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PURIFICATION AND PROPERTIES OF *O*-ACETYL-L-SERINE SULPHYDRYLASE FROM WHEAT LEAVES

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Abstract—*O*-Acetyl-L-serine sulphydrylase (*O*ASS), the enzyme which produces L-cysteine from *O*-acetyl-L-serine (*O*AS) and sulphide, is located in soluble fractions of wheat leaves (*Triticum aestivum* var. Gabo). It has been purified by chromatography on DEAE-cellulose followed by Sephadex G-100 gel filtration. The K_m values for *O*AS and sulphide were 3.7×10^{-5} M and 5.9×10^{-4} M respectively. The purified enzyme was inhibited non-competitively by PCMB, *N*-ethylmaleimide, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), L-cysteine and L-homoserine. Methionine, however, inhibited the enzyme competitively with respect to the S^{2-} group.

INTRODUCTION

The biosynthesis of cysteine in higher plants is similar to that of bacteria, e.g. *Salmonella* and *E. coli* [1, 2]. Two enzymic steps are involved, namely, the acetylation of serine by acetyl-CoA, followed by sulphydrylation to cysteine.

O-acetyl-L-serine sulphydrylase (*O*ASS), is the enzyme which incorporates inorganic S^{2-} into cysteine:

$$OAS + S^{2-} \rightarrow \text{Cysteine} + \text{acetate}$$

The enzyme has been found in bacteria [3], yeast and *Neurospora* [4], and in higher plants [5–10]. There are several reports that L-serine is the normal precursor in yeast [11], spinach [12], and beets [13]. However, there is evidence now that *O*-acetyl-L-serine (*O*AS) is the substrate for the enzyme in plants [7, 17]. *O*AS is derived from the enzymic acetylation of L-serine by serine transacetylase (STA):

$$\text{L-serine} + \text{acetyl CoA} \rightarrow OAS + \text{CoA}$$

In this paper we report on the purification and properties of the *O*ASS enzyme from wheat plants.

RESULTS

Location of OASS activity

Extracts of leaves contain *O*-acetyl-L-serine sulphydrylase (*O*ASS). The data in Table 1 show that the *O*ASS activity was located mainly in the soluble fraction of the leaves (S_{144}). The distribution of serine transacetylase (STA), which is involved in the synthesis of *O*AS in the various fractions, was as follows (μmol thionitrobenzoate formed/min/mg protein): S_{10}, 0.46; S_{144}, 0.03; P_{144}, 0.39 (see Experimental). The P_{144} contained the bulk of the STA activity.

Purification of the enzyme

Details of the enzyme purification are given in Table 2. Leaves (28 g fr. wt) were homogenized in 10 mM Pi buffer (pH 7.2) containing 0.3 M mannitol, 1 mM Na-EDTA, and 0.2 mM dithiothreitol at 2° (see Experimental). The extract was centrifuged at 10 000 *g* for 30 min to obtain Fraction I (S_{10}), which was further centrifuged at 144 000 *g* for 1 hr to obtain Fraction II (S_{144}). Fraction II was loaded onto a DE-11 column (20 × 1.5 cm) previously equilibrated with 10 mM Pi buffer (pH 7.2). The enzyme was eluted between 0.08 and 0.15 M Pi (pH

Table 1. Location of *O*ASS activity in various fractions of leaves (26-day-old wheat plants)

Fraction	Total protein (mg)	Total activity in extract (μmoles cysteine/min)	Specific activity (μmoles cysteine/min/mg protein)	% Activity
S_{10}	189	3.75	0.02	100
S_{144}	150	3.00	0.02	80
P_{144}	25	0.23	0.008	6

Leaves were homogenized in 10 mM Pi buffer (pH 7.2) containing 0.3 M mannitol, 1 mM Na-EDTA, and 0.2 mM dithiothreitol (1 g fr. wt: 3 ml buffer) at 2° (see Experimental). S_{10} is the supernatant fraction left after centrifuging the crude homogenate at 10 000 *g* for 30 min. S_{144} and P_{144} are the supernatant and the pellet fractions, respectively, left after centrifuging the S_{10} at 144 000 *g* for 1 hr. The final pellet (P_{144}) was suspended in 1 ml of 10 mM Pi (pH 7.2). Details of the assay procedure are given in Experimental. The reaction mixture, in a final vol. of 0.28 ml, contained (μmol): 0.1 M Pi buffer (pH 6.8) 20; *O*AS, 10; Na_2S, 4; and 20 μl leaf extract (29 mg protein) in 10 mM Pi buffer (pH 7.2).

