concentration in the treatment solution (Fig. 1). L-Cysteine influx (91 μmol/hr /g fr. wt) was found to proceed at a rate almost four times higher than the influx of D-cysteine (25 μmol/hr/g fr. wt). Therefore, a low rate of influx may explain the low rate of H_2S emission from cucurbit leaf discs in response to D-cysteine despite the high D-cysteine desulphydrase activity present in the leaf. The influx of L-cysteine does, however, not explain the high rate of H_2S emission at low L-cysteine desulphydrase activities. AOA apparently did not affect the influx of L-cysteine into the leaf discs within the first 30 min of incubation, but completely inhibited this process at longer periods of exposure; the influx of D-cysteine was not affected at 100 μm AOA. Therefore, the inhibition of H_2S emission from L-cysteine as well as the lack of inhibition of H_2S emission from D-cysteine by AOA in *in vivo* experiments may be explained by an effect of this compound on the influx of L- and D-cysteine rather than an effect on L- and D-cysteine desulphydrase activity.

Subcellular distribution of cysteine desulphydrase activity

On centrifugation of crude homogenates of cucurbit leaves and photoheterotrophic tobacco cells at 100000 *g*

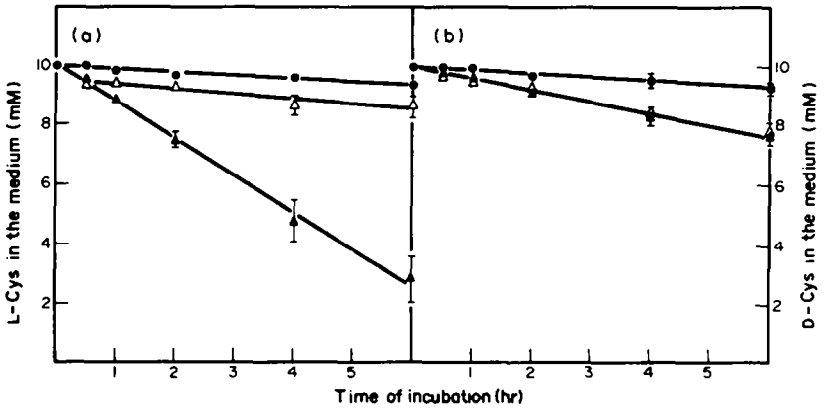

Fig. 1. Effect of AOA on L- and D-cysteine influx into leaf discs of cucurbit plants. Ten leaf discs of 4–6-week-old cucurbit plants were exposed to 10 ml 10 mM L- (a) or D-cystein (b) in the presence (Δ–Δ) or absence (▲–▲) of 100 μM AOA in 100 ml Erlenmeyer flasks. Autoxidation of cysteine was determined in controls without leaf discs (●–●). After the periods of incubation indicated 0.1 ml aliquots of the treatment solutions were removed and diluted with 0.1 M Tris–HCl, pH 7.0. The cysteine-thiol content was determined spectrophotometrically with DTNB. The absence of error bars from a symbol shows that they were smaller in size than the symbol used.

both L- and D-cysteine D-cysteine desulphydrase activity were almost exclusively found in the supernatant fraction (Table 3). This indicated that the degradation of L- and D-cysteine was catalysed by soluble enzymes. Subcellular localization of cysteine desulphydrase activities was investigated by differential centrifugation and monitored by electron microscopy and marker enzymes (Table 4). These experiments were only performed with cultured tobacco cells and not with cucurbit leaves, as the chloroplasts of cucurbit leaves were completely destroyed during centrifugation by the high amounts of assimilatory starch present. Even a dark incubation of the plants for 72 hr did not sufficiently reduce the starch content of the chloroplasts. Glucose-6-phosphate dehydrogenase was used as a cytoplasmic marker, cytochrome-*c*-oxidase as a mitochondrial marker, chlorophyll ($a+b$) and phosphoglyceraldehyde dehydrogenase (NADP-dependent) were used as markers for chloroplasts. D-Cysteine desulphydrase activity declined rapidly during the centrifugation procedure so that only *ca* 60% of the activity in the homogenate was recovered after centrifugation at 600 *g* and 40 000 *g*. The enzyme was predominantly found in the 40 000 *g* supernatant, but small amounts of D-cysteine desulphydrase activity were also observed in the chloroplastic (600 *g* pellet) and mitochondrial (40 000 *g* pellet) fractions. The low level of activity in the 600 *g* pellet may be due to cytoplasmic contamination as shown by the presence of glucose-6-phosphate dehydrogenase activity in this fraction. As low levels of activity of this cytoplasmic marker have been reported in preparations of chloroplasts [22–24], the presence of very low levels of D-cysteine desulphydrase activity in the chloroplasts cannot be excluded. The low D-cysteine desulphydrase activity in the 40 000 *g* pellet is still significantly higher than the activity of the cytoplasmic marker glucose-6-phosphate dehydrogenase. Consequently, small amounts of D-cysteine desulphydrase activity may be present in the mitochondria.

