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Purification of Allantoinase from Soybean Seeds and Production and Characterization of Anti-Allantoinase Antibodies[1]

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Allantoinase catalyzes the hydrolysis of allantoin to allantoic acid, a reaction important in both biogenesis and degradation of ureides. Ureide production in cotyledons of germinating soybean (*Glycine max* L.) seeds has not been studied extensively but may be important in mobilizing nitrogen reserves. Allantoinase was purified approximately 2500-fold from a crude extract of soybean seeds by differential centrifugation, heat treatment, ammonium sulfate fractionation, ethanol fractionation, and fast protein liquid chromatography (Pharmacia) with Mono-Q and Superose columns. The purified enzyme had a subunit size of 30 kD. Polyclonal antibodies produced against the purified protein titrated allantoinase activity in a crude extract of seed proteins. Antibodies recognized the 30-kD band in western blot analysis of crude seed extracts, indicating that they were specific for allantoinase.

Allantoinase (EC 3.5.2.5) catalyzes the hydrolysis of allantoin to allantoic acid in ureide metabolism. These nitrogen-rich organic compounds are used in some plants as nitrogen carriers. Because ureides have a low carbon-to-nitrogen ratio, they are thought to provide efficient transport and storage of nitrogen with minimal expense of reduced carbon (Pate, 1973). The reaction catalyzed by allantoinase plays a dual role in ureide metabolism in plants. It is the final step of ureide biogenesis in tissues that produce allantoic acid and the first step in ureide degradation in tissues that import or store allantoin (Tracey, 1955). Allantoinase has not previously been purified to homogeneity from any plant source.

Ureide metabolism is widespread in higher plants, and in some plants it is an important component of total nitrogen metabolism (Tracey, 1955; Bollard, 1959). Families of higher plants in which ureides are especially prominent include the Leguminosae, Boraginaceae, Platanaceae, Hippocastanaceae, and Aceraceae (Mothes, 1961). Certain legumes, including bean, soybean, and cowpea, change from amide-based nitrogen metabolism to ureide metabolism when they are infected with symbiotic nitrogen-fixing bacteria (Fujihara et al., 1977; Matsumoto et al., 1977a, 1977b; Tajima et al., 1977; Fujihara and Yamaguchi, 1980). Allantoin and allantoic acid are the primary nitrogenous products transported out of soybean root nodules, and they provide the main source of nitrogen for shoot tissues in nodulated soybeans (*Glycine max* L.) and other ureide-transporting legumes (Matsumoto et al., 1977a; Herridge et al., 1978; Thomas and Schrader, 1981b; Schubert, 1981, 1986).

Production of ureides by germinating soybean seeds has not been widely studied. Studies of germinating soybeans (Matsumoto et al., 1977a; Fujihara and Yamaguchi, 1978; Polayes and Schubert, 1984) have shown that ureide metabolism is prominent transiently in cotyledons during early seedling growth. Ureides were produced in cotyledons of nodulating and nonnodulating soybean (Matsumoto et al., 1977a) and in the presence or absence of exogenous nitrate (Polayes and Schubert, 1984), suggesting that ureide formation is independent of symbiotic nitrogen fixation and nitrate uptake by roots.

Ureide metabolism serves different roles and is evolutionarily distinct in plants, animals, and microorganisms. The ureide pathway in animals functions primarily in salvage or excretion of nitrogen from purines (Campbell and Bishop, 1970; Stryer, 1988). In microorganisms, ureide degradation can provide nitrogen from a variety of sources in the external environment (Cooper, 1980). Allantoinase has been isolated from *Pseudomonas* (Jansenn et al., 1982) and from mackerel and frog liver (Noguchi et al., 1986), and the gene encoding allantoinase has been cloned from yeast (Buckholz and Cooper, 1991). However, the plant enzyme has been resistant to previous purification attempts.

The objective of this study was to purify allantoinase from soybean seeds and to produce polyclonal antibodies specific for the enzyme. This paper reports the first purification of allantoinase from a plant source.

