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HOMOGENEOUS ALKYLCYSTEINE LYASE OF *ACACIA FARNESIANA*: FRESH SEEDLINGS VS. ACETONE POWDERS

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Key Word Index—*Acacia farnesiana*; Leguminosae; alkylcysteine lyase; C-S lyase; homogeneous; djenkolic acid; acetone powders.

Abstract—Alkylcysteine lyase (EC 4.4.1.6) was purified essentially to homogeneity from both fresh hypocotyls of 5- to 8-day-old etiolated seedlings of *Acacia farnesiana* and acetone powders of such hypocotyls. The enzyme from the fresh material had twice the specific activity of that from the acetone powder. Sodium dodecylsulphate gel electrophoresis showed that both enzymes were composed of a subunit of M_r *ca* 42 000. The final enzyme solutions were quite different in their absorbance spectra. The fresh hypocotyl enzyme had an absorbance maximum at 425 nm in addition to the 280 nm protein absorbance. This maximum in the visible region is due to bound pyridoxal phosphate. The acetone powder enzyme had the same maxima and in addition peaks at 498 and 340 nm. The fresh enzyme contained 1.8 mol cofactor/mol enzyme and the acetone powder enzyme 1.0 mol/mol. The K_m for the probable natural substrate L-djenkolate was the same for both enzymes, 0.8 mM, but the V_{max} for the fresh was twice that of the acetone powder enzyme. The common practice of using acetone powder preparations for starting material in enzyme purifications would appear to require some caution.

INTRODUCTION

S-Substituted cysteines are found as secondary amino acids in a number of higher plant families [1]. In some cases they are present as the thioether, otherwise commonly as the sulphoxide. Frequently an enzyme is present in the plant which is capable of cleaving the cysteine derivative to volatile sulphur products, pyruvate and ammonia by a beta-elimination reaction [2]. The first of these plant enzymes to be purified to homogeneity was from acetone powders of 5- to 8-day-old etiolated hypocotyls of *A. farnesiana* [3]. It has been named alkylcysteine lyase (EC 4.4.1.6) It will cleave both thioether and sulphoxide cysteine derivatives. This distinguishes this enzyme from alliin lyase (EC 4.4.1.4), which is limited to the sulphoxides. In recent years alliin lyase from onion [4], garlic [5] and broccoli [6] has been purified to homogeneity and found to be composed of glycoproteins. In order to study the properties of the alkylcysteine lyase from *A. farnesiana* in more detail, the enzyme was prepared from acetone powders of etiolated hypocotyls as before [3], and it was also purified from homogenates of the fresh hypocotyls. Significant differences were found in the kinetics, pyridoxal-5'-phosphate content and in the spectra of enzyme protein isolated by these two procedures. These results are the subject of this report.

RESULTS

Purification comparisons

Table 1 summarizes the purification of the enzyme from fresh seedling hypocotyls by the modified purification procedure described in the Experimental. Only a 12-fold purification was achieved, but the enzyme was greater than 97% pure based on scanning of an 11% acrylamide tube gel stained with Coomassie blue after discontinuous gel electrophoresis. One major distinction between use of fresh hypocotyl homogenates as the enzyme source and hypocotyl acetone powder extracts is the difference in the specific activities of the starting material. In Table 1 the starting material had a specific activity of 0.12 μkat/mg. Our original results [3] had a starting specific activity of 0.02 μkat/mg. The same low initial activity compared with fresh homogenates was found in all the acetone powder extracts prepared in the current study. The final purified enzyme from the DEAE column had a specific activity of 1.45 μkat/mg for the fresh hypocotyl preparation compared to 0.7–0.9 μkat/mg for various preparations from acetone powders carried to the same degree of purification.

In some instances, the DEAE enzyme was processed further by elution from a column of hydroxylapatite. This gave no increase in specific activity and only resulted in some loss of enzyme.

