A Cysteine Desulfhydrase Specific for D-Cysteine from the Green Alga *Chlorella fusca*

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Chlorella, D-Cysteine, Cysteine Desulfhydrase

A cysteine desulfhydrase was purified 110-fold from the green alga *Chlorella* using conventional techniques. The isolated cysteine desulfhydrase was specific for D-cysteine having no activity towards L-cysteine. D- and L-cysteine desulfhydrase activities can be separated using DEAE-cellulose chromatography techniques. The isoelectric point of this enzyme was determined to be around a pH of 4.5 using a chromatofocussing column. The pH-optimum for the D-cysteine desulfhydrase was found to be in the range of 8.5 to 9 and the apparent K_M for D-cysteine was determined to 0.16 mM. The enzyme was active without addition of metal ions and EDTA or citric acid did not inhibit this activity.

Introduction

The green alga *Chlorella fusca* will grow on different sulfur sources, including D- and L-cysteine [1]. This initiated a search for enzymes metabolizing D-cysteine. The possibilities for cysteine degradation are analyzed using *Chlorella*-extracts and the properties of a purified cysteine desulfhydrase specific for D-cysteine are described.

Materials and Methods

Organism

Chlorella fusca strain 211-8b (formerly *Chlorella pyrenoidosa* strain 211-8b) of the algal collection of the University of Göttingen was cultured as previously described [2].

Enzyme preparation

200 g of *Chlorella* cells (wet weight) were broken in 50 g batches in a french-press at 12000 PSI in a buffer system containing 0.1 M Tris-HCl pH 8.0 and 10 mM mercaptoethanol. The crude extract was frozen over night, thawed and cleared by centrifugation. To the supernatant Polymin P was added (20 µl of a tenfold diluted Polymin P solution to one ml of crude extract) and the precipitated material was discharged. Solid ammonium sulfate was added to the supernatant and the precipitate between 35% and 80% saturation was collected by centrifugation and dissolved in a small volume of Tris-HCl-buffer pH 8 (0.02 M). This material was separated in 5 runs on a ACA 54 column (2.6 × 70 cm) equilibrated with 0.02 M Tris-HCl buffer pH 8 containing 0.1 M KCl; fractions of 4 ml were collected. Active fractions (Fig. 1) were pooled and concentrated to 20 ml using an Aminco diaflow system with a 10000 cut off filter. This material was placed on a DEAE-cellulose column (2 × 8 cm) equilibrated with 0.02 M Tris-HCl pH 8. The column was developed with a linear gradient (400 ml) from 0 to 0.5 M NaCl in the diluted Tris buffer mentioned. Fractions of 2.5 ml were collected. The separation for D- and L-cysteine specific desulfhydrase activity including cysteine synthase activity is shown in Fig. 2. Active fractions were pooled, concentrated as described above to 3 ml and separated further using a Biogel A 1.5 column (1.5 × 75 cm) equilibrated with the same buffer as mentioned for the ACA 54 column. Fractions of 2.5 ml were collected. Active fractions (82.5 ml to 92.5 ml) were pooled (Fig. 3) and used as the purified enzyme source. By this procedure a 109-fold purification of the D-cysteine specific sulfhydrolase was obtained from *Chlorella* with a yield of 10% (Table I).

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Determination of cysteine degradation

This activity was determined by the release of sulfide from cysteine. The assay system contained in micromoles in a total volume of 1 ml: Tris-HCl pH 9.0, 100; D- or L-cysteine, 0.8; DTE, 2.5, and enzyme as needed. After 30 min at 37 °C the reaction was terminated by adding acidic *p*-methyl-