Table 2. Purification of *O*-acetyl L-serine sulphydrylase from leaves (27-day-old wheat plants)

Fraction	Volume (ml)	Total protein (mg)	Total units	Specific activity*	Yield %	Relative purity
(I) S_{10} Crude extract centrifuged at 10000 *g* 30 min Supernatant fraction	84	2730	294	0.12	100	1
(II) S_{144} S_{10} centrifuged at 144 000 *g* 1 hr Supernatant fraction	55	1809	213	0.12	72	1
(III) Fraction II eluted from DE-11 Column by 0.05–0.2 M Pi buffer (pH 7.2) linear gradient. Activity in 0.08–0.15 M Pi fraction	3	63	89	1.4	30	12
(IV) Fraction III eluted from Sephadex G-100 Column in 10 mM Pi buffer (pH 7.2)	6	4	23	5.7	8	48

* Specific activity: μmol cysteine produced/min/mg protein.

7.2). The most active fractions (70–90 ml) were pooled and concentrated in the Amicon Ultrafiltration Unit (PM 10 filter) to obtain Fraction III, which was loaded onto a Sephadex G-100 column (2.6 × 40 cm) equilibrated with 10 mM Pi (pH 7.2). The column was eluted with the same buffer and actual fractions (25–40 ml) were pooled and concentrated in the Amicon Ultrafiltration Unit to obtain Fraction IV (Fig. 1).

Properties of the enzyme

The production of cysteine was maximal in either 0.01 M or 0.1 M Pi buffer at pH 6.8 and in 0.1 M Tris–HCl buffer at pH 8. The production of cysteine was linear over the range 0.01 to 0.4 mg protein. For the experiments described herein, 0.15 mg protein was used. Maximum activity was recorded at 37° but above this temperature the enzyme was inactivated. The enzyme was stable when stored at −15°, but at 2° it was inactivated after a few days.

The rate of production of cysteine was measured at various *O*AS and S^{2-} concentrations using Fraction IV (Table 2). The results show normal Michaelis–Menten kinetics with a linear relationship between cysteine formed and the two substrates. The K_m values determined from Lineweaver–Burk plots of the data were 3.7×10^{-5} M (*O*AS) and 5.9×10^{-4} M (S^{2-}). *O*AS and S^{2-} above 71.4 and 107 mM, respectively, inhibited the enzyme.

The following compounds did not substitute for *O*AS: L-serine, L-cystine, *O*-acetyl-homoserine, and DL-homoserine. Selenite did not replace S^{2-} in the reaction. The compounds listed on Table 3 were inhibitory. DL-homocystine, L-cystine and L-serine did not affect the enzyme. The K_i values for the various inhibitors are given in Table 3 and illustrated in Figs. 2a–f. Methionine competitively inhibited the enzyme, while PCMB, NEM, DTNB, L-homoserine and L-cysteine were non-competitive inhibitors. GSH (20 μmol) reversed the inhibition by PCMB (10 μmol).

