

# Purification and Initial Kinetic Characterization of Different Forms of O-Acetylserine Sulphydrylase from Seedlings of Two Species of *Phaseolus*

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## ABSTRACT

Purification of O-acetylserine sulphydrylase (OASS) from seedlings of two species of *Phaseolus* reveals the presence in both species of two forms of this enzyme. The isolation and purification procedure gives purification of 7- to 160-fold for individual isoenzymes with specific activities ranging from 33 IU mg<sup>-1</sup> to 775 IU mg<sup>-1</sup> protein.

Detailed study of the basic kinetic parameters of the OASS isoenzymes indicates that both forms from *Phaseolus vulgaris* (which are of about equal specific activity) display substrate inhibition by S<sup>2-</sup> above 1 mM and positive cooperativity at lower concentrations of S<sup>2-</sup>. With respect to O-acetylserine (OAS), the second substrate of the reaction, one *P. vulgaris* isoenzyme shows substrate inhibition by OAS concentrations above 10 mM, while the second is unaffected by OAS concentrations up to 50 mM. The isoenzymes from *Phaseolus polyanthus* (one of which has a specific activity 24 times higher than the other) are slightly and approximately equally inhibited by both S<sup>2-</sup> and OAS.

There is increasing evidence that a variety of plants including spinach (6), kidney bean (20, 22), and species of rape, snapdragon, sunflower, wheat, barley, carrot, aster, flax, and pumpkin (18) synthesize cysteine by a two-step process similar to that known to occur in bacteria (12). First, OAS<sup>1</sup> is formed in a reaction catalyzed by serine transacetylase. Then, cysteine is synthesized from OAS and sulfide by OASS (15, 20-23). Unlike bacteria, there is no evidence for the existence of a multifunctional protein complex of serine transacetylase-OASS in higher plants (20, 22).

This report is concerned with comparison of some of the properties of OASS from 5-day etiolated seedlings of *Phaseolus vulgaris* and *Phaseolus polyanthus*. These species were chosen because the amount of cysteine in seed proteins of *P. polyanthus* was approximately three times that of *P. vulgaris* (2). A relatively simple method of isolation which gives a good yield of the enzymes and which leads to purification to homogeneity as judged by disc gel electrophoresis has been developed. These studies indicate the existence of two isoenzymes of OASS from each species of legume investigated.

## MATERIALS AND METHODS

**Plant Material.** Seeds of *P. vulgaris* L. (Lot 751, Burpee Seed Company, Riverside, Calif.) and *P. polyanthus*, formerly named

<sup>1</sup> Abbreviations: TEMED: N,N,N',N'-tetramethylethylenediamine; FMN: flavin mononucleotide; OAS: O-acetyl-L-serine; OASS: O-acetylserine sulphydrylase.

*P. phyllanthus*, (P.I. No. 190 080) from the USDA Western Plant Introduction Station, Washington State University, Pullman, Wash., were rinsed separately for 30 min in aerated tap water, sown in trays of vermiculite moistened with tap water, and then covered with a thin layer of vermiculite plus more tap water. Germination was for 5 days in the dark at an air temperature of 25 C. The above ground parts were excised and ground for enzyme extraction.

**O-Acetylserine Sulphydrylase Extraction.** All operations were done at 0 to 4 C. Either 100 g of *P. vulgaris* tissue or, due to the limited availability of seed, 45 g of *P. polyanthus* tissue were added to an equal volume of the following grinding medium: 0.1 M tris-HCl (Schwarz/Mann) (pH 8.5), 0.1 mM pyridoxal phosphate (Sigma Co.), 10 mM mercaptoethanol (Calbiochem), 1 mM Na<sub>2</sub> EDTA (Sigma Co.), 1% (w/v) PVP (Polyclar AT, GAF Corp.), and 1 mM phenylmethylsulfonyl fluoride (Calbiochem). Solid PVP and the phenylmethylsulfonyl fluoride (0.1 M stock in 95% ethanol) were added separately just before grinding the tissues. The tissue was ground in a prechilled mortar for 10 min, passed through eight layers of cheesecloth, and centrifuged at 20,000g for 30 min.

Next, activated charcoal (Matheson Co.) and calcium phosphate gel (Nutritional Biochem. Corp.) were added according to the procedures of Granroth (8). Finely ground ammonium sulfate (Schwarz/Mann) was added slowly to 40% saturation. After stirring for 45 min the suspension was centrifuged at 20,000g for 30 min. The pellet was discarded and ammonium sulfate added to bring the supernatant fraction to 90% saturation. After centrifugation, the pellet was resuspended in 0.1 M tris-HCl (pH 8) containing 0.1 mM pyridoxal phosphate, 10 mM mercaptoethanol, and 15% (v/v) glycerol and dialyzed for a 3-hr period against three 1-liter changes of the resuspension solution. The dialysate was centrifuged at 20,000g for 30 min and the supernatant liquid layered on a Sephadex A-50 DEAE (Pharmacia) 2.5 × 30 cm column pre-equilibrated with the deaerated resuspending mixture stated above. The column was washed for 3 hr with the equilibrating buffer before eluting the enzymes with a linear 0 to 0.35 M NaCl linear gradient in resuspending medium (500 ml total) at a rate of 35 to 40 ml/hr. Pooled peak fractions (20 × 2.5 ml) which eluted at approximately 0.15 M NaCl, were dialyzed, added to a second Sephadex A-50 DEAE column (2.5 × 15 cm), and eluted in the same manner as the first DEAE column. Pooled peak fractions were concentrated to 2 ml by UM-10 ultrafiltration membranes (Amicon) and stored at 0 to 4 C in 0.1 M tris-HCl (pH 8) 0.1 mM pyridoxal phosphate, 10 mM mercaptoethanol, and 15% glycerol.