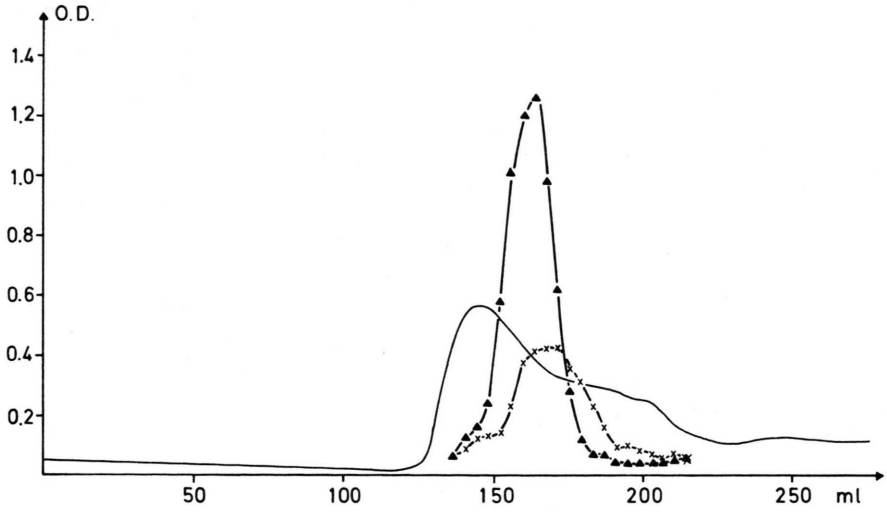

Fig. 1. Separation of D- and L-cysteine desulfhydrase activities on a ACA 54 column. Assay conditions as stated in materials and methods. —— = protein at 280 nm; x—x = sulfide production from L-cysteine at 670 nm; ▲—▲ = sulfide production from D-cysteine at 670 nm.

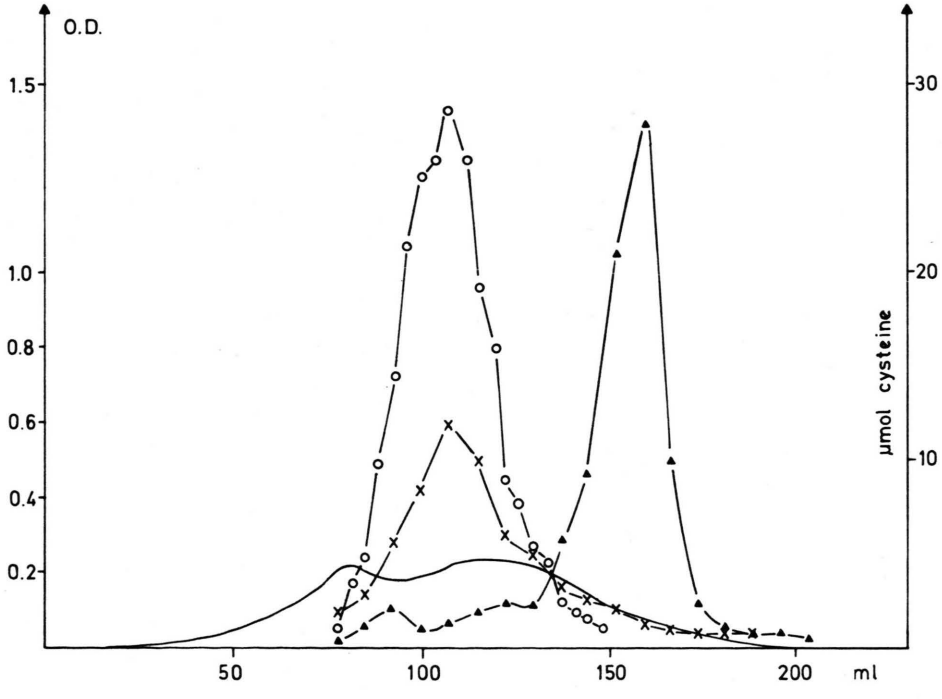

Fig. 2. Separation of D- and L-cysteine desulfhydrase activities on a DEAE-sephacel column. —— = optical density at 280 nm for protein; ▲—▲ = sulfide production from D-cysteine at 670 nm; x—x = sulfide production from L-Cysteine at 670 nm; ○—○ = µmols of cysteine formed from O-acetylserine and H_2S (cysteine synthase).

