Purification and Characterization of O-Acetylserine Sulphydrylase Isoenzymes from Datura innoxia*

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Three isoenzyme forms (designated A, B, and C) of O-acetylserine sulphydrylase were purified from Datura innoxia suspension cultures. Isoenzyme A is the most abundant form, comprising 45-60% of the total activity. Isoenzymes C and B comprise 35-40% and 10-20% of the activity, respectively. The specific activities of the purified isoenzymes are similar (870-893 pmol of cysteine/min/mg of protein). Molecular masses for isoenzymes A, B, and C, estimated by analytical size exclusion high performance liquid chromatography, are 63, 86, and 63 kDa, respectively. Isoenzymes A and B are homodimers; isoenzyme C is a heterodimer. Spectral analysis indicates that these isoenzymes possess a pyridoxal 5'-phosphate cofactor that binds the O-acetylserine substrate. Binding is reversible by addition of the sulfide substrate. The O-acetylserine sulphydrylase isoenzymes are active over a broad temperature range, with maximum activity between 42 and 58 °C. They are active only between pH 7 and 8, with optimal activity at pH 7.6. Kinetic analysis indicates these enzymes are allosterically regulated and exhibit positive cooperativity with respect to both substrates. They are inhibited by sulfide concentrations above 200 μM. The kinetic analysis together with the physical and spectrophotometric characteristics indicate that the O-acetylserine sulphydrylase enzymes have two active sites.

Plants and certain algae are the only eukaryotes to assimilate inorganic sulfur into organic compounds (Schmidt and Jäger, 1992). Incorporation of inorganic sulfur into cysteine by the action of O-acetylserine sulphydrylase is the final step in sulfate reduction and assimilation in plants. Cysteine is synthesized from serine in a two-step process. Serine acetyltransferase (EC 2.3.1.30) catalyzes the acetylation of serine by acetyl-CoA to form O-acetylserine. O-Acetylserine sulphydrylase (EC 4.2.99.8) then catalyzes the addition of inorganic sulfide to O-acetylserine with the release of acetate.

In plants, O-acetylserine sulphydrylase provides cysteine as a precursor for all cellular metabolites requiring reduced sulfur. One of the most abundant is the tripeptide, glutathione. Glutathione is a major cellular reducing agent and as such is instrumental in protecting the cell from oxidative damage caused by a number of stress and injury conditions, including presence of toxic metals, heat shock, and radiation (for reviews, see Larsson et al. (1983); Meister and Anderson (1983); Reed and Fariss (1984); Denke and Fanburg (1989); and Fahey and Sundquist (1991)). Synthesis of glutathione and its precursors may be induced by conditions causing physiological stress.

The presence of O-acetylserine sulphydrylase activity in plants was documented during the 1960s and 1970s (Smith and Thompson, 1971; Masada et al., 1975; Ascaino and Nicholas, 1977; Bertagnoli and Wedding, 1977). However, much of this earlier work was conducted with crude plant extracts, or the purity of the enzyme preparations was not demonstrated. Improvements in separation chemistry have allowed us to obtain highly purified enzyme preparations from plants, and to initiate a more critical evaluation of this enzyme. Plants contain multiple isoenzyme forms of O-acetylserine sulphydrylase that are located in the organelles and the cytosol (Bertagnoli and Wedding, 1977; Ikegami et al., 1987; Nakamura and Tamura, 1989; Lunn et al., 1990; Kuske et al., 1992; Droux et al., 1992; Rolland et al., 1992). The O-acetylserine isoenzymes from Datura innoxia probably perform different metabolic functions in the plant cell and respond differently to different stimuli or requirements for cellular metabolites.

We have purified three O-acetylserine sulphydrylase isoenzymes from D. innoxia and are investigating the roles that these three isoenzymes play in plant cell metabolism, particularly with respect to sulfur assimilation and stress-induced use of cysteine in biosynthesis of glutathione and its metal-binding derivatives (Delhaize et al., 1988; Jackson et al., 1992; Jackson and Kuske, 1993). We present here a comparison of their physical and in vitro catalytic properties.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Except where noted, reagents for enzyme assays, buffers, protein purification, and silver staining were the highest purity obtainable and were purchased from Sigma. Chromatography resins were purchased from Pharmacia LKB Biotechnology Inc. Coomassie dye-binding reagent and protein standards were obtained from Bio-Rad, and the BCA (bicinchoninic acid) assay kit was obtained from Pierce Chemical Co.

**D. innoxia Cell Suspension Cultures**

Cadmium-tolerant (Cd 160ppm and Cd 300ppm) and sensitive (wild-type WDI and a cadmium-sensitive revertant of Cd 300ppm, Cd 300ppm) D. innoxia cell suspension cultures were grown in the dark at 30–33 °C in a modified 1BS medium as described by Jackson et al. (1984). Plant cells for protein purification were grown as 100 ml batch shake cultures in 500-ml De Long flasks at 30–33 °C. Cultures were maintained by dilution of one part cell culture to three parts fresh medium every 3 days. This induced a burst of cell growth. The cadmium-tolerant cells are able to grow continuously in medium containing 250 μM CdCl₂, while the sensitive cell lines die within 48 h following exposure to this CdCl₂ concentration (Delhaize et al., 1989).
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O-Acetylserine Sulphydrylase Purification

Soluble Protein Extraction—Due to equipment and space constraints, we purified the O-acetylserine sulphydrylase proteins in multiple batch runs, each beginning with 250–800 g wet weight of cells from 1-1.5 L of original medium. Cadmium-tolerant D. innoxia (Cd 300³) were harvested 3 days after transfer to fresh medium. Cells were collected by centrifugation at 100 x g for 1 min using a tabletop swinging bucket centrifuge. Growth medium was decanted from the pelleted cells and replaced with an equal volume of extraction buffer (30 mM Tris, pH 7.8, 10 mM β-mercaptoethanol). Cells were thoroughly suspended in this buffer and pelleted as above. The buffer was aspirated from the pelleted cells, and the wet weight of cells was recorded. Pelleted and rinsed cells were quickly frozen in liquid nitrogen and ground to a fine powder. The powder was resuspended in 50°C extraction buffer and cooled in an ice-water bath. All subsequent procedures were carried out at 0–4°C. The blood was centrifuged at 21,000 x g for 20 min, and the supernatant was collected. Batch preparations contained between 2.5 and 5 g of soluble protein. Preliminary experiments indicated that the O-acetylserine sulphydrylase activity was associated with the soluble protein fraction.