MATERIALS AND METHODS

Purification of Allantoinase

Seeds of soybean (*Glycine max* L. cv Williams 82) were surface sterilized with 5% sodium hypochlorite, rinsed well with water, and placed between layers of wet filter paper or in moist vermiculite for 24 h. Hydrated seeds (250 g) were ground first in a mini-blender, then with a mortar and pestle, in 500 mL of 0.1 M Tes, pH 7.5, plus 2.5 g of PVP and filtered

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Abbreviations: bis-Tris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)-methane; FPLC, fast protein liquid chromatography; TBS, Tris-buffered saline; TBST, Tris-buffered saline plus Tween 20.

through Miracloth. The resulting extract was centrifuged at 24,000*g* for 30 min and the supernatant was removed and filtered through Miracloth to remove residual oils. The filtered supernatant was heated in a 75°C water bath for 15 min. The temperature of the extract reached 68°C in 8 min and was held at 68 to 69°C for an additional 7 min. The extract was immediately cooled on ice and centrifuged at 20,000*g* for 20 min to remove precipitated protein. Dry, powdered ammonium sulfate was added to the resulting supernatant to 50% saturation. After the extract was centrifuged at 20,000*g* for 20 min, additional ammonium sulfate was added to the supernatant to 60% saturation, and the extract was centrifuged at 20,000*g* for 20 min. The resulting supernatant was fractionated with absolute ethanol. Ethanol was added to 50% and the extract was centrifuged at 20,000*g* for 20 min. Additional ethanol was added to the supernatant to 70% and the extract was centrifuged at 20,000*g*. The resulting pellet was resuspended in 20 mM bis-Tris, pH 6.2, in a total volume of 11 mL. The extract was then diluted 1:10 (v/v) in 20 mM bis-Tris, pH 6.2, for separation by Mono-Q column chromatography on the FPLC system (Pharmacia).

Extract was loaded onto the column in portions of 10 mL or less, and unbound proteins were eluted with 20 mM bis-Tris. Additional portions of extract were loaded up to a total of 35 mL, allowing elution of unbound protein between each addition. The column was washed with 20 mM bis-Tris until absorption at 280 nm was negligible, and bound protein was then eluted with a linear gradient over 20 mL from 0 to 0.4 M KCl in 20 mM bis-Tris, pH 6.2, at a flow rate of 1 mL/min. Additional runs on the Mono-Q column were done in the same way until all the extract was used. Three fractions from each run with substantial allantoinase activity were pooled, diluted 1:1 (v/v) with 20 mM bis-Tris, pH 6.2, and rerun on the Mono-Q column under the same conditions to concentrate the sample. Fractions with the highest activity were pooled, and 0.5 mL was applied to a Superose 12 column (Pharmacia) on the FPLC system. Proteins were separated and eluted from the column with 20 mM bis-Tris, pH 6.2, at a flow rate of 0.5 mL/min. Superose chromatography was repeated four times with additional sample, and 12 fractions with activity were pooled from the five runs and were diluted to 20 mL in 20 mM bis-Tris, pH 6.2. Diluted sample was then applied to a Mono-Q column. The column was washed until absorption of eluate at 280 nm was negligible, and bound protein was eluted with a linear gradient over 20 mL from 0 to 0.4 M $LiNO_3$ in 20 mM bis-Tris, pH 6.2, at a flow rate of 1 mL/min. Fractions with peak activity were collected and Superose chromatography and Mono-Q chromatography were each repeated once more to remove minor contaminants.

Column fractions were assayed for allantoinase activity as described below. Protein was determined by the method of Bradford (1976) or by the Pierce Micro BCA protein assay. Purified protein was analyzed by SDS-PAGE as described below.

Allantoinase Assay

The assay for allantoinase activity was based on procedures from Vogels and van der Drift (1970) as modified by Schubert (1981). Controls were included to correct for endogenous allantoic acid in the enzyme extract and for nonenzymic breakdown of allantoin as detailed by Schubert (1981).

SDS-PAGE

Purification results were analyzed by SDS-PAGE (Laemmli, 1970). Gels had a bis:acrylamide ratio of 0.8:30 (w/w) and an acrylamide concentration of 12.5% (w/v). For silver staining, gels were fixed in methanol:acetic acid in distilled water (50:10% [v/v], then 10:10% [v/v]), washed in distilled water, and stained with 0.1% $AgNO_3$ (w/v) for 30 min. After a brief wash in distilled water, silver stain was developed in a solution of 0.3 M Na_2CO_3 plus 0.1% formaldehyde (v/v), and development was stopped with one-tenth volume of 2.0 M citric acid.