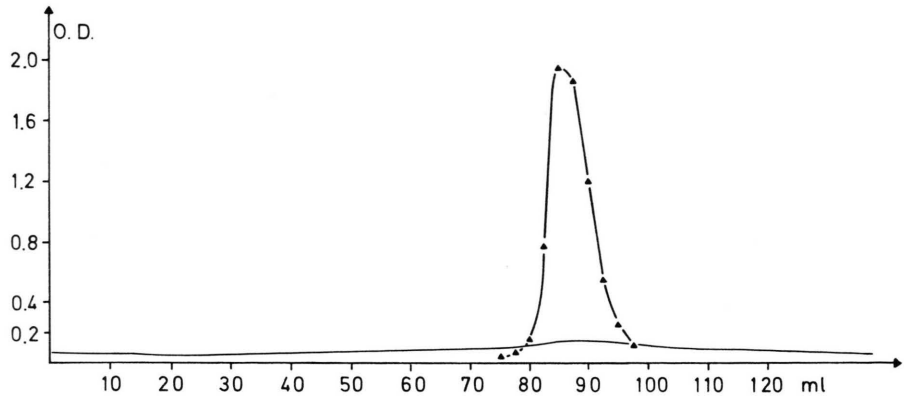

Fig. 3. Separation of the D-cystein desulfhydrase on Biogel A 1.5 M. Assay conditions as in Fig. 1. —— = protein at 280 nm; ▲—▲ = sulfide produced from D-cysteine read at 670 nm.

amine and $FeCl_3$ [3]. Methylene blue formation was determined at 670 nm in a Beckman DU-7 spectral photometer using a millimolar extinction coefficient of 28 [4].

Pyruvate determination

Pyruvate formation was followed by the lactate dehydrogenase method using LDH from pig (Boehringer 127221) and NADH [5].

Ammonia determination

Ammonia was determined using the phenol nitroprusside test from Sigma (solution 640-1 and 640-3) following the procedure supplied by Sigma.

Protein determination

Protein was determined by the Coomassie Brilliant Blue G-250 dye-binding method of Bradford [6] using the dye reagent supplied by Biorad.

Chemicals

D- and L-cysteine and cysteine derivatives were obtained from Sigma (München); N-acetyl-L-cysteine and *p*-methylamine were obtained from Serva (Heidelberg); mercaptoacetic acid was obtained from Fluka (Neu-Ulm); ACA 54 gel from LKB (München); all other chemicals were obtained from Merck (Darmstadt). Polymin P was a generous gift from BASF (Mannheim-Ludwigshafen).

Table I. Purification of the D-cysteine desulfhydrase from *Chlorella fusca*. The starting material was 200 g of wet cells.

	Protein [mg]	Enzyme activity [µmol H_2S formed/h]	Specific activity [µmol H_2S formed/h/mg protein]	Yield	Purification factor
Crude extract	6407	741	0.116	100	1
Polymin supernatant	4474	395	0.088	53	0.76
After ammonium sulfate precipitation	1934	329	0.170	44	1.5
After ACA 54 column chromatography	663	194	0.290	26	2.5
After DEAE-sephacel chromatography	31	121	3.97	16	34
After Biogel 1.5 M chromatography	6	75	12.8	10	109

Chromatofocussing technique

The chromatofocussing set of Pharmacia Fine Chemicals (Freiburg) was used following the instructions supplied. Separation was achieved using the PBE 94 gel and a pH separation from 7 to 4: 10 ml of the gel was used. Since enzyme activity was lost at low pH-buffers, crude extract was used for the iso-electric point determination and enzyme activity was measured immediately after the run was terminated. The column was run at 4 °C and the pH measurements were made at the same temperature.

