Purification and Properties of Soybean Allantoinase[1]

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ABSTRACT

Soybean allantoinase was purified by ammonium sulfate precipitation, diethylaminoethyl cellulose chromatography, and gel filtration. The enzyme, which is present in soybean whey, was purified 671-fold from a pH 4.5 acetate buffer extract. The yield was more than 30%. Allantoinase has a sedimentation coefficient of 4.9S, which corresponds to a molecular weight of about 50,000. The enzyme has an optimum pH of 8.4, K_m of 4.4×10^{-2}M, and an optimum temperature at 70°C. Short heating of the buffer extract activates the enzyme; it is six times more active at 70°C. than at 25°C., but it inactivates twice as fast at temperatures higher than 70°C., its optimum activation point.

Allantoinase (Allantoin amidohydrolase, EC 3.5.2.5) is one of the essential enzymes involved in purine degradation. Early studies (1,2,3) used crude enzyme preparations. In 1961, Nagai and Funahashi (4) obtained 60-fold purity of allantoinase from mung bean seedlings. Lee and Roush (5) used soybean allantoinase of four- to sixfold purity to determine its activity titrimetrically, manometrically, and polarimetrically. In 1965, Franke et al. (6) published a procedure to purify soybean allantoinase that included heat-treatment and adsorption on aluminum hydroxide gel. They obtained a 265-fold purification of the enzyme. The next year, Vogels et al. (7,8) compared the properties of allantoinase from bacterial, plant, and animal sources. Allantoinase from these different sources was classified into four groups according to its properties.

Allantoinase is widely distributed in higher plants (9), microorganisms (10), and animals (11). Soybeans contain the highest allantoinase activity of the many plant seeds examined so far (7). An enzyme of high purity from soybean meal is needed to study its properties. We purified soybean allantoinase 671-fold by diethylaminoethyl (DEAE)-cellulose chromatography and gel filtration. Our procedure for purification of the enzyme and some of its properties are described here.

MATERIALS AND METHODS

Crude Enzyme Preparation

Hawkeye soybeans (1965 crop) were ground in a Weber pulverizer mill (prechilled with ice) and passed through a 100-mesh sieve. All handling of the enzyme was done at 4°C. except during heat-treatment. The freshly ground full-fat meal (100 g.) was extracted with 0.7 liter of 0.01M sodium acetate buffer (pH 4.5) for 2 hr. with continuous stirring. The mixture was centrifuged at 1,500 × g for 15 min. and the supernatant (about 500 ml.) was collected. The pH of the protein

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solution was adjusted to 7.0 with 1N NaOH. The solution was stirred and heated to 75°C. in a water bath and held there for 5 min. After the solution was cooled quickly to 4°C., the denatured protein was removed by centrifugation. The supernatant proteins were fractionated by slow addition of solid ammonium sulfate to a desired percentage of saturation. The protein, which precipitated between 35 and 70% saturation, was collected and dissolved in less than 100 ml. water. The enzyme was dialyzed against 2 to 3 liters of water for 24 hr. with at least five to six changes of water. The precipitate that formed during dialysis was removed by centrifugation and discarded.

Allantoinase present in the supernatant was lyophilized and stored in a freezer for future use. Crude enzyme so stored kept its activity for 6 months.

Diethylaminoethyl Cellulose Chromatography

A DEAE-cellulose column (2 X 43 cm.) was prepared and equilibrated with starting buffer (0.01M phosphate, pH 7.6) as described by Peterson and Sober (12). Lyophilized crude enzyme (0.5 g.) was dissolved in this buffer, and then the solution was dialyzed in the cold against the same buffer for at least 2 hr. to remove residual ammonium sulfate. Precipitate formed during dialysis was removed by centrifugation. After the sample was applied to the column, 10 ml. of starting buffer was run through it to remove any protein that had little or no affinity for the adsorbent. A four-step elution at a rate of 23 ml. per hr. was then used with starting buffer containing 0.034, 0.100, 0.180, and 0.195M sodium chloride. Samples of 5 ml. were collected in a fraction collector. Fractions were checked for enzymatic activity and fractions containing allantoinase were pooled, dialyzed against water, and lyophilized before the gel filtration step which is described later. There was no loss of enzymatic activity on dialysis ahd lyophilization. The addition of 0.01M 2-mercaptoethanol to the starting buffer had no effect on enzymatic activity.