Preparation of Protein for Immunization of Rabbits

Purified protein eluted from the final Mono-Q column was prepared in two ways for immunizing rabbits. Protein in half of the sample was precipitated by adding 7.5 volumes of cold acetone and leaving it overnight at −20°C. The other half of the protein sample was first fixed with glutaraldehyde by adding aqueous glutaraldehyde to the sample to a final concentration of 2% glutaraldehyde and incubating it for 20 min at room temperature. Protein in this sample was then precipitated by adding 7.5 volumes of cold acetone and leaving it overnight at −20°C. Both samples were centrifuged at 10,000*g* for 15 min and redissolved in filter-sterilized 20 mM bis-Tris, pH 6.8.

Immunization and Antibody Preparation

Two rabbits were bled before immunization to collect preimmune sera. Preparations of purified allantoinase were mixed 1:1 (v/v) with Freund's complete adjuvant prior to injections. One rabbit was injected with unmodified protein and a second rabbit was injected with glutaraldehyde-fixed protein (see above). Injections were divided among several subcutaneous and intramuscular sites along the backs of the rabbits. Initial injections contained approximately 200 μg of purified protein. Two additional booster injections were done at approximately 3-week intervals with 100 μg of protein each time. Rabbits were bled for collection of antiserum 3 weeks after the second booster. Antiserum enriched in immunoglobulin G was prepared by precipitation with ammonium sulfate and redissolved in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.6).

Western Blot Analysis

After SDS-PAGE, proteins were electroblotted onto nitrocellulose in transfer buffer (40 mM Trizma base, 5 mM Gly, 20% methanol) with either a Trans-Blot cell (Bio-Rad) or a Semi-Phor dry-blot apparatus (Hoefer). The blot was blocked in TBST plus an additional 0.15% Tween 20 for 20 to 30 min. Blots were incubated in anti-allantoinase antibody diluted 1:500 in TBST plus additional Tween 20 for 2 h, washed twice in the same buffer, incubated in goat anti-rabbit conjugated with alkaline phosphatase in TBST without extra

Tween for 30 min to 2 h, and then washed twice in TBST and once in distilled water. Color was developed with 4-nitroblue tetrazolium chloride (0.25 mg/mL) and 5-bromo-4-chloro-3-indolyl-phosphate (0.125 mg/mL) in substrate buffer (0.1 M Tris-HCl, pH 9.8, 0.1 M NaCl, 5 mM $MgCl_2$).

Immunotitrations

The appropriate amount of antibody was added to 50 μL of crude extract from seeds in either 0.1 M Tes, pH 7.5, or in 20 mM bis-Tris, pH 6.2, in a microcentrifuge tube, and TBS was added as necessary to bring the total volume up to 75 μL. Solutions were mixed and incubated at room temperature for 1 h. Solutions were then centrifuged at 2000*g* for 5 min and 10-μL samples were removed from the supernatant for assay.

RESULTS AND DISCUSSION

Purification of Allantoinase

Allantoinase was purified approximately 2500-fold from a crude protein extract of soybean seeds (Table I). Because thorough grinding of the seeds was essential to obtain the highest activity levels in the crude extract, we used a two-step grinding procedure (see "Materials and Methods"). The subsequent purification procedures were developed from those we applied in attempts to purify allantoinase from soybean root nodules, with some modifications (Webb, 1988; M.A. Webb and K.R. Schubert, unpublished data).

Although specific activity did not increase substantially after the crude extract was heated (Table I), subsequent fractionation steps were not as effective when heat treatment was omitted. The evident lack of increase in specific activity may have been due to imprecision in measuring activity and protein concentration in the crude extract. The crude extract typically contained particulate matter that was not removed by the initial centrifugation, and this may have interfered with assays.