Results

Crude *Chlorella* extracts formed enzymatically sulfide from either D- or L-cysteine; however the rate of sulfide released varied between L- and D-cysteine when the cystein concentrations were varied (Fig. 4). This suggested that different enzyme systems could be involved for D- and L-cysteine catabolism in this alga similar to results obtained for spinach previously [4, 7]. Therefore the purification scheme developed for the spinach system was tested for the *Chlorella* proteins and a 109-fold purified protein fraction obtained as described in materials and methods was used for the characterization of this enzyme.

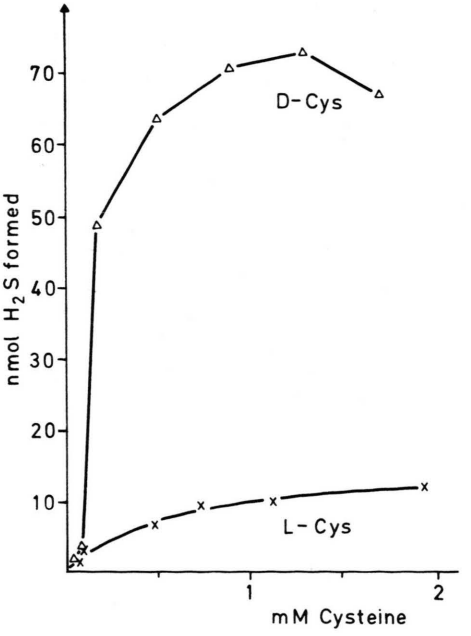

Fig. 4. Sulfide production from D- and L-cysteine using crude *Chlorella* extract. Assay conditions as in Table II; however 2 mM DTE was included; 1.7 mg of crude protein extract was used.

Isoelectric point of the D-*cysteine desulfhydrase*

The isoelectric point of the D-cysteine desulfhydrase was determined using a chromatofocussing technique [8]. Since this enzyme was very unstable at pH values around 5 and below, crude extract was used to determine the isoelectric point as shown in Figure 5. The protein determined as D-cysteine desulfhydrase activity is eluted by this procedure according to its isoelectric point at the resulting pH. Thus the apparent isoelectric point for the *Chlorella* enzyme was found to be around 4.5, whereas the cysteine synthase eluted at a pH of 6.0 (data not shown).

pH-optimum for the D-*cysteine desulfhydrase*

The pH optimum for sulfide production from D-cysteine was found to be around 8.5 to 9 (Figure 6). For routine measurements a pH of 9 was used.

Time and protein dependence of the D-*cysteine desulfhydrase*

The data of Fig. 7 and 8 demonstrate that the desulfhydrase activity is linear over the 30 min period used for routine assays and the rate was linear up to 20 µg of purified protein.

K_M-determination for D-*cysteine*

The K_M for D-cysteine was determined from the data shown in Fig. 9. The apparent K_M was found to be 0.16 mM for D-cysteine; no activity was found with L-cysteine.

Substrate specificity and influence of thiols and salts

The specificity of this purified enzyme fraction was tested with sulfide production from different thiols. These data are summarized in Table II. It is evident that only D-cysteine reacts with this enzyme preparation; there was no activity in the presence of other thiols including L-cysteine and D- and L-cystine, as well as some other L-cysteine derivatives. Certain metals at 2 mM concentration inhibited this activity (Ni, Fe, Zn), whereas others such as Mg, Ca,