The enzyme collected after gel filtration was rechromatographed on a smaller DEAE-cellulose column (2 X 19 cm.) with gradient elution. The gradient was formed by dropwise addition of 0.7M sodium chloride in starting buffer to 200 ml. of starting buffer in a 500-ml. conical mixing flask. Salt concentrations in the column effluents were determined by conductivity measurements. The elution rate was 20 ml. per hr., and 2 ml. of eluate was collected in each fraction. Fractions were analyzed for enzymatic activity, and the active fractions were pooled and stored in a freezer until further studies of their properties could be made.

Gel Filtration

Sephadex G-200 (purchased from Pharmacia) was allowed to swell for 48 hr. in 0.03M phosphate buffer containing 0.4M sodium chloride (pH 7.6, $\mu = 0.5$). A 2 X 215-cm. column was filled with the swollen Sephadex, and the enzyme preparation from stepwise DEAE-cellulose chromatography (in about 10 ml. buffer) was layered on the column. Elution was carried out at a rate of 38 ml. per hr. with the buffer used to expand the Sephadex. Fractions of 5 ml. were collected. Absorbance at 280 mμ was monitored continuously, and every third tube was analyzed for protein and enzymatic activity.

Protein Analysis and Enzymatic Activity

The micro-Kjeldahl method of Johnson (13) and the procedure of Lowry et al.

(14) were used to determine the concentration of protein. Allantoinase activity assayed by the method of Young and Conway (3) exhibited a linear relation with incubation time and amount of enzyme. Unless otherwise stated, the enzymatic assay conditions were as follows: The reaction mixture with a total volume of 2 ml. contained 1.9 mg. of dl-allantoin and an appropriate amount of the enzyme in 0.01M phosphate buffer (pH 7.6). After incubation at 25°C. for 30 min. the hydrolysis was stopped by adding 0.5 ml. of 10% trichloroacetic acid. The extent of allantoin hydrolysis was determined on a 0.1-ml. or larger fraction of the deproteinized supernatant. The unit of allantoinase activity is expressed as μmoles of allantoin hydrolyzed in 30 min. under these conditions, and specific activity is given as allantoinase units per mg. of protein.

The purified enzyme was centrifuged at room temperature with a Spinco Model E at 47,660 r.p.m. The protein solution was equilibrated for 2 hr. with 0.03M phosphate buffer containing 0.4M sodium chloride (pH 7.6, μ = 0.5) before centrifugation.

The effect of pH on allantoinase activity was measured at 25°C. in four buffers, each of 0.1 ionic strength, in four ranges: acetate buffer, pH 4.5 to 5.8; phosphate, 5.8 to 7.8; Tris, 7.8 to 9.0; and glycine, 9.0 to 11.0. A control was included at each point of measurement. Determinations were made in duplicate, and assay conditions were the same as described for enzymatic activity.

The usual procedure was followed in studying the effect of heat on allantoinase by plotting $\log_{10} k = (\mu/2.303R)\ (1/T)$, where k is the reaction velocity, μ the activation energy, R the gas constant, and T the absolute temperature. At each temperature the duration of heating was 5 min., and a control was included.

The K_m of allantoinase was calculated from a Lineweaver-Burk plot of enzymatic activities assayed at different substrate concentrations (pH 8.4 and 60°C.). Such conditions required a control at each concentration but less enzyme for assaying allantoinase activity.