Ammonium Sulfate Precipitation—Granular ammonium sulfate was slowly added to the supernatant with stirring to 20% saturation and the precipitate was collected by centrifugation at 21,000 x g for 20 min. The pellet was discarded and the supernatant was slowly brought to 80% ammonium sulfate saturation. The precipitated protein was pelleted by centrifugation at 21,000 x g for 20 min and gently resuspended in 20–80 ml of 60 mM Tris, pH 7.8, 10 mM β-mercaptoethanol, 10% glycerol.

Gel Filtration Chromatography—Ten- to 12-ml aliquots of the protein sample were applied to Sephacryl 200 HR columns (100 x 2.5 cm) equilibrated in 60 mM Tris, pH 7.8, 10 mM β-mercaptoethanol, 10% glycerol. O-Acetylserine sulphydrylase-active fractions eluted as a broad single peak following the void volume protein peak that contained most of the buffer was exchanged into 10 mM Tris, pH 7.8, and eluted in a 60-ml linear gradient of 0-450 mM NaCl in 10 mM Tris, pH 8.1. One-ml fractions were assayed for O-acetylserine sulphydrylase activity.

Native Molecular Mass and Subunit Structure of the O-Acetylserine Sulphydrylase Isoenzymes

Native molecular masses of the three O-acetylserine sulphydrylase isoenzymes were estimated using gel filtration HPLC on an analytical Bio-Gel SEC-250 column (300 x 7.8 mm) eluted in 70 mM Tris, pH 7.8. Purified isoenzymes were applied to the column, separately and as mixtures of A plus B or A plus C, along with the following protein standards: thyroglobulin, 670 kDa; IgG, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; and vitamin B-12, 1.35 kDa. Flow rate was 1 ml/min. Protein elution was monitored by absorbance at 280 nm, and 200-ul fractions were collected and tested for O-acetylserine sulphydrylase activity as described above. Molecular mass (m) was calculated from the linear relationship between m and relative elution volume (Vc/Vo).

Amino Acid Analysis of O-Acetylserine Sulphydrylase Proteins

Amino acid compositions of the three O-acetylserine sulphydrylase isoenzymes were determined using a Pico-Tag amino acid analysis system (Biodynamics, 1984; Cohen et al., 1989). Purified proteins were hydrolyzed under vacuum in constantly boiling HCl at 112°C for 45 h and derivatized with phenylisothiocyanate. PTC-derivatized amino acids were separated by HPLC on a calibrated Waters C-18 Pico-Tryclosil column (15 cm x 3 mm) at 41°C. The presence of tryptophan was not determined. Cysteine was present in all three proteins but was not derivatized prior to hydrolysis and therefore was not quantified.

UV/Visible Spectral Studies of O-Acetylserine Sulphydrylase Isoenzymes

Ab sorption spectra were measured in a Perkin-Elmer Lambda 3B UV/VIS dual beam spectrophotometer, recording at 120 nm/min and 10 nm/cm, using a 1-cm path length. The O-acetylserine sulphydrylase proteins were analyzed in either 10 mM Tris, pH 8.0 (Fig. 4) or 50 mM Tris, pH 7.8 (Fig. 5), and the spectrophotometer was blanked using the respective buffer solution. Pyridoxal 5'-phosphate was quantified by comparison of absorbance at 412 nm to a standard curve of known concentrations in 10 mM Tris, pH 8.0.

O-Acetylserine Sulphydrylase Activity Assay

O-Acetylserine sulphydrylase activity was assayed by measuring the production of L-cysteine. For initial steps of enzyme purification, 1-ml reaction mixtures contained 100 mM Tris, 20 mM O-acetylserine, 1 mM Na2S, as a reaction pH of 7.6. O-Acetylserine and Na2S were freshly prepared as 1 M and 100 mM stocks, respectively. Absorbance was recorded at 34°C for 2 min, and reactions were initiated by addition of 1-10 µl of protein.
sample. Each reaction was allowed to proceed at 34 °C for exactly 10 min. Reactions were stopped by addition of 200 μl of 1.5 M trichloroacetic acid. Precipitated protein was pelleted by centrifugation for 5 min in a microcentrifuge.

The amount of cysteine present in the supernatant was determined as described by Gaitonde (1967). One ml of the supernatant was transferred to a glass tube and mixed well with an equal volume of acid. The mixture was heated in a boiling water bath for exactly 5 min and then cooled on ice. Two ml of cold 100% EtOH were added to each reaction and mixed well. The cysteine concentration was determined spectrophotometrically by absorbance at 546 nm. This assay is linear for L-cysteine at concentrations between 0.01 and 0.5 μmol. The O-acetylserine sulfhydrylase activity assay was optimized with respect to pH, temperature, and concentration of each component and was linear with respect to the amount of enzyme assayed. One unit of activity is 1 μmol of cysteine/min/mg of protein. For purified and partially purified O-acetylserine sulfhydrylase samples (after size exclusion FPLC, the NaS4 concentration in reaction mixtures was reduced to 200 μM, after determining that this substrate is inhibitory to the purified enzyme at higher concentrations (see below).