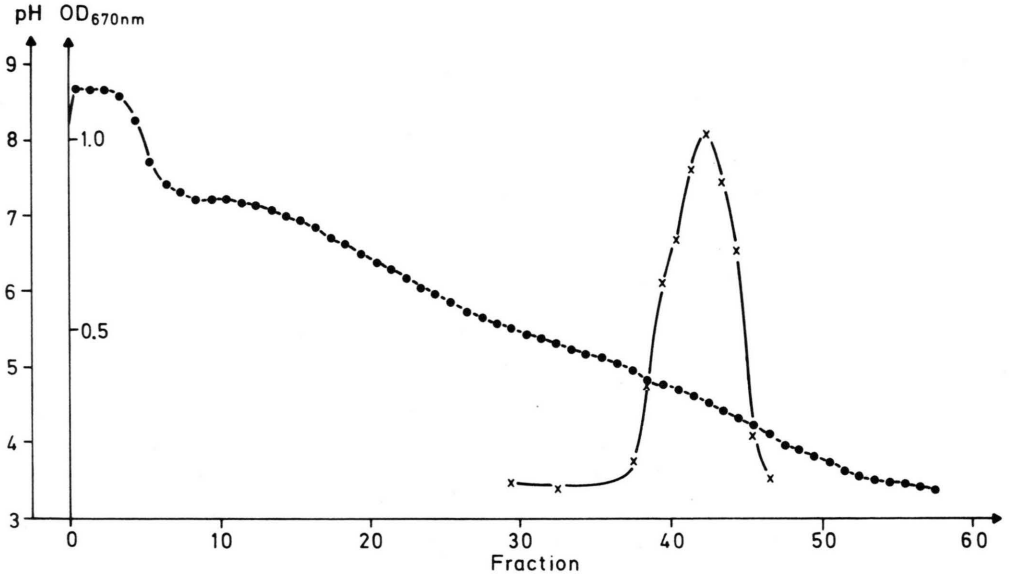

Fig. 5. Chromatofocussing of the D-cysteine desulfhydrase. 24 mg of crude *Chlorella* protein was added to 10 ml of the chromatofocussing gel. The gel was developed using 7–4 buffer. Fractions of 2 ml were collected and 0.8 ml of each fraction was used for the D-cysteine desulfhydrase activity following the conditions given in Table II with addition of 2 mM DTE.

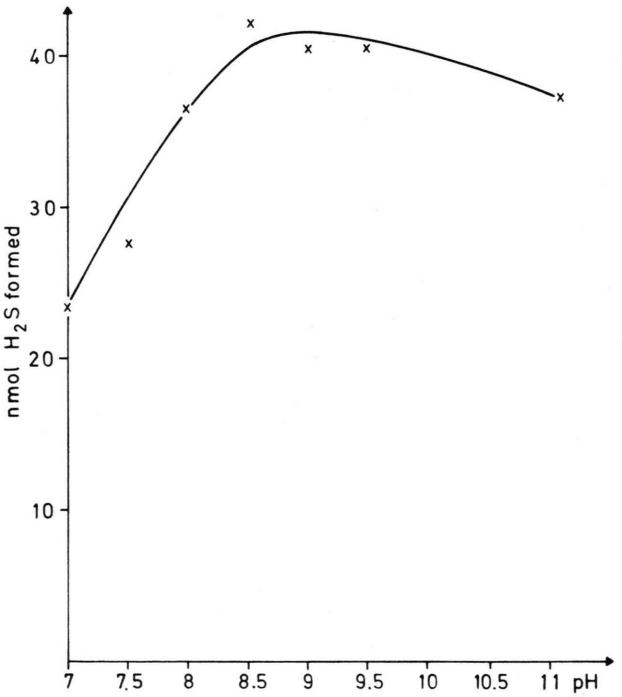

Fig. 6. pH-optimum for the D-cysteine desulfhydrase from *Chlorella fusca*. Conditions as in Table II with addition of 2 mM DTE; 7.2 µg of purified protein was used. Tris-buffer varied as shown.