Fractionations with ammonium sulfate and ethanol significantly increased specific activity, giving 15-fold and 18-fold purifications, respectively. Although yield was substantially reduced with ammonium sulfate fractionation, a narrow fractionation step was deliberately chosen to enhance purification. In contrast, almost no activity was lost after ethanol fractionation (Table I). In purification trials, we compared ethanol and acetone fractionation and found little difference in effectiveness between the two solvents (data not shown).

The remaining purification steps used an FPLC system for column chromatography, including anion-exchange chromatography with a Mono-Q column under two sets of conditions, alternating with gel filtration on a Superose 12 column. Activity profiles are illustrated for chromatography on the first Mono-Q column with a KCl gradient and for the subsequent Superose 12 column (Fig. 1). Allantoinase was bound to the Mono-Q column in low salt and was eluted from the column at approximately 0.2 M salt with either KCl or $LiNO_3$. Yield from the Mono-Q and Superose columns was sufficient enough that these chromatography steps were repeated to remove minor contaminants with little loss of allantoinase activity (Table I).

After the first Mono-Q chromatography, the fraction with peak activity showed a predominant polypeptide at 30 kD and a series of smaller polypeptides (Fig. 2A). After additional column chromatography, SDS-PAGE analysis of the fraction with peak allantoinase activity revealed a single 30-kD polypeptide (Fig. 2B).

This study represents the first complete purification of allantoinase from a plant source. With our procedure, allantoinase was purified from a crude extract of soybean seeds to apparent homogeneity (Fig. 2B) and to a final specific activity of 37.1 μmol min^{-1} mg^{-1} (Table I). A number of partial purifications have been reported from a variety of plants and plant tissues (Nagai and Funahashi, 1961; Nirmala and Sastry, 1975; Mary and Sastry, 1978; Amarjit and Singh, 1985; Rao et al., 1988). Allantoinase also has been partially purified from soybean shoots (Thomas et al., 1983), nodules (Webb, 1988; M.A. Webb and K.R. Schubert, unpublished data), and seeds (Franke et al., 1965; Wang and Anderson, 1969).

Characteristics of Seed Allantoinase

The sequence of the first 25 residues at the amino terminus of purified allantoinase (Fig. 3) were determined by Edman degradation. For several residues there was evidence for more than one amino acid, which may indicate the presence of different isozymes. The amino terminal sequence had no significant similarities with any other sequences in computer

Table I. *Purification of allantoinase from soybean seeds*

Purification Step	Total Activity[a]	Specific Activity	Yield	Purification
	units	*units/mg*	%	*-fold*
Crude extract	305	0.015	100	1.0
Heat (68°C, 7 min)	228	0.016	77.8	1.07
50–60% ammonium sulfate	76	0.23	25.9	15.3
50–70% ethanol	73	4.06	25.0	271
Mono-Q (KCl)	37	20.0	12.7	1335
Superose 12	23	26.4	7.9	1760
Mono-Q ($LiNO_3$)	22	32.7	7.6	2180
Superose 12, Mono-Q	18	37.1	6.2	2473

[a] A unit of activity is defined as the amount of enzyme that catalyzes formation of 1 μmol allantoic acid/min at 30°C.