The subcellular distribution of L-cysteine desulphydrase activity was found to be entirely different from the distribution of the D-cysteine specific enzyme. High levels of L-cysteine desulphydrase activity were found in the 600 *g* pellet, 40 000 *g* pellet, and in the 40 000 *g* supernatant as well. The 600 *g* pellet contained almost half of the homogenate's chlorophyll, but only 25% of its NADP-dependent phosphoglyceraldehyde dehydrogenase activity. From this observation it was concluded that about half of the chloroplasts in the 600 *g* pellet were intact; this assumption was consistant with the results from electron micrographs [*cf.* 25] of the 600 *g* fraction, where 50% of the chloroplasts appeared to be intact. The finding of substantial amounts of L-cysteine desulphydrase activity in the 600 *g* pellet showed that a considerable amount of the activity of this enzyme was present in the chloroplasts. Cytochrome-*c*-oxidase activity in the 40 000 *g* pellet ac-

Table 3. Distribution of L- and D-cysteine DSH activity at 100 000 *g* centrifugation

	Activity [nmol/min/g fr. wt (%)]			
	C. pepo		*N. tabacum*	
	L-Cys DSH	D-Cys DSH	L-Cys DSH	D-Cys DSH
Crude homogenate	0.55 ± 0.02 (100 ± 4)	8.98 ± 0.86 (100 ± 10)	0.13 ± 0.02 (100 ± 12)	5.76 ± 1.31 (100 ± 23)
100 000 *g* supernatant	0.56 ± 0.07 (102 ± 13)	8.34 ± 0.78 (93 ± 9)	0.11 ± 0.01 (85 ± 7)	5.03 ± 0.71 (87 ± 12)
100 000 *g* pellet	0.009 ± 0.004 (2 ± 1)	0.12 ± 0.01 (1.3 ± 0.1)	0.01 ± 0.01 (7 ± 7)	0.09 ± 0.02 (2 ± 0.3)

Photoheterotrophic tobacco cells or leaves of *Cucurbita pepo* cv Small Sugar Pumpkin (12.5 g fr. wt) were homogenized in 50 ml 0.1 M K-phosphate (pH 7.5) for 2 min, filtered and centrifuged for 1 hr at 100 000 *g*. L- and D-Cysteine desulphydrase activity were determined with a flame photometric sulphur analyser.

Table 4. Subcellular distribution of cysteine desulphydrase activity

	D-Cys DSH* (%)	L-Cys DSH† (%)	Glu-6- DH‡ (%)	GAPDH§ (%)	Cyt-*c*-oxidase‖ (%)	Chlorophyll $a+b$¶ (%)
Homogenate	100 ± 23.4	100 ± 8.5	100 ± 16.7	100 ± 13.6	100 ± 7.1	100 ± 3.8
600 *g* pellet	9.5 ± 2.2	34.6 ± 3.4	6.25 ± 3.9	25.4 ± 4.9	0	58.1 ± 1.9
40 000 *g* pellet	7.7 ± 2.7	26.7 ± 3.6	2.5 ± 0.4	0.5 ± 0.2	59.5 ± 14.9	48.6 ± 2.8
40 000 *g* supernatant	43.7 ± 3.9	46.5 ± 5.2	93.1 ± 3.0	169.5 ± 13.2	14.3 ± 2.2	5.7 ± 1.9

* 100% D-cysteine desulphydrase: 4.39 nmol/min/g fr. wt.
† 100% L-cysteine desulphydrase: 0.24 nmol/min/g fr. wt.
‡ 100% glucose-6-phosphate dehydrogenase: 6.00 μmol/min/g fr. wt.
§ 100% phosphoglyceraldehyde dehydrogenase: 5.90 μmol/min/g fr. wt.
‖ 100% cytochrome-*c*-oxidase: 4.20 μmol/min/g fr. wt.
¶ 100% chlorophyll $a+b$: 52.5 μg/g fr. wt.