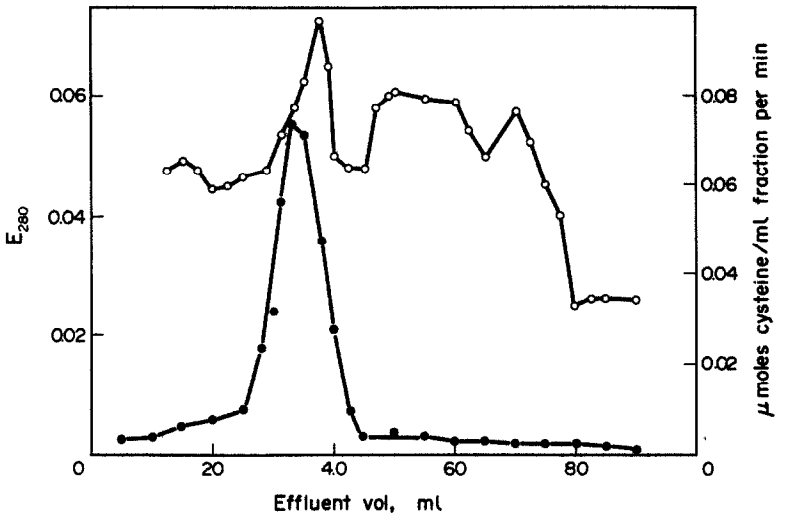

Fig. 1. Elution profile of *O*ASS activity from Sephadex G-100 column (2.6 × 40 cm). The column was packed as described in Experimental and eluted with 10 mM Pi buffer (pH 7.2). Fraction III (Table 2) loaded on to the column, was eluted with the Pi buffer. Fraction IV was obtained by concentrating the active fractions (25 to 40 ml) in an Amicon Ultrafiltration Unit (PM 10). O—O A_{280} nm (1 cm cell); ●—● Enzyme activity: μmol cysteine produced/min/ml fraction.