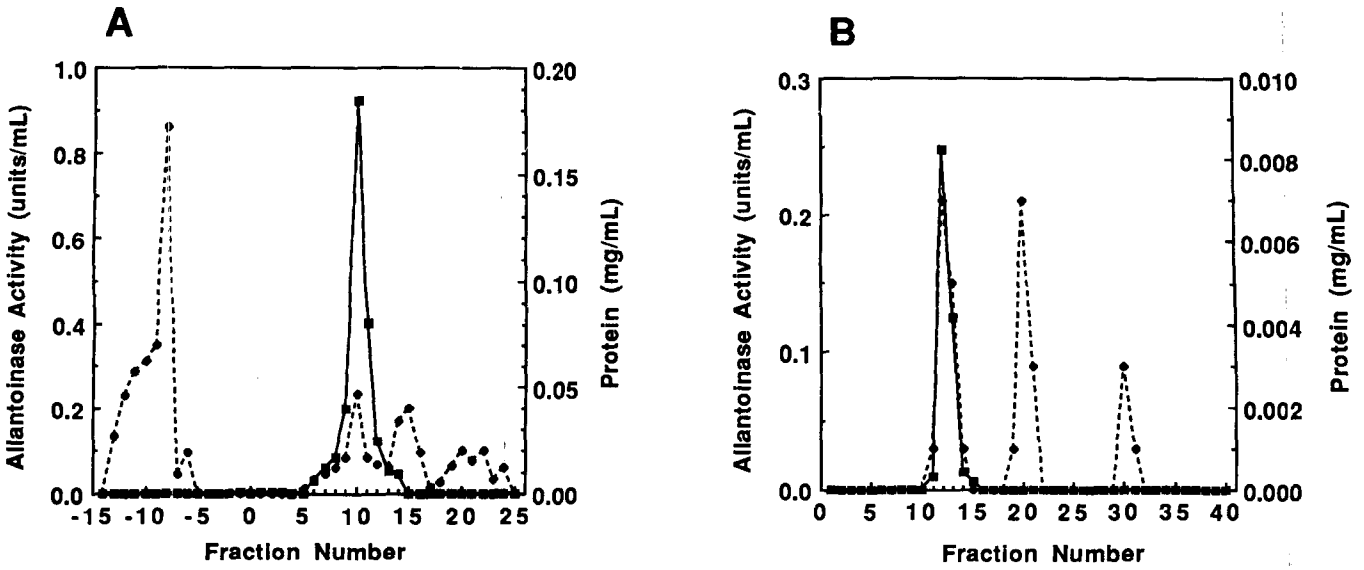

Figure 1. Profiles of allantoinase activity (solid line) and protein concentration (dotted line) from column chromatography during purification. A, Anion-exchange on Mono-Q column, linear gradient of 0 to 0.4 M KCl from fractions 0 to 20; B, gel filtration on Superose 12 column.

data banks and did not share sequence similarity with the amino acid sequence of yeast allantoinase (Buckholz and Cooper, 1991).

Allantoinase activity in soybean seeds was remarkably stable to heat (Table I; Franke et al., 1965), as was found for shoot (Thomas et al., 1983) and nodule allantoinase (Webb, 1988). In this study, 78% of the total activity was retained after seed extracts were heated to 68°C for 7 min (Table I). Franke et al. (1965) found that soybean seed allantoinase was deactivated at 81.5°C. Heat stability has also been reported for allantoinase in seeds of other plants; allantoinase from peanut seeds was active up to 90°C (Singh et al., 1970) and from *Lathyrus sativa* it was active up to at least 60°C (Nirmala and Sastry, 1975). In contrast, allantoinases from several bacterial and animal sources lost activity with heating above 55°C (Vogels et al., 1966).

The seed enzyme also retained activity after anion-exchange chromatography on the Mono-Q column (Table I). In this respect it differs from the enzyme in soybean leaves, which lost activity after DEAE-cellulose column chromatography (Thomas et al., 1983). Severe reductions of activity with anion-exchange chromatography and gel filtration also prevented purification of allantoinase from soybean nodules (Webb, 1988; M.A. Webb and K.R. Schubert, unpublished data).

Allantoinase in Animals and Microorganisms

Allantoinase has been isolated from only a few animal tissues. In mackerel liver, allantoinase is composed of a single subunit of about 54 to 55 kD. In amphibian liver, the enzyme exists as a subunit of 54 kD complexed with allantoicase in the same protein molecule (Noguchi et al., 1986). During evolution, allantoinase and subsequent enzymes in ureide degradation have been lost in some animals, such as most primates, and the end products of purine salvage differ across the animal kingdom.

Many microorganisms, for example *Pseudomonas* (Jansenn et al., 1982) and *Saccharomyces* (Cooper, 1980), are able to use allantoin or other ureide intermediates as a sole source of nitrogen, allowing them to utilize a variety of nitrogen sources from decaying organisms. Allantoinase purified from *Pseudomonas aeruginosa* consisted of four identical 38-kD subunits (Jansenn et al., 1982). The gene encoding allantoinase has been cloned from *Saccharomyces* (Buckholz and Cooper, 1991), in which the enzyme is part of a ureide-degrading pathway induced by allophanate, an intermediate synthesized from allantoin, and repressed by other nitrogen sources such as Asp or Glu (Cooper et al., 1989).