Substrate Saturation Experiments

One-ml reaction mixtures contained 100 mM Tris, different concentrations of O-acetylserine and NaS4, and enzyme at a final reaction pH of 8.0. A stock of O-acetylserine was prepared in 100 mM Tris, pH 8.0, immediately before use and diluted as needed in the same buffer just before addition to the reaction mix. NaS4 was prepared immediately before use as a 100 mM stock in 100 mM Tris, pH 8.1, and diluted to the appropriate concentration immediately before addition to the reaction mix. The concentration of O-acetylserine was varied between 0 and 200 mM NaS4 NaS4 concentration was varied between 0 and 1200 μM O-acetylserine. Twenty substrate concentrations were tested for each series, with data points concentrated at the lower substrate levels (Figs. 7 and 8). Reactions were performed as described above and initiated by the addition of 90 ng (1–3 pl volume) of purified enzyme sample. Each reaction was individually timed and allowed to proceed at 34 °C for exactly 10 min. Reactions were stopped and analyzed for L-cysteine as described above. Assays were linear with respect to cysteine production over 30 min. Substrate saturation experiments were repeated three to five times for each O-acetylserine sulfhydrylase isoenzyme.

Effects of Temperature and pH on Enzyme Activity

Reactions for pH and temperature experiments contained 200 μM NaS4 and 20 mM O-acetylserine in 100 mM Tris buffer. For pH curves, the reaction mix pH was measured at 34 °C in parallel samples (minus enzyme). Reactions were initiated by addition of 1 μl of enzyme in 10 mM Tris, pH 7.6. The effect of different incubation temperatures on enzyme activity was examined by preincubating reaction mixtures for 5 min at the desired temperature, adding enzyme, and continuing incubation at that temperature for 10 min. Reactions were stopped and analyzed for cysteine as described above. All pH and temperature experiments were repeated three times for each O-acetylserine sulfhydrylase isoenzyme.

Effects of Cadmium and Other Metals on Activity

The presence of heavy metals induces biosynthesis of a specific class of metal-binding polypeptides in D. innoxia cell cultures (Robinson et al., 1988; Delhaise et al., 1989; Jackson et al., 1989; Rauzer, 1990). These metal-binding polypeptides are derived from glutathione, and their synthesis requires synthesis of glutathione and its amino acid precursors, cysteine, glycine, and glutamate. We explored the possibility that O-acetylserine sulfhydrylase activity might be influenced by the presence of certain metal ions. The effects of cadmium, copper, nickel, iron, cobalt, and nickel on O-acetylserine sulfhydrylase isoenzyme A were determined using the above standard assay. Metal concentrations of 0.01, 0.1, and 1 μM had no effect on enzyme activity. All metals were inhibitory at 10 μM.

Analysis of In Vitro Kinetic Data

Data from replicate experiments on each isoenzyme were pooled and analyzed by non-linear least squares regression fitting to three models.

Results

O-Acetylserine Sulfhydrylase Isoenzymes in D. innoxia—The same three isoenzymes were separated by DEAE-SPW HPLC from crude soluble proteins extracted from metal-tolerant and -sensitive D. innoxia cell cultures and from metal-tolerant cultures growing in the presence or absence of cadmium (Fig. 1). Isoenzyme A was the most predominant form of the enzyme in all of the D. innoxia cultures, comprising 45–60% of the total activity. Isoenzyme C comprised 35–40% and isozyme B comprised 10–20% of the total activity. The purified isozyme forms were identical to those detected in crude plant cell extracts, indicating that they are not artifacts of purification.

Purification of Three O-Acetylserine Sulfhydrylase Isoenzymes (Table I)—Plant cell cultures used for O-acetylserine sulfhydrylase purification were non-green, nonphotosynthetic cells, and thus did not possess the high concentrations of enzymes involved in photosynthesis in green tissues. In preliminary experiments, we found that O-acetylserine sulfhydrylase activity was present in a broad range of ammonium sulfate concentrations. This is probably due to the presence of multiple isozymes. Although little purification was achieved at this step, ammonium sulfate served to concentrate and stabilize the enzyme. O-Acetylserine sulfhydrylase isoenzymes co-eluted from the Sephacryl 200-HR column as a single broad peak eluting after the void peak that contained most of the protein. In repeated batch runs, no O-acetylserine sulfhydrylase activity was detected in the void protein peak. Isoenzymes A and B were separated by anion-exchange chromatography. Ammonium sulfate gradients of 50–250 mM NaCl in 10 mM Tris, pH 8.1. Isoenzyme A eluted with the bulk of the protein at about 120 mM NaCl, and B eluted at about 230 mM NaCl. Anion exchange HPLC on a Bio-Gel SEC DEAE-5-PW column purified isozyme B (Fig. 2B), and separated isozyme C from isozyme A. Nondenaturing acrylamide gel electrophoresis was used to separate isozymes A and C from a few remaining contaminant proteins (Fig. 2A and C). The three highly purified O-acetylserine sulfhydrylase isoenzymes from D. innoxia had similar specific activities (for example, ranging from 870 to 893 units in the illustrated batch purification).

The final proteins were of very high purity as determined by electrophoresis through SDS-polyacrylamide gels (Fig. 2), two-dimensional gels, and nondenaturing gels (data not shown).
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FIG. 1. Presence of three O-acetylserine sulphydrylase isoenzymes in suspension cell cultures of D. innoxia. Isoenzymes were separated by anion exchange HPLC on a DEAE-5-PW column equilibrated in 10 mM Tris, pH 8.1, by elution in a linear gradient of 0-300 mM NaCl. Fraction number 1 corresponds to 0 NaCl, and fraction number 60 to 300 mM NaCl. Isoenzyme A eluted at approximately 120 mM NaCl, isoenzyme C at 140 mM NaCl, and isoenzyme B at 245 mM NaCl.