Photoheterotrophic tobacco cells (12.5 g fr. wt) were homogenized for 15 sec in a blender, filtered and subjected to a centrifugation at 600 *g*. The supernatant was centrifuged at 40 000 *g*. L- and D-Cysteine desulphydrase activities in the fractions were determined with a flame photometric sulphur analyser. Marker enzymes and chlorophyll content were measured spectrophotometrically.

counted for *ca* 60% of the homogenate's total activity of this enzyme. As cytochrome-*c*-oxidase activity declined during the centrifugation procedure, only 74% of the activity of the homogenate was recovered. Consequently, at least 60% of the mitochondria remained intact during the cell fractionation. The high L-cysteine desulphydrase activity in the 40000 *g* pellet may, therefore, be an indication for high L-cysteine desulphydrase activity in the mitochondria. L-Cysteinedesulphydrase activity in the 40000 *g* supernatant may be a consequence of broken chloroplasts and broken mitochondria and not an indication of a cytoplasmic enzyme. A reliable estimation of the relative distribution of L-cysteine desulphydrase activity among chloroplasts and mitochondria is, however, impossible from the present data. This is because the activity of the enzyme in the homogenate increased during the first 2 hr after homogenization, then significantly declined. Further experiments with isolated chloroplasts and mitochondria are necessary to provide such an estimation.

Inhibitor studies

From the above observations on the subcellular distribution of L- and D-cysteine desulphydrase activity it was concluded that degradation of L- and D-cysteine were catalysed by different enzymes in different compartments of the cell. This conclusion was supported by experiments on the effect of several inhibitors of L- and D-cysteine desulphydrase activity. In these experiments, inhibitors of D-cysteine desulphydrase were tested in crude homogenates and 40000 *g* supernatants; the effect of the same compounds on L-cysteine desulphydrase was analysed in crude homogenates, 600 *g* and 40000 *g* pellets (Figs 2 and 3). The products of cysteine desulphydration, ammonium and pyruvate, inhibited both L- and D-cysteine desulphydrase activity (Fig. 2). With increasing pyruvate concentration inhibition of D-cysteine desulphydrase activity was the same in crude homogenates and 40000 *g* supernatants. At concentrations between 10 and 30 mM, ammonium enhanced D-cysteine desulphydrase activity, but inhibited the enzyme at higher concentrations. With increasing ammonium concentration desulphydration of D-cysteine in crude homogenates and 40000 *g* pellets followed the same pattern, although inhibition was slightly smaller in the 40000 *g* supernatant. As similar observations were made with other inhibitors, namely AOA and OAS at μM concentrations (Fig. 3), D-cysteine desulphydrase in crude homogenates and 40000 *g* supernatants did not show remarkable differences with regard to the inhibitors tested.

Differences in the effect of inhibitors were observed between the L-cysteine specific activities in crude homogenates, 600 *g* and 40000 *g* pellets. Whereas L-cysteine desulphydrase activity in crude homogenates and 40000 *g* pellets was inhibited by pyruvate (Fig. 2) and AOA (Fig. 3)