Production and Characterization of Anti-Allantoinase Antibodies

Polyclonal antibodies against seed allantoinase were raised in rabbits immunized with the purified protein. Immunotitration experiments (Fig. 4) showed that allantoinase activity was reduced more than 80% by addition of anti-allantoinase to crude extracts of soluble seed proteins. Antibodies against both native and glutaraldehyde-fixed allantoinase were equally effective in titrating activity. Addition of preimmune sera to the extracts did not result in significant loss of activity. These experiments showed that the antibodies we produced reacted with seed allantoinase.

Western blot analysis of crude seed extracts probed with anti-allantoinase antisera showed a single immunoreactive band corresponding to an apparent molecular mass of 30 kD (Fig. 5). Blots probed with preimmune serum had no immunoreactive bands. These results show that the antibodies react specifically with the 30-kD subunit of allantoinase and that there are no cross-reactive isozymes in seeds. Together with

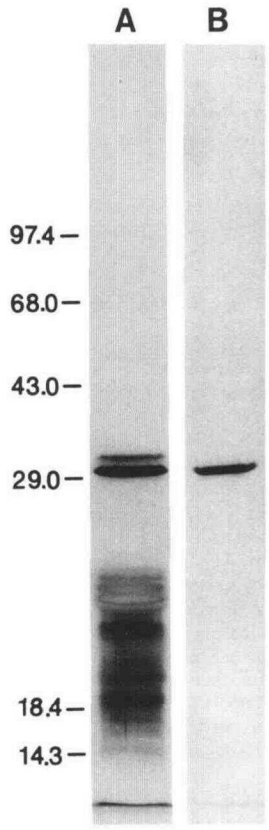

Figure 2. SDS-PAGE analysis of fractions with peak allantoinase activity after selected purification steps (see Table I); gels were stained with silver. A, Initial Mono-Q column; B, final Mono-Q column, 13 μg of protein loaded.

the immunotitration studies, these results suggest that the 30-kD polypeptide we isolated is the predominant polypeptide responsible for allantoinase activity in soybean seeds.

Because the subcellular localization of allantoinase in plants has not been determined, we used the antibodies for immunocytochemical studies. When sections of seed tissues were probed with anti-allantoinase antibodies, only sparse labeling was observed within storage protein bodies under certain conditions (data not shown). Labeling was not observed in sections incubated with preimmune probes. These results were inconsistent with previous biochemical studies, which found that allantoinase activity was associated with glyoxysomal fractions in seeds (St. Angelo and Ory, 1970; Theimer and Beevers, 1971). In our studies we did not observe any labeling of glyoxysomes. We consider the protein body labeling inconclusive, possibly resulting from nonspecific binding of immune sera. Lack of specific labeling could be a consequence of allantoinase denaturation during fixation

Asp/Leu - Lys - Pro - Asp - Ala/Lys - Tyr - Leu - Thr -
Tyr - Leu - Asn - Thr - Arg/His - Pro - Pro - Ser - X -
Glu - Ile/Val - Ala - Ala - X - Lys - Gln - Leu -

Figure 3. Amino terminal sequence of purified allantoinase; residues not determined are designated by X.

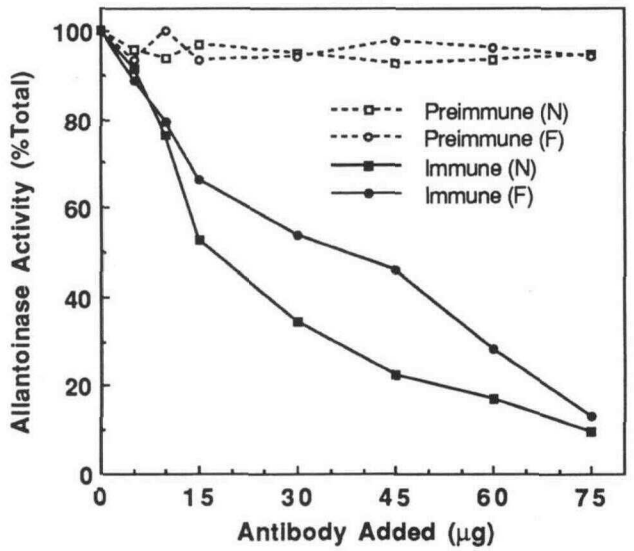

Figure 4. Immunotitration of allantoinase activity in a crude extract (24,000*g* supernatant) of seeds with anti-allantoinase antibodies. N, Antibodies against native protein; F, antibodies against glutaraldehyde-fixed protein.