**Acrylamide Gel Separation.** The disc gel procedures, based on the principles of Ornstein (19), were as shown in Table I.

Gels were run at 4 C for 2.5 hr at 2 mamp/gel with bromophenol blue as the leading dye band. Activities of the OASS

Table I. Disc Gel Procedures, Based on the Principles of Ornstein

	Volume ratios	Components in 100 ml	pH (25 C)
Resolving gel*	1	(a) 45 g acrylamide 1.6 g bisacrylamide	
	1	(b) 24 ml of 1 N HCl 18.15 g tris 0.2 ml TEMED	8.9 ± .2
Stacking gel	2	(c) 0.2 g ammonium per-sulfate	
	1	(a) 5 g acrylamide 1.2 g bisacrylamide	
Upper buffer	1	(b) 12.8 ml 1M H <sub>3</sub> PO <sub>4</sub> 2.85 g tris 0.2 ml TEMED	7.2 ± .2
	1	(c) 3 mg FMN 40 g sucrose	
Lower buffer	(a)	0.632 g Tris 0.394 g Glycine	8.9 ± .2
	(b)	1.21 g tris 5 ml 1 N HCl	8.1 ± .2

\*Native 8% resolving gels were prepared by adding 2.5 ml of (a) to 1.25 ml glass distilled water and 3.75 ml of (b) and degassing the mixture for 3 to 5 min. Then, 7.5 ml of (c), which was degassed separately, was combined with the (a) plus (b) mixture to form the gel.

were located by taking 1.5-mm slices of the resolving gel (7 × 0.5 cm) with an Ames model 3542 lateral gel slicer (Ames Co., Elkhart, Ind.) and placing each individual slice into 0.4 ml of 0.1 M tris-HCl (pH 8) (0–4 C), 0.1 mM pyridoxal phosphate, 10 mM mercaptoethanol, and 15% (v/v) glycerol. Elution of the enzymes from the slices was carried out for 24 hr at 0 to 4 C. Aliquots of each eluate were assayed by the sulfide electrode assay of Cook and Wedding (5) at 25 C in 1 ml containing 0.1 M tris-HCl (pH 7.6), 0.1 mM pyridoxal phosphate, 20 mM OAS (Calbiochem), 0.25 mM sodium sulfide, and starting each reaction with enzyme. For gel scans at 600 nm, unsliced gels were stained with Coomassie blue in 7% acetic acid and destained in acetic acid-methanol-water (1,800 ml methanol plus 370 ml acetic acid made to 4 liters with glass-distilled H<sub>2</sub>O).

**Enzyme Assay.** Activity was monitored as rate of decrease in sulfide ion concentration during the enzymic formation of cysteine from sodium sulfide and OAS according to procedures given by Cook and Wedding (5). A 1-ml total volume of the standard assay mixture contained as final concentrations: 0.1 M tris-HCl (pH 7.6) plus 0.1 mM pyridoxal phosphate, 20 mM OAS (added separately from 1 M stock in glass-distilled H<sub>2</sub>O), 0.9 to 1 mM sodium sulfide (also added separately from stock in 0.1 M tris-HCl [pH 7.6] plus 0.1 mM pyridoxal phosphate), and initiating each reaction with enzyme.

**Protein Determinations.** Protein concentrations were determined by first dialyzing 0.4-ml aliquots against 50 mM K-phosphate buffer (pH 8) (0–4 C) before analysis by either the method of Lowry *et al.* (13) or, for very low protein concentrations, by the fluorometric assay of Bohlen *et al.* (4). BSA (fraction V, Sigma Co.) was used as the standard and all samples were read against blanks containing the phosphate buffer.

**Estimation of Molecular Weight.** Estimations were made by both gel filtration on an agarose Bio-Gel A-0.5m (100–200 mesh) column (2.5 × 50 cm) (Bio-Rad) and by the polyacrylamide gel method of Hedrick and Smith (9). In the latter case, OASS was localized by the same procedures described under "Acrylamide Gel Separation."

**Assays for Serine Transacetylase-O-Acetylserine Sulfhydrylase Complex.** Activity of the multienzyme complex of OASS with serine transacetylase was assayed at 25 C by the sulfide ion electrode assay method (5) as before with the addition of 10 mM L-serine (Nutritional Biochemical Corp.), 1 mM acetyl-CoA (Boehringer-Mannheim Corp.), and 0.25 mM sodium sulfide, starting the reaction with enzyme. Complex activity was also tested for by the spectrophotometric assay of Kredich and Tomkins (12) with final concentrations of 0.1 M tris-HCl (pH 7.6), 0.1 mM pyridoxal phosphate, 10 mM L-serine, 1 mM acetyl-CoA, 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (Aldrich Chemical Co.), and enzyme, monitoring change in absorbance at 412 nm at 25 C.