### Purification of O-acetylserine sulphydrylase isoenzymes from D. innoxia

Values presented are from one of several batch purifications. Three isoenzymes were purified as described under "Experimental Procedures."

| Fraction          | Volume | Total Protein | Total Activity | Specific Activity | Yield | Purification |
|-------------------|--------|---------------|----------------|-------------------|-------|--------------|
| Crude             | 1800   | 6516          | 8518           | 1.3               | 100   | 1            |
| Supernatant       | 1680   | 5156          | 7683           | 1.5               | 90    | 1.2          |
| Ammonium sulfate  | 60×8   | 2370          | 5877           | 2.5               | 69    | 1.9          |
| Sephacryl 200-HR  | 90     | 501           | 2981           | 6.0               | 35    | 4.6          |
| DEAE CL-6B        |        |               |                |                   |       |              |
| A                 | 75×6   | 77            | 1022           | 13.3              | 12    | 10           |
| B                 | 38×6   | 9             | 199            | 22.1              | 2.3   | 17           |
| Bio-Sil SEC-250   |        |               |                |                   |       |              |
| A                 | 63×6   | 2             | 980            | 490               | 11.5  | 377          |
| B                 | 29×6   | 0.142         | 97             | 688               | 1.1   | 525          |
| DEAE-5-PW         |        |               |                |                   |       |              |
| A                 | 9.0    | 0.620         | 426            | 687               | 5.0   | 599          |
| B                 | 1.2    | 0.012         | 10.6           | 883               | 0.13  | 679          |
| C                 | 9.0    | 0.479         | 301            | 628               | 3.5   | 483          |
| Native gel        |        |               |                |                   |       |              |
| A                 | 3.0    | 0.122         | 109            | 893               | 1.3   | 687          |
| B                 | 3.5    | 0.100         | 87             | 870               | 1.0   | 669          |

* One international unit (IU) is 1 pmol of cysteine produced/min/ml.

### Specific Activity

The specific activities of O-acetylserine sulphydrylase enzymes purified here are comparable to those of O-acetylserine sulphydrylases identified from *Raphanus sativus* roots (Tamura et al., 1976), *Brassica chinensis* leaves (Masada et al., 1975), and *Salmonella typhimurium* (Becker et al., 1969); and isoenzymes A and B from *Pisum sativum* (Ikegami et al., 1987), which range from 892 to 1100 units. These values are 1.5–10 times higher than the specific activities of this enzyme from other plants (Murakoshi et al., 1985; Ikegami et al., 1987, 1988; Droux et al., 1992).

### Native Molecular Mass and Subunit Composition

The native molecular mass for both isoenzyme A and C estimated by size exclusion HPLC was 63 kDa (Fig. 3). This value falls in the mid-range of native molecular masses reported for O-acetylserine sulphydrylase proteins from other plants (Masada et al., 1975; Tamura et al., 1976; Bertagnolli and Wedding, 1977; Murakoshi et al., 1985; Ikegami et al., 1987, 1988; Droux et al., 1992), a eukaryotic alga (León et al., 1987), and bacteria.

Purified proteins were stable (i.e., no loss in specific activity) for 6 months when stored at −70 °C in 10–30 mM Tris, pH 8.1, containing 10% glycerol. They readily degraded when stored at 4 or −20 °C in buffered solutions, and with repeated freezing-thawing. Isoenzyme B was less stable than isozymes A and C, possibly because it was stored at a less concentrated solution.

Addition of the pyridoxal 5'-phosphate cofactor to the purification buffers or activity assay did not improve enzyme recovery or enzyme activity. At saturating O-acetylserine levels, additional pyridoxal 5'-phosphate had no effect on enzyme activity. At subsaturating levels of O-acetylserine, addition of 100 mM pyridoxal 5'-phosphate reduced O-acetylserine sulphydrylase activity to 81% at 10 mM O-acetylserine and to 50% at 0.5 mM O-acetylserine (data not shown).

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A B

FIG. 2. a, purified isoenzymes A, B, and C (Panels A, B, and C, respectively) of O-acetylserine sulfhydrylase from Datura innoxia were reduced by treatment with SDS and dithiothreitol and separated by electrophoresis in 12.5% acrylamide resolving gels. Molecular mass markers in lanes adjacent to each protein sample are phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), lactic dehydrogenase (36.5 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and cytochrome c (14 kDa). b, comparison of isoenzyme subunit structure. Isoenzymes A and B migrate as single bands with molecular mass of 32 and 43 kDa, respectively. Isoenzyme C migrates as two bands of approximate mass 32 and 31.2 kDa. The molecular mass markers illustrated are bovine serum albumin (68 kDa), lactic dehydrogenase (36.5 kDa), and carbonic anhydrase (29 kDa).

(Becker et al., 1969; Boronat et al., 1984). In addition to these abundant forms of O-acetylserine sulfhydrylase, we purified a less abundant form, isoenzyme B, that has similar specific activity to the other two isoforms, but has a molecular mass of 86 kDa (Fig. 3). This is the first report of an O-acetylserine sulfhydrylase protein this large.

The O-acetylserine sulfhydrylase proteins that have been described from plants, bacteria, and an alga are homodimers. Under reducing, denaturing electrophoresis conditions, the D. innoxia isoenzyme A ran as a single band at 32 kDa, indicating that the protein is a homodimer (Fig. 2A). Similarly, isoenzyme B ran as a single band at 43 kDa, indicating that it also is a homodimer (Fig. 2B). Purified isoenzyme C consistently produced two bands of equal intensity. The larger species was 32 kDa in size, and the second species was about 800 Da smaller. To determine whether this second protein band was a contaminant protein, samples of purified isoenzyme C were subject to electrophoresis through a series of nondenaturing acrylamide gels with acrylamide concentrations of 5, 7.5, 10, 12, 15, and 17.5% and silver-stained. The native protein was visible as a single band in all gels. The single protein band present in nondenaturing gels consistently gave rise to two silver-stained bands on SDS-polyacrylamide gels, after electroelution of the native protein from the nondenaturing gel. These results indicate that isoenzyme C is a heterodimer, a subunit structure that is unique among the O-acetylserine sulfhydrylase proteins that have been described.

Amino Acid Composition—The amino acid profiles of the three isoenzymes are similar. Histidine and serine contents varied between the isoenzymes, but there was little variation in the other amino acids (data not shown).