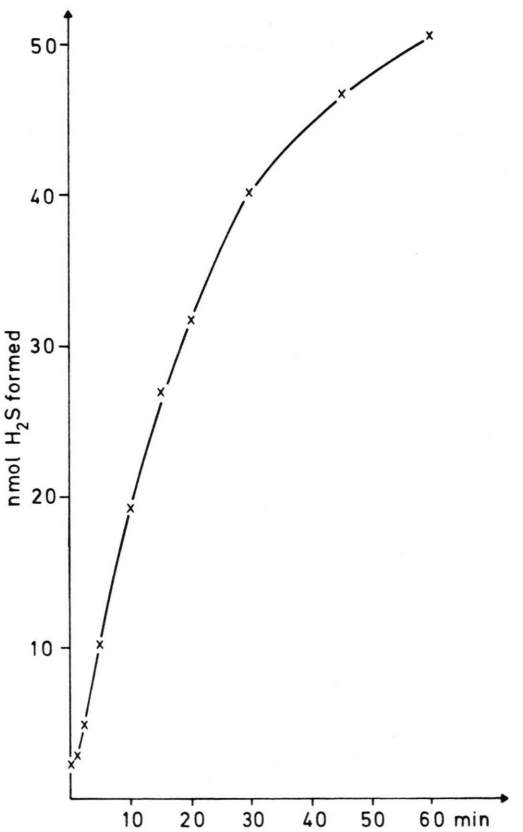

Fig. 7. Time dependence of the D-cysteine desulfhydrase reaction. Conditions as in Fig. 6.

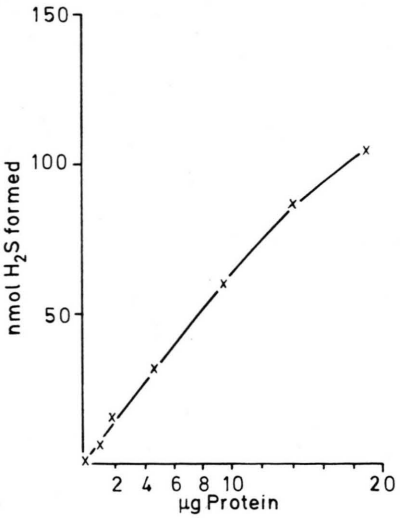

Fig. 8. Protein-dependence of the D-cysteine desulfhydrase activity. Conditions as in Table II with addition of 2 mM DTE. The protein was varied as indicated.

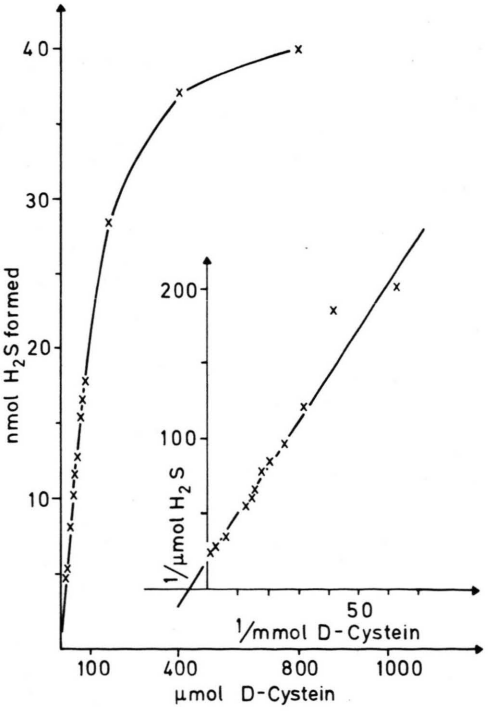

Fig. 9. K_M-determination for D-cysteine with the D-cysteine desulfhydrase. Conditions as in Table II using 6.5 µg of purified protein with the addition of 2 mM DTE; the D-cysteine concentration was varied as indicated.

Table II. Specificity of the D-cysteine desulfhydrase for different thiols. Assay conditions: Each tube contained in µmoles: Tris-HCl pH 9: 100; thiol as indicated: 0.8; protein: 6.5 µg in a total volume of 1 ml. Incubation for 30 min at 37 °C. The reaction was stopped by adding the reagents for sulfide determination. The methyleneblue reading at 670 nm was converted to nmol sulfide.