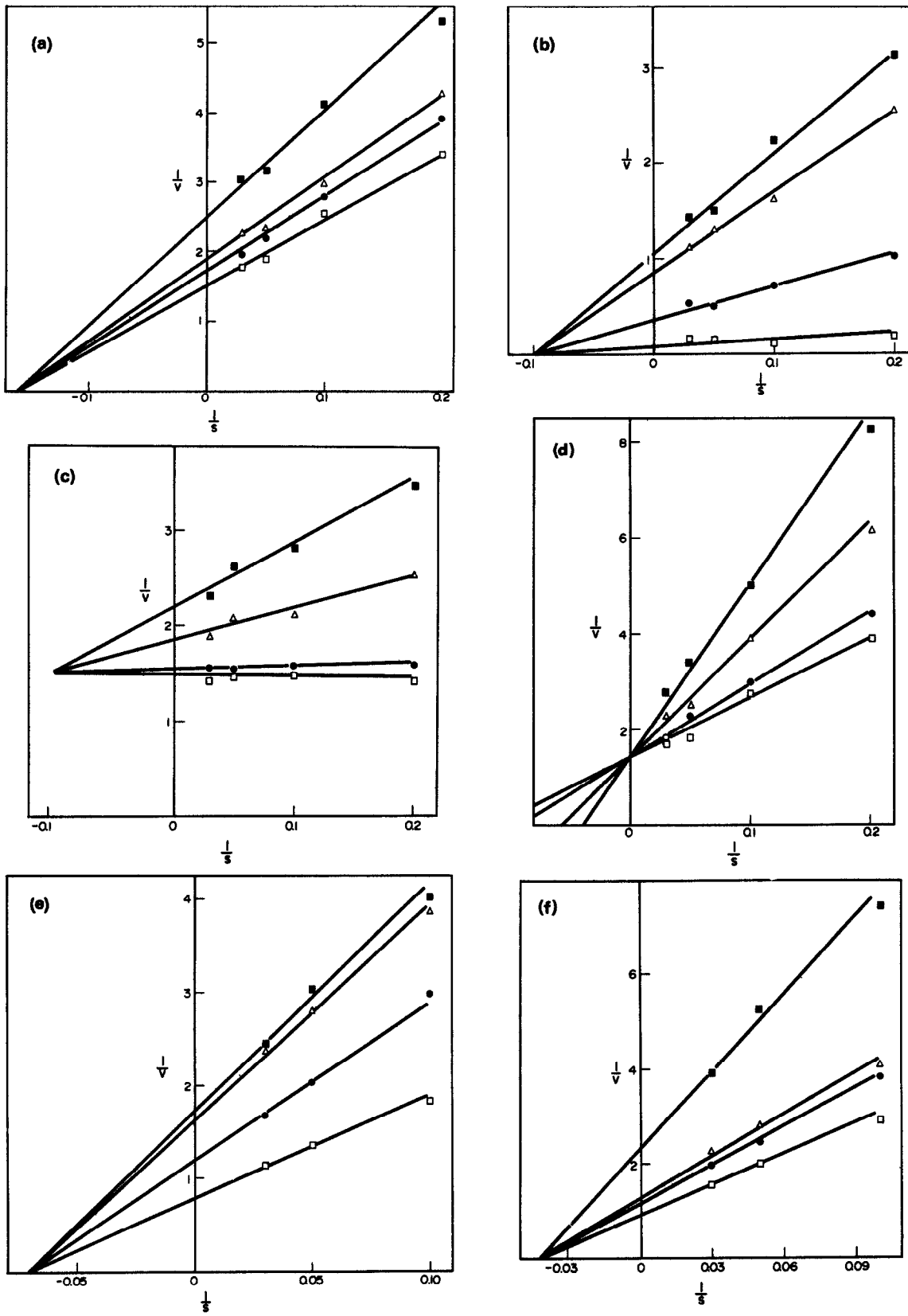

Fig. 2. K_i values for various inhibitors. (μmol/0.28 ml reaction mixture): (a) PCMB, □ ○; ● 0.002; △ 0.02; ■ 0.2; (b) NEM, □ ○; ● 0.4; △ 1; ■ 2; (c) DTNB, □ ○; ● 0.04; △ 0.2; ■ 0.4; (d) Methionine, □ ○; ● 0.2; △ 0.4; ■ 1; (e) L-Homoserine, □ 0; ● 0.2; △ 0.4; ■ 0.8; (f) L-Cysteine, □ 0; ● 0.2; △ 0.4; ■ 0.66. The velocity of reaction (μmol cysteine/10 min) is measured against the following concentrations of S^{2-}: 17.8, 35.7, 71.4 and 107 mM. See Experimental for assay conditions.

Table 3. Effects of various inhibitors on *O*ASS activity

Inhibitor	Conc (mM)	% Inhibition	K_i values (M)
PCMB	0.1	21	2.86×10^{-3}
	1.0	38	
NEM	0.1	30	1.43×10^{-3}
	1.0	81	
	5.0	84	
DTNB	0.01	19	6.3×10^{-4}
	0.1	38	
	0.5	56	
	1.0	63	
L-Cysteine	3.5	69	2.27×10^{-3}
	4.0	84	
L-Methionine	1	32	1.75×10^{-3}
	2	47	
	5	61	
L-Homoserine	1	45	1.32×10^{-3}
	2	48	

The enzyme was preincubated with the inhibitor for 20 min at 25° before adding the substrates. The reaction mixture in a final vol. of 0.28 ml contained (μmol): *O*AS, 10; Na_2S, 4; 0.1 M Pi buffer (pH 6.8), 20; and 0.02 ml Fraction IV, Table 2 (0.15 mg protein). This was incubated at 37° for 10 min and the reaction terminated with TCA to a final concn of 4.4% (w/v). The following compounds did not inhibit the enzyme: DL-Homocystine, L-Cystine, L-Serine. The velocity of reaction was measured against the following concentrations of S^{2-}: 17.8, 35.7, 71.4 and 107 mM.

DISCUSSION

The enzymes involved in the synthesis of L-cysteine in *Salmonella* are associated with a soluble protein complex which dissociated reversibly into STA and *O*ASS in the presence of *O*AS [3]. In *P. aeruginosa* [14] and higher plants [8, 9] a firmly bound STA was found to be attached to mitochondria, whereas *O*ASS was located in the soluble fractions [9]. The results reported herein show that the bulk of STA activity was found in the particulate fraction of extracts of wheat leaves, while the *O*ASS activity was located in the supernatant fractions. The purified enzyme (Fraction IV, Table 2) was devoid of STA activity.

The K_m value for *O*AS was obtained in these studies for wheat (3.7×10^{-5} M). This K_m value for *O*AS, reflecting the strong affinity of the enzyme for its substrate, may account for the difficulty in isolating these trace amounts of *O*AS from plants. It is noteworthy that Ngo and Shargool [10] also recorded low K_m value (1.74×10^{-6} M) for *O*AS in rapeseed. Kinetic data for *O*AS and S^{2-} were not obtained for kidney bean seedlings by Smith and Thompson [8] because of the unreliable method used to determine S^{2-}. By using specific methods to stabilize the Na_2S (see Experimental), it was possible to determine the K_m value for S^{2-} in wheat (5.9×10^{-4} M). This is similar to those for rapeseed (4.3×10^{-4} M), *E. coli* and *Salmonella* ($< 1 \times 10^{-4}$ M), or *P. aeruginosa* (5×10^{-4} M).