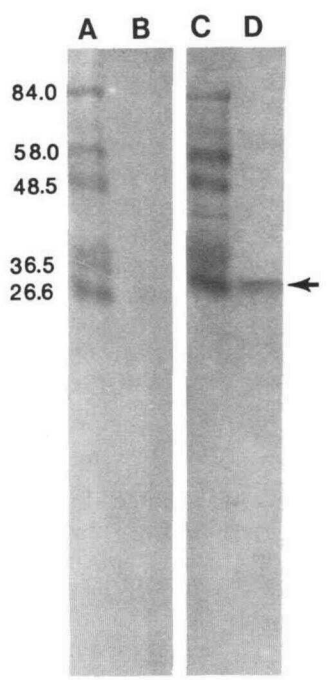

Figure 5. Immunoblot of crude extract from seeds after separation by SDS-PAGE. A and C, Prestained mol wt markers; B and D, seed extract (24,000*g* supernatant). A and B, Probed with preimmune serum; C and D, probed with anti-allantoinase antibodies against native protein.

and embedding procedures, which can disrupt antibody-antigen binding. Several methods of tissue preparation have been tried so far without resolution of these technical difficulties.

Allantoinase in Seeds

Allantoinase activity may vary over the course of seed germination and seedling growth. In germinating mung bean (Nagai and Funahashi, 1961), castor bean (Ory et al., 1969), *L. sativa* (Nirmala and Sastry, 1975), and *Dolichos biflorus* (Mary and Sastry, 1978), allantoinase activity increased in seeds or seedlings. Different patterns of activity increase and decrease were observed in the different species in relation to germination. In peanut, allantoinase activity was constant for the first 10 d after seed germination.

Allantoinase activity levels in soybean seeds were comparable with those in nodules, suggesting that ureide metabolism is an important component of nitrogen metabolism in seeds. However, ureide production by seeds has not been studied extensively, and the role of ureides in germinating seeds is not known. In general, ureides are thought to function as efficient carriers of nitrogen, since they require relatively little reduced carbon in comparison with amides, such as Glu and Asp, which are also used in nitrogen transport.

In germinating soybeans, Polayes and Schubert (1984) found that allantoinase activity in the cotyledons increased to a peak 4 d after germination and then decreased. Content of allantoin and allantoic acid in the cotyledons also increased to a peak at about 5 d after germination (Matsumoto et al., 1977a; Polayes and Schubert, 1984). These patterns are consistent with a function for ureides in mobilizing storage reserves during seed germination and early seedling growth. Soybean seeds do not have substantial starch reserves, and they begin to mobilize storage protein before cotyledons have become photosynthetic. Incorporation of storage nitrogen into ureides for export from the cotyledons could be important for energy conservation during early seedling establishment. Although it has not been established that ureides are exported from cotyledons, it is clear that shoot tissues can use ureides as a nitrogen source (Thomas and Schrader, 1981a; Costigan et al., 1987; Winkler et al., 1987). The importance of ureide metabolism in the overall nitrogen metabolism of seedlings remains to be established.

SUMMARY

In this study we purified allantoinase 2500-fold from soybean seeds to a final specific activity of 37.1 μmol min^{-1} mg^{-1} and to apparent homogeneity. The purified enzyme had a subunit size of 30 kD. The first 25 amino acids of the amino terminus were determined and had no significant sequence similarity with other known protein sequences, including the sequence for yeast allantoinase. Immunological studies suggested that the 30-kD polypeptide comprised the predominant enzyme responsible for allantoinase activity in seeds. Allantoinase activity in seeds was relatively high, suggesting that ureide metabolism may play an important physiological role in seed germination.

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