Compound added	Sulfide formed [nmol]	% of control
D-cysteine	30.5	100
DL-homocysteine	0	0
Mercaptoacetic acid	0.3	1
Mercaptoethanol	0.2	0.6
D-cystine	0.2	0.6
L-cystine	0.1	0.3
L-cysteine	0	0
Cysteamine	0.8	2.6
L-cysteinemethylester	0.1	0.3
L-cysteineethylester	0	0
N-acetyl-L-cysteine	0	0

Table III. Effects of salts, metals and complexing agents on the D-cysteine desulfhydrase activity. Conditions as in Table II, however 2 mM DTE was included in the assay system and metals and salts were added as indicated.

Compound added	Sulfide formed [nmol]	% of control
none	43.6	100
2 mM $MgCl_2$	43.8	100.6
2 mM $CaCl_2$	40.1	92.1
2 mM KCl	40.8	93.6
2 mM NaCl	41.3	94.7
2 mM $FeCl_2$	8.1	18.5
2 mM $AlCl_3$	42.3	97.1
2 mM $NiCl_2$	2.4	5.5
1 mM $NiCl_2$	12.7	29.2
0.4 mM $NiCl_2$	24.8	56.9
0.2 mM $NiCl_2$	39.6	90.9
0.1 mM $NiCl_2$	39.0	89.5
2 mM $ZnCl_2$	3.3	7.5
1 mM $ZnCl_2$	22.9	52.5
0.4 mM $ZnCl_2$	34.9	80.1
0.2 mM $ZnCl_2$	39.9	91.5
0.1 mM $ZnCl_2$	46.7	107
2 mM Titriplex III (EDTA)	39.8	91.2
2 mM Tri-sodiumcitrate	43.5	99.8

Table IV. Production of sulfide, pyruvate, and ammonia by the D-cysteine desulfhydrase from *Chlorella*. Conditions as in Table II, however 2 mM DTE was included and 24 mg of purified enzyme was used at the pH indicated.

		nmol formed
a) pH 9.0	sulfide	187
	ammonia	54
	pyruvate	21
b) pH 8.0	sulfide	169
	ammonia	44
	pyruvate	16

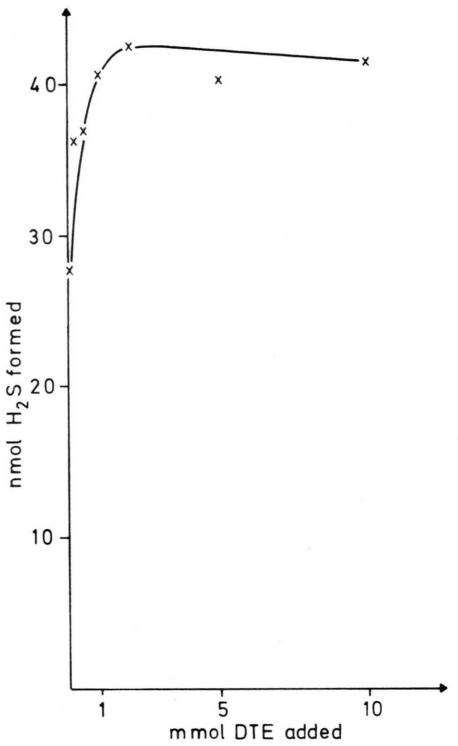

Fig. 10. DTE-dependent activation of the D-cysteine desulfhydrase from *Chlorella fusca*. Conditions as in Fig. 6 at the pH of 9.0. DTE was added as indicated.

Na, or K did not inhibit this reaction. The enzyme was active without addition of monovalent or divalent metals and chelating agents did not inhibit (Table III). Addition of DTE enhanced this activity to about 150% (Fig. 10) of the control.

Products formed

The D-cysteine desulfhydrase activity was analyzed for the possible formation of pyruvate and ammonia besides sulfide. These data are summarized in Table IV. It is evident that the sulfide formed is much higher than pyruvate or ammonia formed, suggesting that other products not found by this technique are formed as well.