Spectral Properties of Purified O-Acetylserine Sulfhydrylase Proteins—Absorbance spectra for the three D. innoxia isoenzymes over wavelengths 190–850 nm were very similar with identical absorption maxima (Fig. 4). The spectra of the purified enzymes exhibited two major peaks with absorbance maxima at 278 nm, due to aromatic amino acids in the protein, and at 412 nm. The absorbance at 412 nm is characteristic of the aldoxime form of pyridoxal phosphate (Cook and Wedding, 1976; Kallen et al., 1985) and is due to a Schiff base formed between the pyridoxal phosphate cofactor and the ε-amino group of an enzyme lysine. Concentrated solutions of O-acetylserine sulfhydrylase protein are yellow due to the presence of this cofactor. In general, bound pyridoxal phosphate is present in many transaminase enzymes (Wilson and Crawford, 1965; Kaplan and Flavin, 1966; Schnackerz et al., 1979), with characteristic absorbance between wavelengths of 400 and 430 nm (Zeffren and Hall (1973) and Cook et al. (1992), for examples). This cofactor has also been identified in O-acetylserine sulfhydrylase from bacteria and other plants (Becker et al., 1969; Masada et al., 1975; Tamura et al., 1976; Murakoshi et al., 1985; Ikegami et al., 1987, 1988; Droux et al., 1992). The molar ratio of pyridoxal phosphate (A412) to protein (A278) was 2:1, suggesting that there is 1 molecule of pyridoxal 5'-
phosphate associated with each protein subunit. The O-acetylserine sulphydrylase protein appeared to be saturated with tightly bound pyridoxal 5'-phosphate, since the activity of the pure enzyme was not increased by addition of free cofactor.

The effects of the two substrates, O-acetylserine and sulfide, on the absorbance spectrum of isoenzyme A were determined. The enzyme (28 μg/ml in 50 mM Tris, pH 7.6) was first titrated with O-acetylserine at concentrations of 0.2, 1.0, 2.0, 4.0, 10.0, and 20.0 μM. Addition of the O-acetylserine substrate to the purified enzyme results in considerable change in the absorbance spectrum, especially with regard to the absorbance due to pyridoxal 5'-phosphate. In the presence of O-acetylserine at pH 7.6, there is a concentration-dependent spectral shift from 412 to 460 nm, accompanied by an increase in absorbance as a broad shoulder around 330 nm (Fig. 3). The enzyme preparation was saturated at 4.0 μM O-acetylserine. A similar spectral shift was observed in the pyridoxal 5'-phosphate containing enzyme, ω-serine dehydratase, by Schnackerz et al. (1979). They demonstrated that this type of spectral shift was due to the formation of a Schiff base intermediate between ω-aminoacylrate and the pyridoxal 5'-phosphate cofactor. Formation of this ω-aminoacylrate intermediate was reversible upon addition of the second substrate, sulfide. Similarly, the observed changes in absorbance of the O-acetylserine sulphydrylase-O-acetylserine solution could be reversed by titration with Na₂S (data not shown). In an O-acetylserine-saturated solution, addition of 4.0 μM Na₂S regenerated the original spectrum with the absorbance maxima at 412 nm, probably by its reaction with the intermediate to transfer the acetyl group of O-acetylserine to sulfide, forming the L-cysteine product (Cook and Wedding, 1976). The observed changes in O-acetylserine sulphydrylase absorbance spectra upon addition of O-acetylserine and reversion with addition of Na₂S illustrate the involvement of the pyridoxal 5'-phosphate cofactor in the catalytic activity of this enzyme. The spectral shift to 460 and 330 nm also spontaneously reverted to the original spectrum after several hours of storage, probably due to the instability of O-acetylserine at neutral pH (Kredich and Tomkins, 1966; Becker et al., 1969).

Effects of Temperature and pH on O-Acetylserine Sulphydrylase Activity—The three O-acetylserine sulphydrylase isoenzymes responded very similarly to changes in reaction temperature and pH. They were active over a broad range of temperature (Fig 6A). Activity generally increased from 20 to 30 °C and leveled off between 30 and 40 °C. Maximum activity was observed at temperatures between 42 and 58 °C. O-Acetylserine sulphydrylase activity was highly dependent on reaction pH. The enzymes were only active in the range of 7 to 8, with optimal activity of all isoenzymes at pH 7.6. Even slight changes in pH above or below this value dramatically reduced activity. For this reason, pH was carefully monitored at all concentrations of O-acetylserine and Na₂S used in enzyme assays. The purified O-acetylserine sulphydrylase proteins were irreversibly inactivated in buffers at pH below 6.8 and during isoelectric focusing, despite attempts to regain activity by addition of cofactor and pH adjustment. Similar inactivation has been observed in the O-acetylserine sulphydrylase from other organisms (Ikegami et al., 1988; Cook et al., 1992). Above pH 8.0, the O-acetylserine substrate undergoes an O- to N-shift, and is no longer a substrate for the enzyme (Murakoshi et al., 1985; Ikegami et al., 1988). By spectral analysis, Cook et al. (1992) have shown that at pH greater than 8.1 the ε-amino group of the active site lysine from bacterial O-acetylserine sulphydrylase becomes deprotonated resulting in deacylation activity. This generates pyruvate and ammonia from O-acetylserine, instead of cysteine.

Substrate Saturation Curves—The in vitro catalytic ability of the three O-acetylserine sulphydrylase forms was similar
overestimate this parameter. The amount of cooperativity, and the MMF equation appeared to be at least two active sites in these enzymes, with 1 molecule of pyridoxal 5'-phosphate per site. The values of \( n \), predicted from this isoenzyme in Table II. Results are illustrated for isoenzyme C only (Figs. 7 and 8).

**O-Acetylserine**—The saturation curves for O-acetylserine were sigmoid (Fig. 7b), suggesting the enzymes are allosteric and exhibit positive cooperativity with respect to this substrate. Note that all three equations used to fit the sigmoid portion of the O-acetylserine saturation curve have similar fits, indicated by the similar mean square error (M.S.E.) and coefficient of determination \( (R^2) \) values in Table II. The MMF model is a generalization of the Hill equation in that it allows for a non-zero intercept. Since we have no information on the shape of the curve very close to zero, it may be incorrect to assume the curve is strictly sigmoidal from zero to the observed data points. Allowing a non-zero intercept allows the model to fit more closely to the data and not extrapolate from the data down to zero. Since the Hill equation forces the curve down through zero, the \( S_0 \) tends to be reached sooner (smaller value) than using the MMF equation. The logistic equation, as a very general growth equation, produces estimates of \( V_{max} \) and \( S_0 \), that are comparable to the other two models for the O-acetylserine substrate. The data obtained from O-acetylserine saturation experiments of isoenzymes A and C provided better fits to the three models than isoenzyme B. The data set for isoenzyme B contained a great deal of scatter at O-acetylserine concentrations above 10 \( \mu \)M. This is reflected in the MSE and \( R^2 \) values for this isoenzyme in Table II.