**Assays for Cysteine Desulfhydrase.** The sulfide electrode assay was used with a 1-ml volume consisting of 0.1 M tris-HCl (pH 7.6) (25 C), 0.1 mM pyridoxal phosphate, 10 mM L-cysteine (Sigma Co.), enzyme, and monitoring appearance of sulfide. A coupled spectrophotometric assay was also used with final concentrations of 0.1 M tris-HCl (pH 7.6) (25 C), 0.1 mM pyridoxal phosphate, 10 mM L-cysteine, 50 μM NADH, lactate dehydrogenase, and enzyme. Oxidation of NADH was monitored at 340 nm as the pyruvate formed from cysteine by cysteine desulfhydrase is converted to lactate by lactate dehydrogenase.

## RESULTS

**Isolation and Physical Properties.** For good initial yield of enzyme activity a mortar and pestle were used to disrupt the tissues in the presence of PVP and the grinding medium was adjusted to pH 8.5 so that after homogenization, the mixture reached a near optimum pH of 8. Approximately 15,000 units of OASS activity were obtained from only 100 g fresh wt of *P. vulgaris* seedlings (Table II) and 4,600 units from 45 g of *P. polyanthus* (Table III). These values are about an order of magnitude greater than enzyme preparations from mature rape leaves (14) or radish roots (23) and several orders of magnitude larger than that obtained from kidney bean seedlings (22). It should be noted that the investigations on the mature rape leaves, radish roots, and kidney bean seedlings employed a K-phosphate buffer (pH 8) for the grinding medium and homogenization of the tissues in a Waring Blendor. The initial specific activities of 4.47 and 4.75 (Tables II and III) are also greater than the 0.68 for mature rape leaves (14), 0.0158 for kidney bean seedlings (22), and 3.7 for radish roots (23).

Addition of pyridoxal phosphate was made to all isolation media and assay buffers since dialysis of partially purified *P. vulgaris* enzyme preparations against 0.1 M tris-HCl (pH 8) (0–4 C) in the absence of pyridoxal phosphate resulted in a 40% loss of enzyme activity compared to dialysis in the presence of 0.1 mM pyridoxal phosphate. Dialysis against 0.05 M K-phosphate buffer (pH 8) (0–4 C) resulted, however, in a loss of 50% of the activity regardless of whether pyridoxal phosphate was present. This loss of activity during dialysis against phosphate could not be reversed by subsequent addition of pyridoxal phosphate, even in concentrations up to 50 mM. Furthermore, if the first DEAE column in the isolation procedure (step 7, Tables II and III) was preequilibrated with K-phosphate buffer plus 0.1 mM pyridoxal phosphate and the enzymes eluted with a 0.05 to 0.5 M K-phosphate gradient, 83% of the activity was lost.

Preparations of OASS from kidney bean seedlings (20) as well as mature rape leaves (14) have been reported to be soluble rather than particulate. This appears to be the case in the present study as well, since centrifugation of the crude homogenate at 100,000g for 50 min sediments less than 1% of the total activity.

The necessity for a broad ammonium sulfate cut to precipitate most of the OASS activities from both legume species studied is in accord with results reported for enzyme preparations from spinach leaves (7), mature rape leaves (14), kidney bean seedlings (22), and mature radish roots (23). The ammonium sulfate step also indicated some variability in the tolerance of the enzymes to ammonium sulfate, with preparations from *P. vulgaris* losing 39% of their activity when the ammonium sulfate saturation was increased from 40 to 90% while preparations from *P. polyanthus* lost only 6% of their activity. The enzymes appear to be stable at low ionic strength as indicated by the fact that incubation of partially purified enzyme preparations for 4 hr in a range of pH 7.6 tris-HCl buffer concentrations of 0.2 M to 8.9 mM resulted in no loss of activity.

Measurement of enzyme activities was restricted to pH 7.6 since at more acid pH values, sulfide was lost by volatilization

Table II. Isolation of O-Acetylserine Sulfhydrylases from *P. vulgaris* Seedling Tissues

Step	Vol	Activity	Total protein	S.A. <sup>b</sup>	Yield
	ml	units <sup>a</sup>	mg		%
1. Supernatant fraction from centrifugation of crude homogenate	118	14679	3280	4.47	100.0
2. Activated charcoal (1.0 g)	114	14683	2964	4.95	100.0
3. Calcium phosphate gel (2.0 g)	110	14256	2640	5.40	97.1
4. 0-40% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> supernatant fraction	110	16192	1925	8.41	110.3
5. 40-90% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> pellet resuspended	26	9880	1404	7.04	67.3
6. Dialysate after 20,000 g centrifugation	34	8908	1306	6.55	60.7
7. Pooled peak fractions from first Sephadex DEAE A-50 column	110	5478	81.95	66.85	37.3
8. Pooled peak fractions from second Sephadex DEAE A-50 column followed by concentration with UM-10 membrane	2.0	3487	13.9	250.80	23.8
9. Acrylamide gel separation					
Slice #22 isoenzyme A (see Fig. 1)	0.4	3.48	.01333	261	— <sup>c</sup>
Slice #26 isoenzyme B (see Fig. 1)	0.4	2.87	.01333	215	—

<sup>a</sup>  $\mu\text{mol min}^{-1}$  as determined by sulfide electrode assay (0.1 M Tris-HCl pH 7.6 25°C plus 0.1 mM pyridoxal phosphate, 20 mM O-acetyl-L-serine, 0.85-0.9 mM sulfide) start reaction with enzyme.