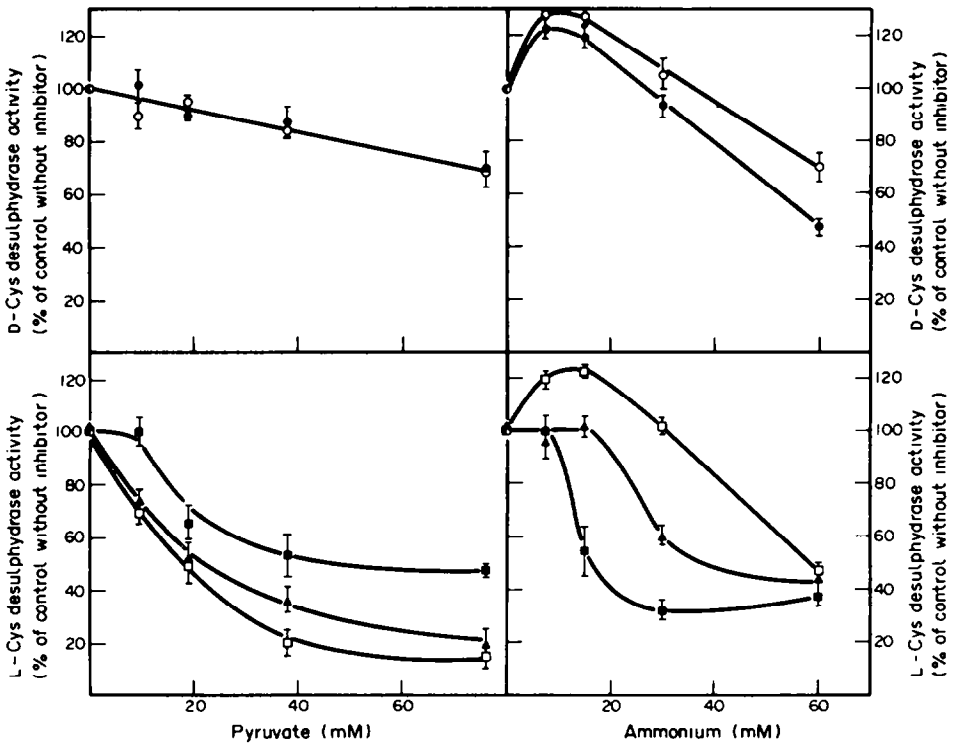

Fig. 2. Effect of ammonium and pyruvate on L- and D-cysteine desulphydrase activity of cultured tobacco cells. Photoheterotrophic tobacco cells (12.5 g fr. wt) were homogenized in 50 ml 0.03 M KPi buffer (pH 7.5) with 4 g Polyclar AT (Serva) for 15 sec in a blender, filtered and subjected to a centrifugation at 600 *g*. The supernatant was centrifuged at 40000 *g*. L- and D-Cysteine desulphydrase activity in the fractions were determined at the pyruvate or ammonium concentrations indicated with a flame photometric sulphur analyser. D-Cysteine desulphydrase: o–o crude homogenate; ●–● 40000 *g* supernatant. L-Cysteine desulphydrase: □—□ crude homogenate; ■—■ 600 *g* pellet; ▲–▲ 40000 *g* supernatant.