RESULTS

Extraction of Enzyme

Of the three buffers and distilled water used as solvents, water extracted the highest amount of allantoinase, as well as other protein (Table I). The water extract yielded a specific activity of 0.41. The specific activity was lowest with 0.01M Tris buffer, pH 7.0, which lowered the yield of total units of enzyme by 38% compared to that with water. Although lowering the pH of the solvents from 7.0 to 4.5 decreased solubility of total protein markedly, enzymatic activity decreased only slightly. Acetate buffer at 0.1M, pH 4.5, gave a specific activity of 0.76. Since the same buffer at 0.01M concentration had the highest specific activity (1.05), it was used for all subsequent experiments.

Procedure of Enzyme Purification

Table II summarizes results from the purification of allantoinase. In agreement with the findings of Franke et al. (6), heat-treatment caused a loss of about 30% of the enzyme, but the increase in specific activity was significant. Ammonium sulfate fractionation following the heating step doubled the specific activity. An additional

TABLE I. EXTRACTION OF ALLANTOINASE FROM SOYBEAN MEAL (100 g.) BY DIFFERENT SOLVENTS

Solvent	pH	Volume	Total Units of Enzyme	Protein Concentration	Specific Activity of Enzyme
		ml.		mg./ml.	unit/mg. protein
Water		400	4,480	27.05	0.41
Tris buffer, 0.01M	7.0	460	2,760	24.55	0.24
Acetate buffer, 0.1M	4.5	510	2,240	5.75	0.76
Acetate buffer, 0.01M	4.5	580	3,620	5.94	1.05

30-fold purification resulted after DEAE-cellulose chromatography. A typical chromatographic elution pattern (Fig. 1) indicates that the enzyme activity appeared in a single peak. However, ultracentrifugal analysis of the enzyme fraction showed that the preparation was a mixture of 2S and 5S materials. The mixture was fractionated by gel filtration and the activity was found to be associated with the 5S protein. The impurities were removed by gel filtration as shown in Fig. 2. A fivefold increase in specific activity was also noted after this step. Although the enzyme activity eluted in a single symmetrical peak, the protein did not. The enzyme was, therefore, rechromatographed on a DEAE-cellulose column with gradient elution (Fig. 3).

Results in Table II indicate that the enzyme was purified an additional 1.7-fold with the second DEAE-cellulose chromatographic step. The enzyme preparation appeared homogeneous by ultracentrifugation (Fig. 4). The protein moved as a

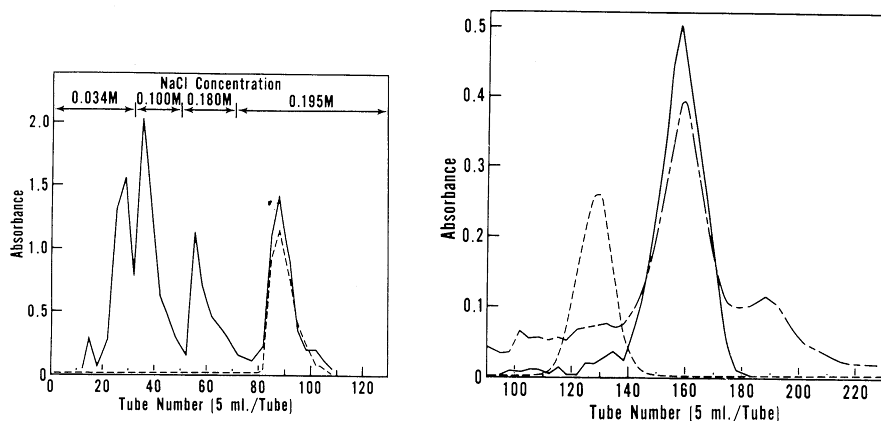
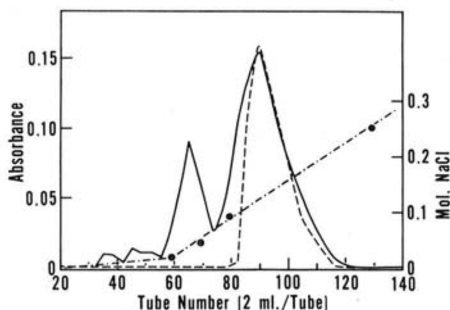