<sup>b</sup>  $\mu\text{mol min}^{-1}$  mg protein<sup>-1</sup>.

<sup>c</sup> 35  $\mu\text{l}$  of the enzyme stock in step 8 (i.e. about 60 units of activity) loaded onto the gel and 6.35 total units for the two isozymes recovered, giving yield of 10.6%.

Table III. Isolation of O-Acetylserine sulfhydrylases from *P. polyanthus* Seedling Tissues

Step	Vol.	Activity	Total protein	S.A. <sup>b</sup>	Yield
	ml	units <sup>a</sup>	mg		%
1. Supernatant fraction from centrifugation of crude homogenate	55	4554	957	4.75	100.0
2. Activated charcoal (0.5 g)	53	4844	826.8	5.85	106.4
3. Calcium phosphate gel (1.0 g)	50	4580	680.0	6.73	100.6
4. 0-40% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> supernatant fraction	50	3940	500.0	7.88	86.5
5. 40-90% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> pellet resuspended	10	3680	410.0	8.97	80.8
6. Dialysate after 20,000 x g centrifugation	12	3305	408.0	8.10	72.6
7. Pooled peak fractions from first Sephadex DEAE A-50 column	70	1407	29.4	47.85	30.9
8. Pooled peak fractions from second Sephadex DEAE A-50 column followed by concentration with UM-10 membrane	2.0	1248	6.6	189.09	27.4
9. Acrylamide gel separation					
Slice #21 isoenzyme A (see Fig. 1)	0.4	0.74	.0224	33.03	— <sup>c</sup>
Slice #25 isoenzyme B (see Fig. 1)	0.4	16.15	.0208	776	—

<sup>a</sup>  $\mu\text{mol min}^{-1}$  as in Table II.

<sup>b</sup>  $\mu\text{mol min}^{-1}$  mg protein<sup>-1</sup>.

<sup>c</sup> 170  $\mu\text{l}$  of enzyme from step 8 (i.e. about 106 units of activity) loaded onto the gel and 16.89 total units for the two isozymes recovered, giving a yield of 15.9%.

and irreversible enzyme inactivation at pH values below 7.4. At pH values higher than 7.6, OAS is rapidly converted to N-acetylserine which is not a substrate for the enzyme from bacteria (12) or plants (14).

For the storage of the enzyme stocks, the best results were obtained when peak fractions eluted off the second DEAE column (step 8, Tables II and III) were concentrated and stored at 0 to 4°C in 0.1 M Tris-HCl (pH 8), 0.1 mM pyridoxal phosphate, 10 mM mercaptoethanol, and 15% (v/v) glycerol. Slower loss of enzyme activity by storage of stocks at 0 to 4°C rather

than -20°C has also been reported for other plant sources (14, 22, 23).

Molecular weights of the purified enzyme preparations were estimated by agarose gel filtration and polyacrylamide gel electrophoresis to be in the range of 65,000 to 70,000. This is in good agreement with the 62,000, 66,000, and 68,000 reported for preparations from mature rape leaves (14), radish roots (23), and bacteria (1), respectively. Hendrickson and Conn (10) reported 41,000 for the enzyme from blue lupine seedlings.

Before the kinetic studies were carried out, tests for possible

interfering reactions were made. In bacterial systems, a multifunctional protein complex of serine transacetylase-O-acetylserine sulfhydrylase as well as a cysteine desulfhydrase, have been reported (5, 11, 12). No activity of these enzymes was detected in these preparations. Similar results have been reported for these interfering reactions in enzyme preparations from mature rape leaves (14) and kidney bean seedlings (20, 22). Tests for the reverse reaction of OASS gave very slow and variable rates, about 1,000 times slower than the forward reaction.

**Separation of Isoenzymes.** When aliquots of the pooled and concentrated enzyme fractions from step 8 of the purification procedure (Tables II and III) were subjected to polyacrylamide gel separation, activity profiles as shown in Figure 1 were obtained. When the *P. vulgaris* enzymes eluted from gel slices 22 and 26 were reelectrophoresed on two separate gels which were not sliced but stained, one gel showed a single band with  $R_F$  of 0.48 while the other gel showed a single band with  $R_F$  of 0.58. Reelectrophoresis of a mixture of the two eluted enzymes on a third gel revealed two distinct bands with  $R_F$  values of 0.49 and 0.57 (Fig. 2). Similar results were obtained for *P. polyanthus*

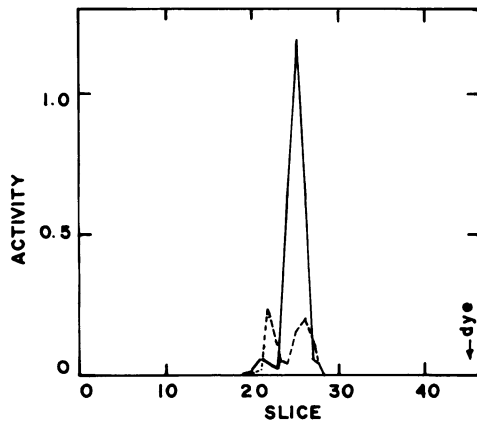


FIG. 1. Separation of OASS isoenzymes by gel electrophoresis. Gels sliced and assayed as described under "Materials and Methods." (—): isoenzymes from *P. polyanthus*; (---): isoenzymes from *P. vulgaris*. Activity in  $\mu\text{mol min}^{-1}$ .