Based on our findings for subunit size and cofactor presence in the O-acetylserine sulphydrylase proteins, we expect there to be at least two active sites in these enzymes, with 1 molecule of pyridoxal 5'-phosphate per site. The values of \( n \), predicted from the Hill and MMF equations, are consistent with this hypothesis. The Hill equation characteristically underestimates the amount of cooperativity, and the MMF equation appeared to overestimate this parameter.

In initial experiments, O-acetylserine sulphydrylase activity appeared to be inhibited by O-acetylserine concentrations above 25 \( \mu \)M (Fig 7a). Cook and Wedding (1976) reported a similar finding for O-acetylserine from *S. typhimurium*. Measurements of reaction pH in these assays demonstrated that the high concentrations of O-acetylserine in these assays were reducing the reaction pH to below the narrow optimum range for O-acetylserine sulphydrylase activity (Fig. 6B). To compensate for this, the pH of the 100 mM Tris reaction buffer was increased incrementally by 0.2 increment, so that addition of the \( Na_2S \) and O-acetylserine substrates resulted in a final reaction pH of 7.6. When assays were conducted under these conditions, no inhibition was observed.

**Sodium Sulfide**—The early portion of the sodium sulfide saturation curves, up to 200 \( \mu \)M, were sigmoid (Fig. 8b) but the curves did not smoothly approach an asymptote. Instead they peaked and decreased abruptly. The addition of an inhibition curve to the sigmoid saturation curve made these curves more difficult to fit. The logistic model was able to fit the early part of the curve, and appears to provide the best estimates of \( V_{max} \) and \( S_0 \), in the presence of the inhibition curve. The Hill and MMF equations did not fit the early part of the curve as well and there is more uncertainty in the estimates of the asymptote \( V_{max} \) and \( S_0 \) using these models. Because of the inhibition portion of the curve, it is difficult to extrapolate physiological conclusions about the three isoenzymes based on these *in vitro* results. The estimates of \( n \) from the \( Na_2S \) data were between 1 and 3, which is consistent with the results of the O-acetylserine saturation curves, and our physical data on these proteins. The \( Na_2S \) substrate was inhibitory to all three enzymes at concentrations above 200 \( \mu \)M. Similar inhibition has been described in O-acetylserine sulphydrylase from *Phaseolus* sp. (Bertagnolli and Wedding, 1969) and *S. typhimurium* (Cook and Wedding, 1976).

**DISCUSSION**

Multiple forms of O-acetylserine sulphydrylase have been identified in plants using nondenaturing polyacrylamide gel electrophoresis (Bertagnolli and Wedding, 1977) and anion exchange chromatography (Ikegami et al., 1987, 1988; Nakamura...
and Tamura, 1989; Lunn et al., 1990; Kuske et al., 1992; Rolland et al., 1992). The number of isoenzymes identified from different plant species ranges from one to five. Most plants probably contain multiple forms of this enzyme, and some of the early studies of plant O-acetylserine sulfhydrylase enzymes were probably accomplished using mixtures of isoenzymes. We separated and purified three forms of the enzyme from D. innoxia with similar in vitro catalytic abilities but different physical properties. These three isoenzymes probably perform different functions in the plant cell.

In the bacterium, S. typhimurium, O-acetylserine sulfhydrylase activity is present as a free form and in association with serine transacetylase as a 309-kDa multienzyme complex comprised of 2 molecules of O-acetylserine sulfhydrylase and 1 molecule of serine transacetylase (Kredich and Tomkins, 1966; Becker et al., 1969; Kredich et al., 1969). We found no evidence of a similar complex in non-photosynthetic D. innoxia cell cultures, either by separation of crude protein extracts on anion exchange HPLC, or gel filtration chromatography. Similarly, Bertagnolli and Wedding (1977) found no evidence for such a complex in green tissue from two Phaseolus sp. using activity assays for both O-acetylserine sulfhydrylase and serine transacetylase. In contrast, Droux et al. (1992) recently identified that 3–5% of the O-acetylserine sulfhydrylase activity in Spinacia oleracea chloroplasts eluted as a 310-kDa complex that also contained serine transacetylase activity. If a similar complex exists in D. innoxia chloroplasts, it is possible that it is not formed in the non-photosynthetic plastids of the cell cultures used in this study. We are currently investigating the subcellular location of O-acetylserine sulfhydrylase isoenzymes from cell cultures and photosynthetic and non-photosynthetic tissues of D. innoxia plants.

Most investigators have assigned Michaelis-Menten kinetics to O-acetylserine sulfhydrylase enzymes (Becker et al., 1969; Masada et al., 1975; Ascano and Nicholas, 1977; León et al., 1987; Murakoshi et al., 1985; Ikegami et al., 1987, 1988; Nakamura and Tamura, 1989; Droux et al., 1992). With multiple data points and replicated experiments we have shown that this is clearly not the case for the three O-acetylserine sulfhydrylase proteins from D. innoxia. Our work is in agreement with the results of Bertagnolli and Wedding (1977), who found that O-acetylserine sulfhydrylase from Phaseolus sp. exhibited positive cooperativity at lower concentrations of sulfide. The D. innoxia isoenzymes exhibit positive cooperativity with both the O-acetylserine and sulfide substrates. The O-acetylserine sulfhydrylases from D. innoxia were not inhibited by the sulfide substrate. However, in contrast to the S. typhimurium O-acetylserine sulfhydrylase (Cook and Wedding, 1976; Bertagnolli and Wedding, 1977), they were not inhibited by O-acetylserine as long as the reaction pH was maintained in the narrow, optimal range required for activity.