Fig. 1 (left). Diethylaminoethyl(DEAE)-cellulose column chromatography of heated and ammonium sulfate-fractionated (35 to 70% saturation) soybean protein. A sample with protein concentration of 0.56 g. in 25 ml. buffer was added to a column (2 X 47 cm.) and eluted stepwise with increasing NaCl concentration in 0.01M phosphate buffer. Solid line, protein; dashed line, allantoinase activity.

Fig. 2 (right). Chromatography of allantoinase on a Sephadex G-200 column (2 X 215 cm.). Solid line, protein; dashed and solid line, 280 mμ; dashed line, allantoinase activity.



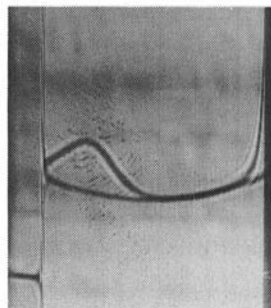


Fig. 3 (left). Final chromatography of allantoinase on a DEAE-cellulose column (2 X 19 cm.) with gradient elution. Solid line, protein; dashed line, allantoinase activity; dash-and-dot line, NaCl concentration.

Fig. 4 (right). Ultracentrifugal patterns of enzyme from Fig. 3 after centrifugation at 47,660 r.p.m. for 80 min.

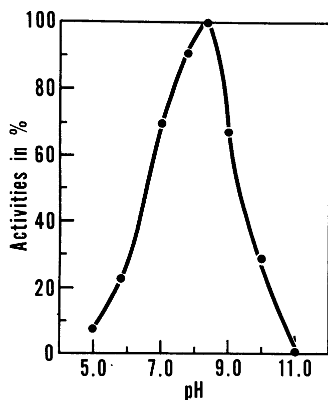
single peak with a sedimentation coefficient of 4.9S. This preparation was used in further studies of the enzyme's properties.

Properties of Soybean Allantoinase

The pH optimum of the enzyme was 8.4 (Fig. 5). Different values have been reported for various enzyme preparations. Brunel (2) recorded a pH optimum of 7.6; Franke et al. (6), 7.8; and Nagai and Funahashi (4), 8.0 with allantoinase from mung bean seedlings. The optimum pH of allantoinase is obviously above 7. Since allantoin undergoes rapid nonenzymatic hydrolysis at pH values greater than 8.5, we included appropriate controls in assaying enzymatic activities at pH values greater than 7.8

TABLE II. PROCEDURE OF ALLANTOINASE PURIFICATION

Purification Step	Total Protein	Enzyme Total Units	Specific Activity	Recovery
	mg.		unit/mg. protein	%
0.01M Acetate buffer extract pH 4.5 from 100 g. meal	3,888	3,413	0.88	100
Heated extract	1,989	2,156	1.08	63
Ammonium sulfate fractionation	970	2,117	2.18	62
Stepwise DEAE-cellulose chromatography	29	1,850	64.2	54
Sephadex G-200 gel filtration	3.8	1,310	345	38
Gradient DEAE-cellulose chromatography	1.8	1,060	590	31



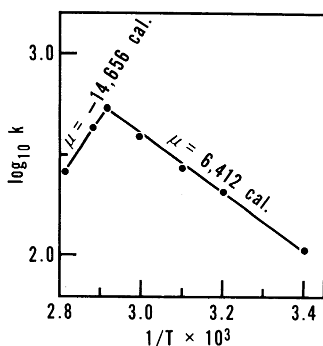


Fig. 5 (left). Effect of pH on allantoinase activities. (Acetate buffer, pH 5.0 to 5.8; phosphate, 5.8 to 7.8; Tris, 7.8 to 9.0; glycine, 9.0 to 11.0.)