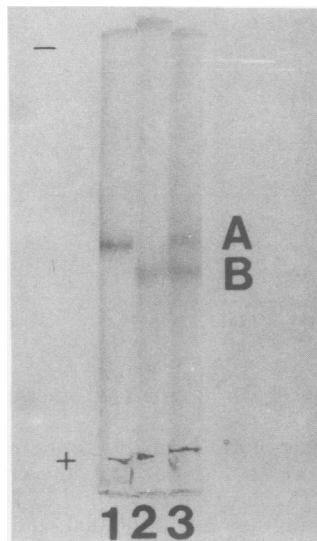


FIG. 2. Disc electrophoresis of OASS isoenzymes A and B from *P. vulgaris*. Gels run and stained as described in text. Gel 1: purified isoenzyme A; gel 2: purified isoenzyme B; gel 3: mixture of purified isoenzymes A and B.

enzymes eluted from gel slices 21 and 25 (Fig. 1) in that reelectrophoresis on separate gels resulted in a single band on each gel with  $R_F$  values of 0.47 and 0.55, while a mixture of the two enzyme forms run on a third gel gave two distinct bands of  $R_F$  values 0.48 and 0.56.

For mature rape leaf enzyme preparations, Masada *et al.* (14) reported that gel electrophoresis of a mixture of the two enzyme forms in the presence of pyridoxal phosphate in the gels and buffers resulted in apparent coincidence of the bands. However, we find that electrophoresis of a mixture of the two enzyme forms from either *Phaseolus* species in the presence of 0.1 mM pyridoxal phosphate (or 5 mM mercaptoethanol) in both the gels and buffer systems resulted in two distinct bands with the same  $R_F$  values as obtained in the absence of pyridoxal phosphate (or mercaptoethanol).

**Comparative Kinetic Studies of O-Acetylserine Sulfhydrylase Isoenzymes.** The isoenzymes separated as in Figure 1 were designated A (for the slower moving form,  $R_F$  0.47–0.49) and B (for the more mobile form,  $R_F$  0.55–0.58). These four isoenzymes were used in a preliminary kinetic study of the response of each to the substrates ( $S^{2-}$  and OAS) of the reaction.

Since OASS from *Salmonella* has been shown to have double competitive bi-bi ping-pong reaction mechanism with both substrates displaying substrate inhibition (5), it was expected that the enzymes from bean probably would also be inhibited by their substrates. With respect to  $S^{2-}$ , this expectation is borne out for *P. vulgaris* isoenzymes A and B as shown in Figure 3. In this case, concentrations of  $S^{2-}$  higher than 1 mM produce a progressive inhibition of the reaction, although there the inhibition was limited to about 50% of the maximal velocity attained. The curves in this figure are drawn from point to point because it was found impossible to obtain a satisfactory fit by the gradient least squares method (3) to any simple model of substrate inhibition. For this reason accurate kinetic constants for these isoenzymes have not been obtained by this method. The best fit, obtained as described below, indicates that the apparent  $K_m$  values for  $S^{2-}$  are approximately 1 mM, and that the inhibition constants for  $S^{2-}$  are also about 1 mM for both isoenzymes. As may be seen in Figure 4, more accurate estimates of  $K_m$  may be calculated by other methods.

The poor fit to the data of Figure 3 given by the usual substrate inhibition equation:  $v = VS/K_m + S^2/K_i + S$ , where the symbols have their usual meaning, indicates that a more complex mechanism may be involved. In fact, a model which assumes that sulfide binds at three sites on these enzymes, one

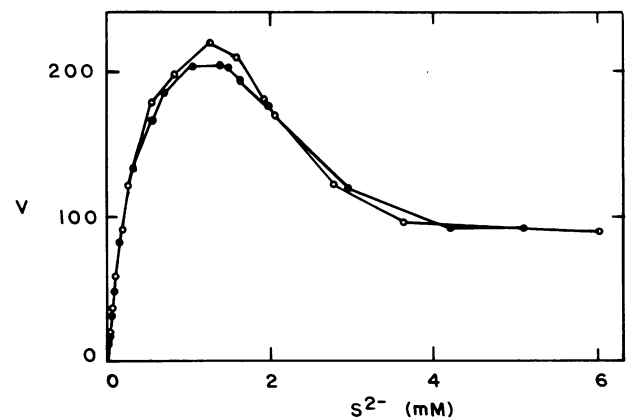


FIG. 3. Response of the activity of OASS isoenzymes from *P. vulgaris* to  $S^{2-}$  concentration. Assays by sulfide ion selective electrode using a constant 20 mM concentration of OAS and variable  $S^{2-}$  concentrations. Specific activity expressed as  $\mu\text{mol } S^{2-}$  disappearing from reaction medium  $\text{min}^{-1} \text{mg protein}^{-1}$ . ●: isoenzyme A; ○: isoenzyme B. V in  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ .

the active site, and two other sites which produce inhibition, gives a better fit. Data to confirm such a mechanism are not presently available. An additional complexity in the mechanism which mitigates against fitting the results to a simple model becomes apparent if we examine the response of the *P. vulgaris* isoenzymes A and B to sulfide concentrations below 2 mM (Fig. 4).