In the plant cell, cysteine synthesis is sensitively regulated.

![Graph A](https://example.com/graphA.png)

**Fig. 6.** Effect of (A) reaction temperature and (B) pH on activity of O-acetylserine sulfhydrylase isoenzyme C. Isoenzymes A, B, and C responded similarly to reaction temperature and pH in replicated experiments.

### Table II

**Kinetic constants from substrate saturation experiments with O-acetylserine sulfhydrylase isoenzymes A, B, and C**

Comparative results obtained from analysis using the Hill equation, the MMF model, and the logistic equation (Log) are presented. Equations used for analysis are described under "Experimental Procedures." The M.S.E. indicates the degree of fit to each model, with smaller numbers indicating better comparative fit. The coefficient of determination values ($R^2$) indicate the proportionate reduction of the total variation associated with the model. Larger $R^2$ values indicate a better comparative fit.

| Isoenzyme | \( V_{\text{max}} \) | \( S_{0.5} \) | \( n \) |
|-----------|-----------------|-------------|------|
|           | Hill | MMF | Log | Hill | MMF | Log | Hill | MMF | Log | Hill | MMF | Log | Hill | MMF | Log | Hill | MMF | Log |
| A          |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| B          |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| C          |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

### Table III

**Kinetic constants from sodium sulfide inhibition experiments with O-acetylserine sulfhydrylase isoenzymes A, B, and C**

Comparative results obtained from analysis using the Hill equation, the MMF model, and the logistic equation (Log) are presented. Equations used for analysis are described under "Experimental Procedures." The M.S.E. indicates the degree of fit to each model, with smaller numbers indicating better comparative fit. The coefficient of determination values ($R^2$) indicate the proportionate reduction of the total variation associated with the model. Larger $R^2$ values indicate a better comparative fit.

| Isoenzyme | \( V_{\text{max}} \) | \( S_{0.5} \) | \( n \) |
|-----------|-----------------|-------------|------|
|           | Hill | MMF | Log | Hill | MMF | Log | Hill | MMF | Log | Hill | MMF | Log | Hill | MMF | Log | Hill | MMF | Log |
| A          |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| B          |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| C          |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

* Entire dataset was used to fit each model.
* To avoid the substrate inhibition portion of the curve (Fig. 8), analysis was restricted to sodium sulfide concentrations between 0 and 200 μM for isoenzymes A and C, and between 0 and 150 μM for isoenzyme B.
through O-acetylserine sulfhydrylase activity. This activity is specific for and highly responsive to the enzyme substrates. O-Acetylserine and sulfide affect the enzyme in a positive manner at low concentrations, and sulfide inhibits activity as concentration of this substrate increases. Previous studies have shown substrate specificity for both the alanyl moiety and the sulfide donor in plant O-acetylserine sulfhydrylases (Murakoshi et al., 1985; Ikegami et al., 1988). Free sulfide (in the form of Na₂S) is the only sulfide source that has been shown to be a substrate for O-acetylserine in vitro, and whether a sulfide carrier is involved in the in vivo synthesis of cysteine is still unknown (Schmidt and Jäger, 1992).

The three isoenzymes in our in vitro analysis were most highly active at temperatures higher than physiologically normal. Glutathione concentrations increase in plants exposed to heat shock (Nieto-Sotelo and Ho, 1986), suggesting an increase in biosynthesis of this tripeptide. While cysteine levels do not increase, this amino acid is a required precursor for glutathione and it is reasonable that cysteine synthesis would increase in response to heat shock. The observed high activity of the O-acetylserine sulfhydrylase isoenzymes at temperatures between 42 and 58 °C suggests a mechanism for increased cysteine synthesis in response to elevated temperatures.

O-Acetylserine sulfhydrylase enzymes play several metabolic roles in plants. They are responsible for the final step in the assimilation of inorganic sulfur into an organic molecule and therefore play an essential role in primary plant metabolism. In addition, O-acetylserine sulfhydrylases provide one of the necessary precursors (cysteine) for the synthesis of glutathione and the metal-binding polypeptides involved in cellular response to oxidative stress induced by toxic metals, heat, radiation, and other perturbations. We and others (Lunn et al., 1990; Rolland et al., 1992) have demonstrated that in plants, different O-acetylserine sulfhydrylase isoenzymes are predominant in the cytosol, chloroplasts, and mitochondria. We have initiated studies to examine the involvement of the O-acetylserine sulfhydrylase isoenzymes in different aspects of plant cell metabolism, to determine whether the O-acetylserine sulfhydrylase proteins localized in different regions of the plant cell play different roles in sulfur assimilation and transport, protein synthesis, glutathione accumulation and responses to external abiotic stresses. With the purified O-acetylserine sulfhydrylase proteins described here, antisera against these proteins, and the genes encoding O-acetylserine
sulfhydrylase in D. innoxia, we can begin to address these questions.