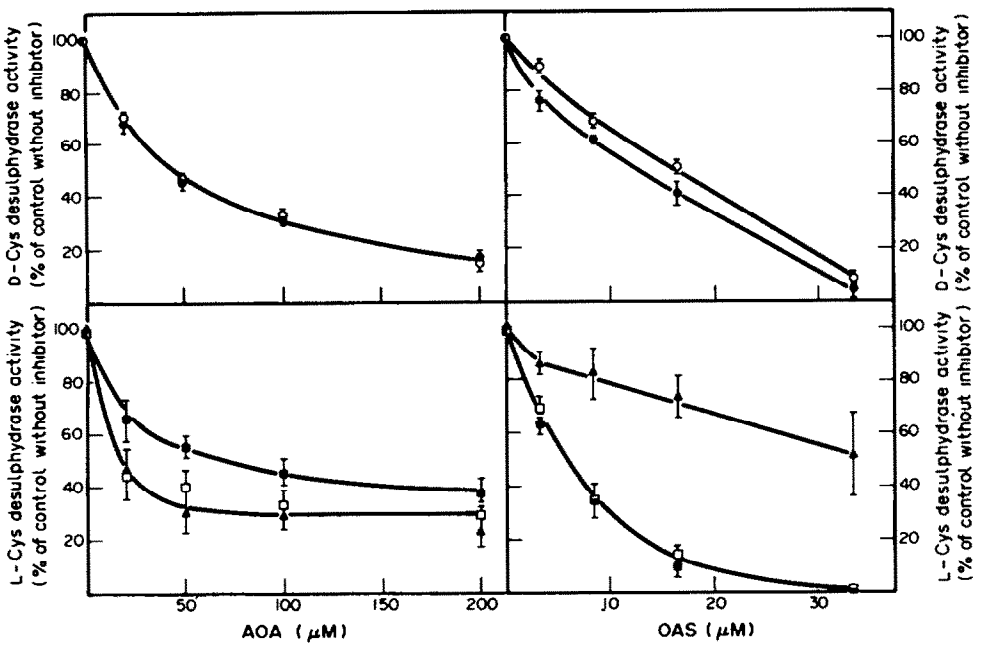

Fig. 3. Effect of AOA and OAS on L- and D-cysteine desulphydrase activity of cultured tobacco cells. Photoheterotrophic tobacco cells (12.5 g fr. wt) were homogenized in 50 ml 0.03 M KPi buffer (pH 7.5) with 4 g Polyclar AT (Serva) for 15 sec, filtered and subjected to a centrifugation at 600 *g*. The supernatant was centrifuged at 40 000 *g*. L- and D-Cysteine desulphydrase activity in the fractions were determined at the AOA or OAS concentrations indicated with D-Cysteine desulphydrase: o–o crude homogenate; ●–● 40 000 *g* supernatant. L-Cysteine desulphydrase; □–□ crude homogenate; ■–■ 600 *g* pellet; ▲–▲ 40 000 *g* supernatant.

in much the same way, the L-cysteine specific activity in the 600 *g* pellet was reduced by these compounds to a smaller extent. OAS inhibited L-cysteine desulphydrase activity in crude homogenates and 600 *g* pellets much stronger than in 40 000 *g* pellets (Fig. 3). Ammonium concentrations of 10–30 mM enhanced L-cysteine desulphydrase activity in crude homogenates; higher concentrations inhibited the activity in this fraction. With the 600 *g* and 40 000 *g* pellets stimulation of L-cysteine desulphydrase activity by ammonium was not observed; inhibition of the enzyme at higher concentrations was much stronger with the 600 *g* than with the 40 000 *g* pellet. These data clearly show that L-cysteine desulphydrase activity in 600 *g* and 40 000 *g* pellets respond to several inhibitors in an entirely different way. It may therefore be concluded that degradation of L-cysteine in chloroplasts and mitochondria is catalysed by different L-cysteine desulphydrases. Further investigations with enzyme preparations from isolated chloroplasts and mitochondria will show whether these enzymes also exhibit different catalytic and physical properties.

DISCUSSION

The present experiments show that leaf cells of cucurbit plants as well as cultured tobacco cells emit hydrogen sulphide not only in response to L-cysteine but also in response to D-cysteine. In previous experiments, performed in a flow-through system [26], emission of hydrogen sulphide in response to D-cysteine was not observed. In this system, dilution of the hydrogen sulphide emitted may have reduced its concentration below the detection limit of the sulphur analyser. The finding of hydrogen sulphide emission in response to both enantiomers of cysteine *in vivo* is consistent with *in vitro* experiments performed in the present investigation and by other authors [*cf.* 17, 27, 28] showing that L- and D-cysteine desulphydrase activity is present in plant cells. The lack of synthesis of pyruvate from L-cystine under the conditions used for the determination of cysteine desulphydrase activity rules out the possibility that an unspecific cystine lyase in tobacco or cucurbit cell homogenates is responsible for the observed desulphydration of L- and D-cysteine. Such a C–S lyase, degrading cystine, L- and D-cysteine, *O*-acetylserine and several *S*-substituted cysteines by an α,β-elimination reaction has recently been purified from cabbage [20]. C–S lyases from other Brassica species exhibited a higher substrate specificity and did not degrade cysteine [*cf.* 29].

The present studies on the subcellular localization of cysteine desulphydration in tobacco cells suggest that L- and D-cysteine are degraded by different enzymes in different compartments of the cells: D-cysteine desulphydrase is a soluble, cytoplasmic enzyme and L-cysteine desulphydrase a soluble enzyme predominantly localized in the chloroplasts and mitochondria. Inhibitor studies with different fractions of tobacco cells further revealed differences between chloroplastic and mitochondrial L-cysteine desulphydrase. Therefore, tobacco cells apparently contain at least three different L- or D-cysteine desulphydrases in different compartments of the cells (Fig. 4). These observations are consistent with previously published experiments with homogenates from spinach leaves, where L- and D-cysteine desulphydrase activity were separated by column chromatography [28].