The inflection in the Woolf plot which occurs at about 0.3 mM S<sup>2-</sup> is indicative of positive cooperativity. The change in slope is significant for both isoenzymes at *P* < 0.01, although there is no significant difference between the slopes of the lines for isoenzymes A and B in either the low substrate or high substrate phases of the plots. As may be seen in Table IV, the effect of the allosteric response to S<sup>2-</sup> is to change the *K<sub>m</sub>* for S<sup>2-</sup> from about 1 mM to about one-fourth of that value. The over-all effect is exemplified in the Hill numbers for the same data which are 1.4 for isoenzyme A and 1.5 for isoenzyme B. These values are probably low because the inhibitory effect of S<sup>2-</sup> is somewhat manifested at these concentrations reducing the apparent sigmoidicity of the response.

The response of isoenzymes A and B from *P. vulgaris* to OAS is shown in Figure 5. Unlike the responses to S<sup>2-</sup> seen in Figures 3 and 4, there is in this case a clear difference between the isoenzymes in the response to substrate. Isoenzyme A shows no indication of inhibition by OAS at concentrations up to 50 mM and the line is fitted with the standard Michaelis-Menten relationship, indicating a *K<sub>m</sub>* of about 4 (see Table IV). Isoenzyme B, on the other hand, has a *K<sub>m</sub>* for OAS of only 1.6 mM and is moderately inhibited by this substrate. The fitting process indicates a *K<sub>i</sub>*<sub>OAS</sub> of about 80 mM. This difference is not an artifact of a single enzyme preparation, as similar results have been ob-

tained with the isoenzymes separated from *P. vulgaris* in several different preparations.

The isoenzymes of OASS from *P. polyanthus* display quite different relative activities, but their responses to S<sup>2-</sup> are essentially identical (Fig. 6 and Table IV). Both isoenzymes A and B have a *K<sub>m</sub>* for S<sup>2-</sup> of about 0.2 mM, in the range of the *P. vulgaris* *K<sub>m</sub>* values (Fig. 3) found at intermediate sulfide concentrations. The inhibition by sulfide, however, is less pronounced than with the *P. vulgaris* enzymes and the *K<sub>i</sub>* indicated by fitting to the substrate inhibition equation is about 10-fold

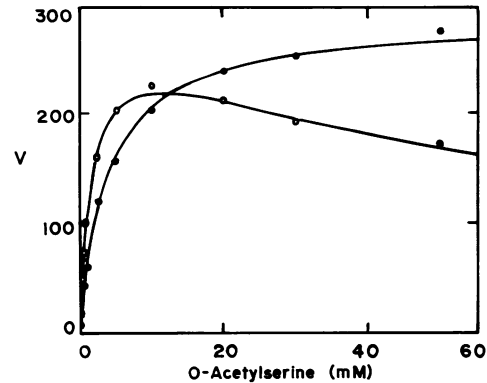


FIG. 5. Response of OASS isoenzymes from *P. vulgaris* to OAS concentration. Assay procedures as in Figure 2 with a constant 1 mM concentration of S<sup>2-</sup> and variable OAS concentrations. ●: isoenzyme A, line fitted by weighted fit procedure to a rectangular hyperbola; ○: isoenzyme B, line fitted by gradient least squares procedure (3) to the substrate inhibition equation (see text). V in μmol min<sup>-1</sup> mg<sup>-1</sup>.

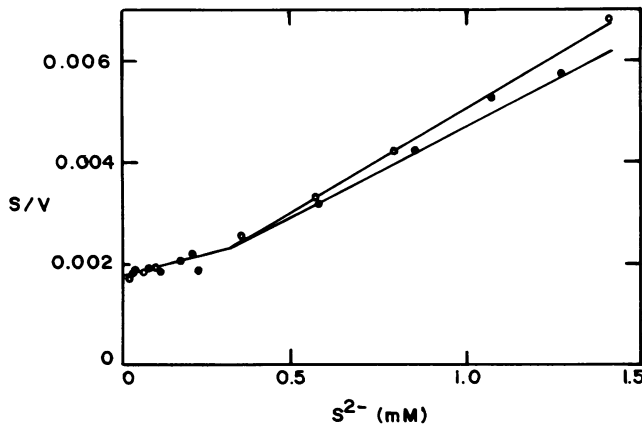


FIG. 4. Response of OASS isoenzymes from *P. vulgaris* to S<sup>2-</sup> at concentrations below 2 mM. The lines of the Woolf plot, fitted by the method of least squares, show an upward inflection indicating positive cooperativity. Assay procedures as in Figure 2. ●: isoenzyme A; ○: isoenzyme B.