Discussion

Chlorella fusca strain 211-8b will grow on D-cysteine as only sulfur source [1]. This indicated clearly that this organism was capable to handle D-cysteine in such a manner that the sulfur requirements for protein synthesis (L-cysteine) and sulfo lipid formation (oxidation to $R-SO_3H$) are possible. A D-cysteine specific desulfhydrase activity had been isolated from spinach leaves [4]. Therefore the possibility that such an enzyme was present in *Chlorella* cells as well was investigated using the analytical methods developed for the spinach enzyme. The data of this publication clearly demonstrate that a D-cysteine specific desulfhydrase is present in *Chlorella fusca* and a 109-fold purification of this desulfhydrase activity was achieved as shown in materials and methods. DEAE-cellulose chromatography separated between D-cysteine specific and L-cysteine specific desulfhydrase activities. It is evident from Fig. 2 that the L-cysteine specific desulfhydrase activity and cysteine synthase activity cochromatographed on the DEAE-cellulose column, which suggests that H_2S release from L-cysteine could be possible with the cysteine synthase by intermediate formation of an aminoacrylic acid and addition of L-cysteine instead of H_2S for the back reaction, which would be in accord for the data of isotopic exchange reactions obtained for the chlorella enzyme previously [11]. The D-cysteine desulfhydrase from spinach eluted before the L-cysteine specific desulfhydrase and cysteine synthase, whereas with the *Chlorella* system the opposite behaviour was found, *i.e.* the cysteine synthase and L-cysteine specific desulfhydrase activities eluted before the D-cysteine desulfhydrase. Cysteine synthase and D-cysteine desulfhydrase from *Chlorella* could be separated also by the chromatofocussing technique where the cysteine synthase was eluted at a pH of 6 and the D-cysteine desulfhydrase at a pH of 4.5, demonstrating different apparent isoelectric points [8]. The properties of the spinach and *Chlorella* D-cysteine desulfhydrases are more or less similar. The pH-optima in both cases are found around 8.5 to 9; these enzymes were found to be specific for D-cysteine, the apparent K_M-data for D-cysteine are similar (0.16 mM for *Chlorella* and 0.14 mM for spinach), and addition of DTE enhanced this activity to about 150% of the control [4]. Neither the spinach nor the *Chlorella* enzyme needed metals for activity and EDTA did not inhibit. Both activities were however inhibited by Ni and Zn, probably by complexing cysteine with these metals [9]. The spinach enzyme was stable when stored at $-20\,^{\circ}C$, whereas the *Chlorella* enzyme lost its activity during a period of about 3 month.

The D-cysteine desulfhydrases from spinach and *Chlorella* did not consumpt oxygen during the release of sulfide when analyzed with a clark type oxygen electrode, thus this activity is not related to D-amino acid oxidases, which is in accord with our measurements that little ammonia is formed during the reaction. We are aware that an unknown compound has to be formed during the desulfhydrase reaction, since the relation of sulfide to ammonia and pyruvate did not follow stochiometric relations. One possibility would be that an amino acrylic acid is formed [10] which than could react with either another thiol such as D-cysteine, EDTA or even water to give rise to the corresponding compounds. We could not detect the formation of serine or of another amino acid using an amino acid analyzer. Therefore a second compound (which has to be formed besides sulfide) is unknown at present.

The D-cysteine desulfhydrase activity was not altered significantly when cells were grown on D-cysteine, L-cysteine, L-methionine, or thiosulfate demonstrating that this enzyme activity seems to be constitutive under the conditions analyzed. The presence of a constitutive D-cysteine desulfhydrase in spinach and *Chlorella* suggests that it should have a function. Some possibilities for a role of D-cysteine (and possibly D-amino acids) have been discussed recently including separation of synthesis and degradation, separation for pool size discrimination, a possible signal theory, or as a specific precursor in certain biosynthetic routes [4]. None of these possibilities has been analyzed in detail so far, therefore more information is needed to understand the role of the D-cysteine specific desulfhydrases from plant and algal cells.

Acknowledgements

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