Fig. 6 (right). Arrhenius plot of data from temperature effects on allantoinase activities (pH 7.0, substrate concentration 2 mg. dl-allantoin per ml.).

The optimum temperature for allantoinase activity was 70°C. (Fig. 6). An Arrhenius plot of the data indicated gradual activation in the range from 25° to 70°C. A μ value (activation energy) of 6,412 cal. was calculated from the slope of the line. The enzyme was six times more active at 70°C. than at 25°C. and was rapidly inactivated at temperatures above the optimum. From 70° to 80°C. allantoinase lost its activity twice as fast as the rate of activation at lower temperatures (μ = −14,656 cal.).

Allantoinase had a K_m of 4.4×10^{-2}M at pH 8.4 and 60°C. (Fig. 7). Results were similar at pH 7.0 and 23°C.

DISCUSSION

Soybeans contain the highest allantoinase activity of the various plant seeds examined so far. These seeds include mung bean, *Phaseolus hysterinus*, lupin, gherkin, barley, and pea (7). Selection of a suitable extractant to solubilize the enzyme from soybean meal is important in its purification. Soybean allantoinase and soybean globulins are both extracted with water at pH 6.5 to 6.7 (the pH of aqueous meal extracts). Since the major soybean globulins have an isoelectric point

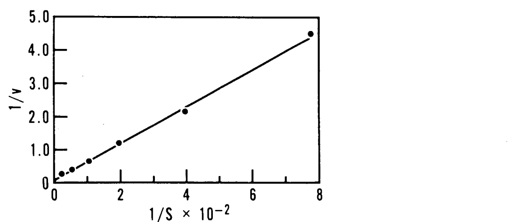


Fig. 7. Lineweaver - Burk plot of data from substrate concentrations on allantoinase activities (pH 8.4 and enzymatic activities assayed at 60°C.).

of 4.5, a buffer at this pH will minimize the solubility of globulins. Allantoinase extracted with sodium acetate buffer at pH 4.5 did give a higher specific activity than either water or Tris buffer at pH 7.0. The type of salt and its concentration are also important. Lee and Roush (5) indicated that phosphate inhibited allantoinase activity. Our results (Table I) also showed that extracts with sodium acetate at low concentration contained higher specific activity of allantoinase than extracts with Tris buffer or sodium acetate at high concentrations.

Attempts to isolate allantoinase by alcohol or acetone precipitation were only partially successful. Purification of the enzyme was less than 100-fold by these precipitation methods. DEAE-cellulose chromatography with stepwise salt elution appeared to be more effective. However, ultracentrifugation and gel filtration showed that the enzyme was contaminated with 2S materials. Although contaminants were not identified, they reacted with protein reagent and at the same time gave absorptions higher at 260 than at 280 $m\mu$. Gel filtration removed these impurities effectively. The enzyme appeared nearly homogeneous by ultracentrifugation after a second DEAE-cellulose column chromatography. The sedimentation coefficient of the purified enzyme was 4.9S which we estimate to correspond to a molecular weight of about 50,000 to 75,000 by comparison with sedimentation coefficients for other proteins (15).

Our studies yielded a K_m value of 4.4×10^{-2}M with dl-allantoin as substrate. This value is greater than the K_m value of 6.7×10^{-3}M by Franke et al. (6) but agrees with values for allantoinase from mung bean seedlings (4) and *Arthobacter allantoicus* (7). Franke et al. used dl-allantoin as substrate and calculated their results on the basis of d-allantoin. The difference in K_m values, whether reported in terms of the dl- or d- form of the substrate, seems less important than the fact that the enzyme has a low affinity for the substrate. Allantoinase reportedly reacts 20 times faster with d-allantoin than with the l-form (6).

Allantoinase exhibited an unusually high optimum temperature (70°C.). Since the enzyme is obviously very active in the temperature range at which soybeans are stored, the effect of allantoinase on soybean meal in storage should be investigated.

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