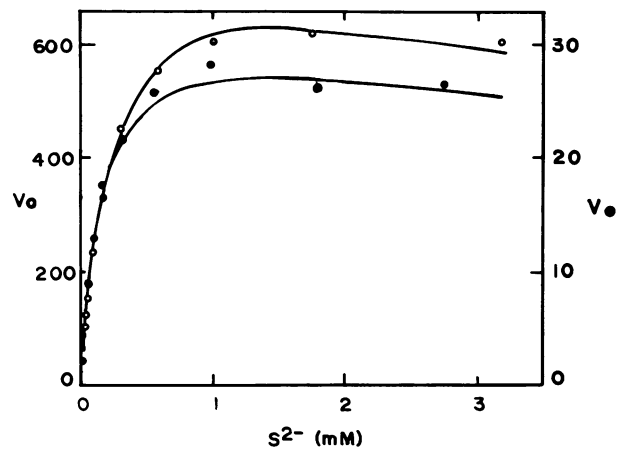


FIG. 6. Response of OASS isoenzymes from *P. polyanthus* to S<sup>2-</sup> concentration. Assay procedures as in Figure 2. Lines fitted by gradient least squares procedure (3) to substrate inhibition equation (see text). ●: isoenzyme A; ○: isoenzyme B. V in μmol min<sup>-1</sup> mg<sup>-1</sup>.

Table IV. Kinetic Constants for Isoenzymes of O-acetylserine Sulphydrylase from *Phaseolus vulgaris* and *P. polyanthus*. Constants obtained as described in text.

Isoenzyme	<i>K<sub>m</sub></i> <sub>S<sup>2-</sup></sub>	<i>K<sub>m</sub></i> <sub>OAS</sub>	<i>K<sub>i</sub></i> <sub>S<sup>2-</sup></sub>	<i>K<sub>i</sub></i> <sub>OAS</sub>	Relative Activity <sup>a</sup>
<i>P. vulgaris</i> A	0.93	0.28	3.83	1 <sup>b</sup>	17,700
<i>P. vulgaris</i> B	0.99	0.33	2.28	1 <sup>b</sup>	14,600
<i>P. polyanthus</i> A	0.18	0.75	10.1	— <sup>c</sup>	2,200
<i>P. polyanthus</i> B	0.28	3.89	7.5	152	52,700

<sup>a</sup> On the basis of a molecular weight of 68,000 daltons  
<sup>b</sup> Approximate value, see text  
<sup>c</sup> No inhibition

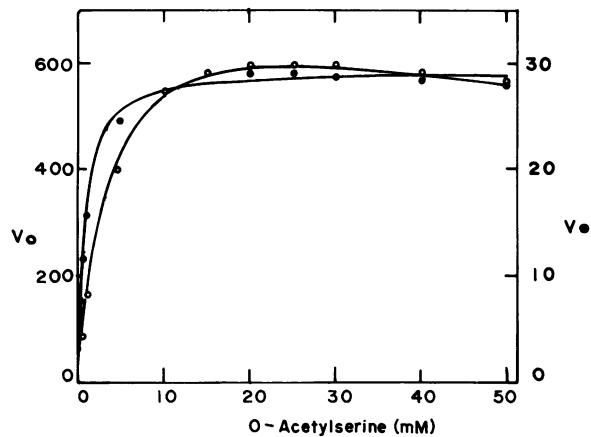


FIG. 7. Response of OASS isoenzymes from *P. polyanthus* to OAS concentration. Assay procedures as in Figure 4. ●: isoenzyme A, line fitted by weighted fit procedure to a rectangular hyperbola; ○: isoenzyme B, line fitted by gradient least squares procedure (3) to substrate inhibition equation (see text).  $V$  in  $\mu\text{mol min}^{-1} \text{min}^{-1}$ .

larger than the estimated  $Ki_{S^{2-}}$  found with the *P. vulgaris* enzymes.

With respect to OAS, the responses of the *P. polyanthus* isoenzymes are similar to those of from *P. vulgaris* except for the difference in relative activities (Fig. 7). The  $Km_{OAS}$  of isoenzyme A is smaller than that for isoenzyme B and there is little or no inhibition by OAS in isoenzyme A, while isoenzyme B has a  $Ki_{OAS}$  value about twice as large as that of the *P. vulgaris* isoenzyme B.

In summary, the kinetic characteristics of the four OASS isoenzymes from *P. vulgaris* and *P. polyanthus* show differences both between the isoenzymes from the same species and between those from the two species. The *P. vulgaris* isoenzymes are about equally active, both show positive cooperativity with respect to sulfide and strong and approximately equal substrate inhibition by sulfide. Toward OAS, however, one has a smaller  $Km$  than the other and the one with the small  $Km$  is inhibited by OAS, while the other is not. In the case of the *P. polyanthus* isoenzymes, no sigmoidicity is apparent in response to sulfide, but both are somewhat inhibited by this substrate. The  $Km$  for OAS is smaller for the slow moving (A) isoenzyme, but little or no substrate inhibition is seen with this enzyme form, while isoenzyme B is inhibited by OAS, although with a larger  $Ki$  value than with the *P. vulgaris* isoenzyme B. The primary difference between the *polyanthus* isoenzymes is in their specific activity, with isoenzyme B having a specific activity 20-fold higher than A. This last characteristic could, of course, represent an enzymically inactive contaminating protein which runs with isoenzyme A, and which does not occur in the *P. vulgaris* preparations.