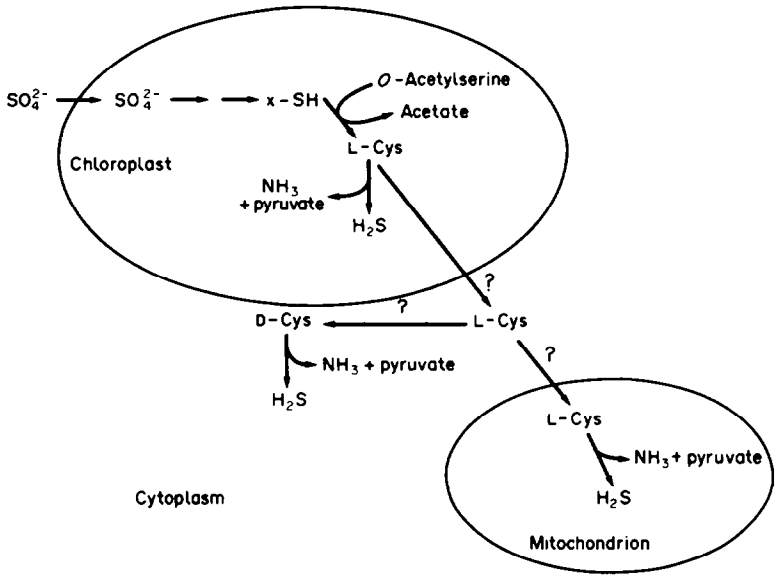

Fig. 4. Proposed paths of cysteine desulphydration in green cells of higher plants.

At present the physiological role of cytoplasmic D-cysteine desulphydrase in plants is obscure as the origin of the substrate of this enzyme is unknown. D-Cysteine may, however, be produced by a cysteine racemase from L-cysteine in a similar way to that in which D-alanine is synthesized from L-alanine in pea seedlings [30]. Such a racemase may provide an additional regulatory device to coordinate the flux of L-cysteine into protein synthesis and into its degradative path, when excess L-cysteine is present in the cytoplasm (Fig. 4).

EXPERIMENTAL

Plant material. Seeds of cucurbit plants (*Cucurbita pepo* L. cv Small Sugar Pumpkin) were planted directly into a mixture of soil and compost and watered daily with tapwater. Plants were grown for 4–6 weeks in a greenhouse with night temps of 20° and day temps of 22–30°. Fertilization was provided weekly with Stodiek blue (Stodiek, Löhne) or Polycrescal (Schering, Berlin) fertilizer. The plants were kept at a day-length of at least 15 hr; in the winter, additional illumination was provided by HRI lamps (400 W; Osram) at 15–25 $\mu E\,m^{-2}\,sec^{-1}$. The plants used in the experiments had seven to nine visible leaves.

The photoheterotrophic suspension culture of *Nicotiana tabacum* L. var. 'Samsun' used in the present investigation originated from a callus culture isolated by Bergmann [31]. Suspensions were grown in a modified Murashige and Skoog medium [32] and kept at 25 ± 2° and 60–70% air humidity under continuous illumination (48–56 $\mu E\,m^{-2}\,sec^{-1}$; fluorescent lamps Philips type TL 40W/55, TL 65W/29 or Astra 40W white 25). Suspensions were subcultured in 1l. Erlenmeyer flasks containing 300 ml medium; every 10 days of culture 20 ml suspension were transferred into 300 ml fresh medium.

In vivo experiments. Ten leaf discs (0.1 cm; 10.8 mg fr. wt each) from 4–6-week-old cucurbit plants were exposed to 10 ml 10 mM L- or D-cysteine (pH 6.0) in 100 ml Erlenmeyer flasks. Exponential phase tobacco cells (60 mg fr. wt) were inoculated under sterile conditions into 5 ml culture medium in 25 ml Erlenmeyer flasks containing 0.8 mM L- or D-cysteine as sole sulphur source. Flasks were sealed as previously described [19]. After 3 hr (cucurbit discs) or 22 hr (tobacco cells) of incubation flasks were rapidly connected [18] to a flame photometric sulphur analyser (Monitor Labs, San Diego, Cal., U.S.A.) to determine the emission of volatile sulphur. Previous experiments [18, 26] revealed that the volatile sulphur emitted into the headspace of the flasks was entirely H_2S.