O-Acetylhomoserine did not serve as substrate for the wheat enzyme, in agreement with the data for kidney bean seedlings [8] and spinach [6]. However, the lack of specificity for the —SH acceptor has been reported for the enzyme from higher plants [6, 7] and bacteria [1]. The following reaction was proposed to account for these results:

*O*AS + Methyl mercaptan → Methyl cysteine

The *O*ASS activity is reported to be repressed by cysteine in *E. coli*, *R. spheroides* and *Salmonella* [14] or controlled by intracellular concentrations of this amino acid in *E. coli* [15], but no information is available for higher plants. Competitive inhibition was detected for methionine, while a non-competitive type was observed for the other compounds listed in Table 3.

EXPERIMENTAL

Plant material. Wheat seeds (*Triticum aestivum* var. Gabo) were surface-sterilized with 0.02% $HgCl_2$, washed several times with autoclaved H_2O and germinated in black plastic trays containing vermiculite. Seeds were grown in continuous light at 20° in the phytotron unit with an intensity of 17 600 lx. One-tenth-strength Hoagland's soln was added daily for 1 week, followed by half-strength thereafter. The leaves were used as the source of enzyme.

Extraction procedure. The leaves were homogenized at 2° in a chilled mortar with the aid of pre-cooled acid-washed sand [1 g fr. wt, 3 vol. of 10 mM Pi buffer (pH 7.2) containing 0.3 M mannitol, 1 mM Na-EDTA, and 0.2 mM dithiothreitol]. The homogenate was squeezed through muslin cloth and centrifuged at 10 000 *g* for 30 min to obtain the S_{10} (Fraction I), then at 144 000 *g* for 1 hr to produce the S_{144} (Fraction II).

Purification of extract. Fraction II was loaded onto a DE-11 column (20 × 1.5 cm) prepared according to the manufacturer's instructions and previously equilibrated with 10 mM Pi buffer (pH 7.2). The column was eluted with a linear gradient (0.05–0.2 M Pi buffer, pH 7.2), and 5 ml fractions were collected at a flow rate of 2.5 ml/min. Active fractions were pooled and concnd in the Amicon Ultrafiltration Unit using PM 10 membrane filter to obtain Fraction III. Proteins were determined by A_{280}. Fraction III was loaded onto a Sephadex G-100 column (2.6 × 40 cm) previously equilibrated with 10 mM Pi buffer (pH 7.2) and eluted with the same buffer. Portions (5 ml) were collected and the active fractions pooled and concnd in the Amicon Ultrafiltration Unit (PM 10 filter) to obtain fraction IV.

Protein assay. Protein content of various fractions were checked from A values at 260 and 280 nm, and then by a routine assay using the microbiuret method [16].

Enzyme assay. (a) *L-serine transacetylase assay.* The reaction mixture in a final vol. of 1 ml contained in μmol: Tris(hydroxymethyl)–amino methane (Tris–HCl) buffer (pH 7.6), 50; dithionitrobenzoate, 1; acetyl CoA, 0.2; and L-serine, 1. The reaction was initiated by adding the enzyme, and the activity measured by determining the increase in A_{412}. (*b*) *OASS assay.* Activity was measured by the method of ref. [14]. The substrates were freshly prepared just before use. *O*AS crystals were dissolved in 0.1 M Pi buffer (pH 5) containing 10 mM Na-EDTA and 50 μM pyridoxal phosphate. Thoroughly washed and dried Na_2S crystals were dissolved immediately in 0.1 M Pi buffer (pH 11.5) containing 10 mM Na-EDTA and 50 μM pyridoxal phosphate in vials which had been flushed with N_2. The reaction mixture in a final vol. of 0.28 ml at pH 6.8 contained (μmol): *O*AS, 10 in 0.05 ml; Na_2S, 4 in 0.02 ml; 0.1 M Pi buffer (pH 6.8), 20 in 0.19 ml; and the extract (20 μl). KCN was omitted from the reaction mixture. The reaction was carried out at 37° and was initiated by adding Na_2S. After 10 min, TCA was added to a final concn of 4.4% (w/v) to terminate the reaction. Cysteine was determined by the method of ref. [14]. To a test-tube (100 × 11 mm) containing 0.5 ml 17 M HOAc and 0.5 ml concnd ninhydrin (Reagent 2) [12], the total incubation mixture (0.56 ml) was added, and the tubes covered with aluminium caps. The mixture was thoroughly shaken in a Vortex mixer, heated to 100° for 10 min, and rapidly cooled. 8.5 ml of 100% EtOH was added and mixed immediately to stabilize the pink colour formed, and the A_{560} determined in a 1 cm glass cuvette against a reagent blank.

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