**Alternate Substrates.** Although the exact nature of the substrates metabolized *in vivo* is uncertain, it has been reported for OASS from rape seedlings (18) as well as mature radish roots (23) that OAS is the much preferred substrate. Negative results were obtained with L-serine, N-acetyl-L-serine, and O-acetyl-L-homoserine for purified enzyme preparations from mature rape leaves although the concentration ranges tested were not stated (14). No other role than in cysteine or S-methylcysteine biosynthesis has been postulated for OAS in *Phaseolus* (20). In the enzyme preparations under consideration here, L-serine over a concentration range of 0.25 to 100 mM gave rates 5 to 10% that of saturating OAS while no rates were observed with  $\beta$ -chloroalanine; however, 20 mM O-propionyl-L-serine gave the same activity as 20 mM OAS.

## DISCUSSION

**Isolation and Physical Properties.** Some differences seem to

exist between the enzyme preparations reported here and other investigations. First, it has been claimed that not only significant purification but also stabilization of the enzymes from mature rape leaves (14) and radish roots (23) occurred by subjecting the initial crude homogenates to 70 C for 3 min. Enzyme activities eluted from the first DEAE column in these studies decrease rapidly at temperatures > 40 to 45 C. Even the purified enzyme preparations following polyacrylamide gel separation lose 10 to 15% of their activity on standing at room temperature (23–25 C) for 24 hr. Second, there is evidence that pyridoxal phosphate is a component of OASS from both bacteria (5, 12) and mature plant tissues (14, 23). Although no stimulation of enzyme activity by exogenous pyridoxal phosphate was reported for enzymes from kidney bean seedlings (22) or mature rape leaves (14), dialysis experiments on the preparations in the present studies indicated that the holoenzyme is somewhat labile. Both dialysis and DEAE chromatography in K-phosphate buffer enhance this lability, perhaps through phosphate suppressing reassociation of the pyridoxal phosphate. Since these experiments used preparations containing both enzyme forms it may be that one form has a more labile pyridoxal phosphate than the other. There is some evidence for OASS from mature rape leaves that this may be the case (14). For these reasons, a tris-HCl buffer system rather than phosphate was used for all isolation and assay buffers in these studies. Loss in enzyme activity in phosphate buffer whether pyridoxal phosphate is present or not may partly explain the poor recovery of units of enzyme activity and the lower specific activities found in investigations employing phosphate buffers (14, 22, 23).

### Separation and Kinetic Properties of Two Enzyme Forms.

The broad distribution of activity in ammonium sulfate cuts in addition to two peaks of activity eluting from the DEAE columns hinted at the possible existence of more than one enzyme form in the aerial parts of the legume seedlings tested in this report. Giovanelli and Mudd (7) reported earlier two ammonium sulfate fractions which contained OASS activity while Ngo and Shargool (18) had visualized compartmentalized isoenzymes with one for producing cysteine for synthesis of thioglucosides and the other for cysteine incorporated into protein. We have obtained direct evidence for the existence of two enzymes by polyacrylamide gel electrophoresis which resulted in separation of two forms from each legume species tested in this report. Purified enzyme preparations from mature rape leaves (14) were also found to consist of two enzyme forms which have similar molecular size, subunit composition, and  $Km$  for OAS but which differ in electrophoretic mobility, stability at 0 to 4 C, fluorescence under UV light, and specific activities. Masada *et al.* (14) referred to the slower moving band during gel electrophoresis as a "modified" OASS, the modification by implication being due to some alteration in the pyridoxal phosphate chromophore, and stated that the activity of this enzyme form could not be raised to that of the faster moving "native" enzyme by preincubation with pyridoxal phosphate but that the electrophoretic mobility of the two enzyme forms "coincided well" if electrophoresed in the presence of pyridoxal phosphate. This is clearly not the case for enzymes from *Phaseolus* seedlings. Pyridoxal phosphate (0.1 mM) was present in all isolation media and assay buffers and, in one experiment, in the gels and buffers during electrophoresis, and there still resulted two distinct enzyme forms although the activities of the two enzymes eluted from the gel slices were about the same for *P. vulgaris* but markedly different for *P. polyanthus*. Whether or not these differences are due to some alteration in the state of pyridoxal phosphate in the enzymes remains to be determined. It is interesting to note in this context that Tamura *et al.* (23) found only one enzyme form in preparations from mature radish roots.

The  $Km$  values for sulfide obtained for either enzyme form from either *Phaseolus* species studied in this report are well within the range of 0.13 to 0.43 mM reported for bacteria (5)

and rape seedlings (16), respectively. The  $K_m$  values for O-acetylserine also fall in the range 2.1 to 6.2 mM reported for *Salmonella* (5), mature rape leaves (14), rape seedlings (17), and mature radish roots (23).

The sulfide electrode assay as used in this report has permitted a more detailed and precise evaluation of the kinetic parameters of enzyme forms separated from two legume species than has been reported for OASS from other plant sources. The differences with respect to sulfide inhibition between the enzyme forms separated from *P. vulgaris* and *P. polyanthus* and the more subtle but consistent kinetic differences with respect to the other substrate, OAS, may be one way that levels of cysteine in plant tissues are controlled and the observed characteristics may be related to the differing levels of cysteine found in seed protein of the two species studied here (2).

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