Cysteine desulphydrase activity. 12.5 g fr. wt leaves of 4–6-week-old cucurbit plants or exponential phase tobacco cells were homogenized in 50 ml 0.1 M K-phosphate, pH 7.5, for 2 min in a blender with the equipment described by Kannangara *et al.* [32]. When tobacco cells were used, 4 g Polyclar AT (Serva) were added to the buffer at least 12 hr prior to its use in the homogenization procedure; alternatively, 1% (w/v) ascorbic acid was added immediately before homogenization. The crude homogenates were filtered (Selecta 520b 1/2; Macherey, Nagel & Co., Dueren) and stored for 60 min at 4°. Enzyme activity was almost doubled during this time, then remained stable for several hours. L- and D-cysteine desulphydrase activity were determined from the rate of H_2S emission in response to L- or D-cysteine as previously described [33].

Influx experiments. Ten leaf discs (0.1 cm; 10.8 mg fr. wt each) from 4–6-week-old cucurbit plants were exposed to 10 ml 10 mM L- or D-cysteine, pH 6.0, in the presence or absence of 100 μM AOA in 100 ml Erlenmeyer flasks. The cysteine content of the treatment soln was analysed over a period of 6 hr in 0.1 ml aliquots. The aliquots were diluted with 0.1 M Tris–HCl, pH 7.0, and the thiol content determined spectrophotometrically with DTNB [34]. Exponential phase tobacco cells (60 mg fr. wt) were exposed to 5 ml culture medium with 0.8 mM L- or D-cysteine as sole sulphur source. The cysteine content of the treatment soln was analysed in 0.05 ml aliquots as described above.

Differential centrifugation. Exponential phase tobacco suspensions were filtered, the cells washed with sulphur-free Murashige and Skoog medium [19] and suspended (12.5 g fr. wt tobacco cells/50 ml buffer) in 0.03 M KPi buffer (4 mM $MgCl_2$, 0.3 M sucrose, 0.1% BSA), pH 7.5. 4 g Polyclar AT (Serva) was added to the buffer at least 12 hr before the experiment was started. Suspensions were homogenized for 15 sec in a blender with the equipment described by Kannangara *et al.* [32], filtered (Selecta

520b 1/2; Macherey, Nagel & CO., Dueren) and subjected to differential centrifugation as previously described [25]. Pellets were resuspended in 0.1 M KPi, pH 7.5.

Marker enzymes. Chlorophyll ($a+b$) was extracted with 90% Me_2CO and determined spectrophotometrically as described by Jeffrey and Humphrey [35]. NADP-dependent phosphoglyceraldehyde dehydrogenase activity was measured in the back reaction as reported by Latzko and Gibbs [36] with the modifications that DTT was used instead of GSH and that 45 U 3-phosphoglycerate kinase were present in the enzyme assay. Glucose-6-phosphate dehydrogenase determination was a modification of the method of Pearce and Carr [37]: synthesis of NADPH was analysed spectrophotometrically in an assay mixture containing 400 μmol KH_2PO_4, 10 μmol $MgCl_2$, 0.5 μmol NADP and 10 μmol glucose-6-phosphate in a total vol. of 3 ml at pH 7.0. The reaction was started by addition of glucose-6-phosphate. Cytochrome-*c*-oxidase was determined as previously described [38, 39] with the following modification: cytochrome *c* (50 mg/100 ml 0.01 M KPi, pH 7.0) was reduced by addition of sodium dithionite. Excess dithionite was removed by dialysis; the reduced cytochrome *c* soln was stored at −25°. Enzymatic oxidation of cytochrome *c* was measured spectrophotometrically at 550 nm in an assay mixture containing 1 ml 0.1 M KPi, pH 6.0, and 50 μl reduced cytochrome *c* soln.

Electron microscopy. Fractions from the differential centrifugation procedure were fixed, dehydrated and embedded as previously described [25]. After thin-sectioning the preparations were contrasted with lead citrate and examined with a Siemens 101 electron microscope, operating at 80 kV.

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