REFERENCES

Assato, A., and Nicholas, D. J. D. (1977) Phytochemistry 16, 889–893
Becker, M. A., Kredich, N. M., and Tomkins, G. M. (1969) J. Biol. Chem. 244, 2418–2427
Bertogelli, B. L., and Wedding, R. T. (1977) Plant Physiol. 60, 115–121
Biddingmeyer, B. A., Cohen, S. A., and Tarvin, T. L. (1984) J. Chromatogr. 336, 93–104
Blum, H., Bier, H., and Gross, H. J. (1987) Electrophoresis 8, 93–99
Borenat, A., Britton, P., Jones-Mortimer, M. C., Kornberg, H. L., Lee, L. G., Murfitt, D., and Parra, F. (1984) J. Gen. Microbiol. 130, 673–685
Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
Cohen, S. A., Meys, M., and Tarvin, T. L. (1989) in The Pico-Tag Method: A Manual of Advanced Techniques for Amino Acid Analysis (Waters, W. M., ed) pp. 1–123, Millipore Corp., Bedford, MA
Cook, P. F., and Wedding, R. T. (1976) J. Biol. Chem. 251, 2023–2029
Cook, P. F., Hara, S., Nalabolu, S., and Schnackerz, K. D. (1992) Biochemistry 31, 2298–2303
Delhaize, E., Jackson, P. J., Lujan, L. D., and Robinson, N. J. (1989) Plant Physiol. 88, 700–706
Deneske, S. M., and Farnburg, B. L. (1989) Am. J. Physiol. 257, L163–L173
Droux, M., Martin, J., Sajus, P., and Douce, R. (1992) Arch. Biochem. Biophys. 285, 370–390
Fahy, R. C., and Sundquist, A. R. (1991) Adv. Enzymol. Relat. Areas Mol. Biol. 64, 1–53
Fersht, A. (1985) Enzyme Structure and Mechanism, pp. 263–277, W. H. Freeman & Co., New York
Gaitonde, M. K. (1967) Biochem. J. 104, 627–633
Hanau, B. D. (1981) in Gel Electrophoresis of Proteins: A Practical Approach (Hames, B. D., and Rickwood, D., eds) pp. 1–86, IRL Press, Oxford
Hill, R. (1925) Proc. R. Soc. Ser B 100, 419
Ikegami, F., Kaneko, M., Lambein, F., Koo, Y.-H., and Murakoshi, I. (1987) Phytochemistry 26, 2699–2704
Ikegami, F., Kaneko, M., Kamiyama, H., and Murakoshi, I. (1988) Phytochemistry 27, 607–609
Jackson, P. J., and Kuske, C. R. (1993) in Iron Chelation in Plants and Soil Microorganisms (Barton, L. L., and Hemmings, B. C., eds) pp. 157–175, Academic Press, San Diego, CA
Jackson, P. J., Roth, E. J., McClure, P. R., and Naranja, C. R. (1984) Plant Physiol. 75, 914–918
Jackson, P. J., Unkefer, C. J., Doole, J. A., Watt, K., and Robinson, N. J. (1989) Proc. Natl. Acad. Sci. U.S.A. 84, 6619–6623
Jackson, P. J., Delhaize, E., and Kuske, C. R. (1992) Plant Soil 146, 281–289
Kallen, R. G., Korpela, T., Martell, A. E., Matsumiya, Y., Metzler, C. M., Metzler, D. E., Moroson, Y. V., Ralet, I. M., Savin, F. A., Torchinsky, Y. M., and Ueno, H. (1985) in Transaminases (Christen, P., and Metzler, D. E., eds) pp. 37–108, John Wiley & Sons, New York
Kaplan, M. M., and Flavin, M. (1966) J. Biol. Chem. 241, 5781–5789
Kennedy, W. J., Jr., and Gentle, J. E. (1980) Statistical Computing, pp. 480–483, Marcel Dekker, Inc., New York
Kredich, N. M., and Tomkins, G. M. (1969) J. Biol. Chem. 245, 4955–4965
Kredich, N. M., Becker, M. A., and Tomkins, G. M. (1969) J. Biol. Chem. 244, 2428–2439
Kuske, C. R., Ticknor, L. O., Rice, M. E., and Jackson, P. J. (1992) J. Cell. Biochem. 16D, 122 (abstr.)
Lein, J., Remoro, L. C., Galvan, F., and Vega, J. M. (1987) Plant Sci. 53, 93–99
Lewey, O. H., Rosenbrough, N. J., Parr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
Lunn, J. E., Droux, M., Martin, J., and Douce, R. (1990) Plant Physiol. 94, 1345–1352
Masada, M., Fukushima, K., and Tamura, G. (1975) J. Biochem. 77, 1107–1115
Meister, A., and Anderson, M. E. (1983) Annu. Rev. Biochem. 52, 711–760
Morgan, P. H., Mercier, F., and Flodin, N. W. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4327–4331
Murakoshi, I., Ikegami, F., and Kaneko, M. (1985) Phytochemistry 24, 1907–1911
Nakamura, K., and Tamura, G. (1989) Agric. Biol. Chem. 53, 2557–2568
Nieto-Sotelo, J., and Hu, T.-H. D. (1986) Plant Physiol. 82, 1031–1035
O’Farrel, P. H. (1975) J. Biol. Chem. 250, 4007–4012
Ratkowsky, D. A. (1983) Nonlinear Regression Modeling, pp. 61–83, Marcel Dekker, Inc., New York
Ratkowsky, D. A. (1986) Biochem. J. 240, 357–360
Ratkowsky, D. A. (1990) Handbook of Nonlinear Regression Models, pp. 128–136, Marcel Dekker, Inc., New York
Rao, W. B. (1990) Annu. Rev. Biochem. 59, 61–86
Reed, D. J., and Farsa, M. W. (1984) Pharmacol. Rev. 36, Suppl. 2, 255–335
Roberts, D. V. (1977) Enzyme Kinetics, Cambridge University Press, New York
Robinson, N. J., Ratliff, R. L., Anderson, P. J., Delhaize, E., Berger, J. M., and Jackson, P. J. (1988) Plant Sci. 66, 191–204
Rolland, N., Droux, M., and Douce, R. (1992) Plant Physiol. 98, 927–935
Schmidt, A., and Jager, K. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 325–349
Schnackerz, K. D., Ehrlich, J. H., Giesemann, W., and Reed, T. A. (1979) Biochemistry 18, 3557–3563
Smith, I. K., and Thompson, J. F. (1971) Biochem. Biophys. Acta 227, 288–295
Tamura, G., Iwasawa, T., Masada, M., and Fukushima, K. (1976) Agric. Biol. Chem. 40, 637–638
Tryptase, A. (1990) Lisp-Stat: An Object-Oriented Environment for Statistical Computing and Dynamic Graphics, John Wiley & Sons, New York
Wilson, D. A., and Crawford, I. P. (1965) J. Biol. Chem. 240, 4801–4808
Zeffren, E., and Hall, P. L. (1973) The Study of Enzyme Mechanisms, pp. 133–163, John Wiley & Sons, New York