SDS-PAGE analysis

Purified enzyme from both types of preparation were analysed by gel electrophoresis under denaturing conditions as described in the Experimental. There was no difference in the results from either preparation. A single band having an M_r of *ca* 42 000, based on comparison with the mobilities of proteins of known M_r, was present. Our previous report [3] had indicated that the holoenzyme was composed of two dissimilar subunits. The smaller one was reported as having a M_r of 48 000, which is in fair agreement with the current results. In the course of the present studies, it was found that the temperature at

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Table 1. Purification summary of fresh hypocotyl enzyme

	Volume (ml)	Protein (mg)	Total activity (μkat)	Specific activity (μkat/mg)	Purification	Recovery (%)
Homogenate	280	295	34.9	0.12	1.0	100
Protamine sulphate supernatant	295	224	29.3	0.13	1.1	84
$(NH_4)_2SO_4$ precipitate	5.0	59	18.2	0.31	2.6	52
Biogel A fractions	16.0	12.6	9.5	0.75	6.3	27
DEAE-Biogel A fraction	8.8	4.6	6.7	1.45	12.2	19

which the sample was heated in SDS prior to electrophoresis had an effect on the number and M_r of the bands perceived on the gel after staining. Our previous results were based on a method in which heating took place for 10 min at 65°. In the present work, if the sample was heated for 5 min at 100° prior to electrophoresis only a single band was found on the gel, whereas heating at 75° in 0.2% SDS yielded two bands as described previously [3].

Presence of pyridoxal phosphate

The effect of added pyridoxal phosphate on both types of enzyme preparation was examined. Table 2 shows the results of adding exogenous pyridoxal phosphate to the assay mixture with each type of enzyme. In agreement with the earlier results [3] there was a significant increase in specific acitivity in the presence of the cofactor with the enzyme purified from the acetone powder. The increase was 37% compared with only a 7% increase with the enzyme from the fresh hypocotyls. In the absence of pyridoxal phosphate, the purified acetone powder enzyme was less than 50% as active as the fresh enzyme. Upon addition of exogenous cofactor the activity of the acetone powder enzyme, although increased, was still only 60% as active as the enzyme from fresh tissue.

Spectra of the purified proteins from each preparation were obtained using a scanning spectrophotometer. The acetone powder enzyme showed the same peaks as reported previously [3], namely one at 498 nm and another at 425 nm, plus a new one as a shoulder at about 340 nm on the major protein absorbance peak. The enzyme solution prepared from an acetone powder is brown in colour. On the other hand, the enzyme solution obtained from fresh hypocotyls is light-green. The latter has a spectrum with the usual protein absorbance maximum at 280 nm and only one other peak at 425 nm which is due to bound pyridoxal phosphate. Figure 1 is a reproduction of the scan from both preparations.

The amount of pyridoxal phosphate bound to the protein was determined chemically (see Experimental). The purified enzyme from fresh hypocotyls contained 12.6 nmol of pyridoxal phosphate/mg of enzyme compared to 7.3 nmol/mg for the acetone powder enzyme. This is only 58% of that found in the fresh hypocotyl enzyme. Assuming that the extinction coefficient for the bound cofactor is the same in both enzyme preparations, the spectral data (Fig. 1) show that the acetone powder enzyme contains only 55% as much cofactor bound on an equal weight basis. From these data and assuming that the holoenzyme had an M_r of 142 000 [3], there are 1.8 mol of cofactor per mol fresh hypocotyl enzyme compared to 1.0 mol/mol acetone powder enzyme. This latter ratio agrees well with the earlier results [3].

Table 2. Effect of added pyridoxal phosphate

Assay conditions	Reaction rate (μkat/mg)	
	Acetone powder	Fresh hypocotyls
Minus cofactor	0.71 ± 0.02	1.51 ± 0.05
Plus cofactor	0.97 ± 0.01	1.62 ± 0.03
% Increase in activity	37	7

Pyridoxal phosphate was present at 25 μM where added. The error is expressed as the standard deviation based on five replications.

Kinetic differences

The usual kinetic parameters of K_m and V_{max} were determined for the enzyme from each type of starting material. The results in Table 2 show a marked difference in the specific activity for the enzymes from each preparation. Determinations of the K_m value for L-djenkolate for each type of isolated enzyme showed very little difference in the value. By linear regression analysis of the $1/v$ vs. $1/S$ form of the Michaelis–Menten equation the K_m was 0.8 mM for the fresh enzyme and 0.7 mM for the acetone powder enzyme. The correlation coefficients for the data were 0.99 and 1.00, respectively. However, the V_{max} for the fresh enzyme was 2.0 μkat/mg protein and 1.0 for the acetone powder enzyme. These results were consistent for three separate purifications for each type of enzyme.

DISCUSSION

One of the classical procedures for obtaining a standard preparation of starting material for enzyme isolation has been the preparation of an acetone powder of the tissue or organism selected. Among the advantages are the ability to prepare uniform material from a large amount of tissue and to circumvent the seasonal nature of many plant materials. The assumption has been made that the enzyme protein isolated from this powder is essentially the native protein of the living tissue. The results presented above suggest that the process of preparation of the powder has

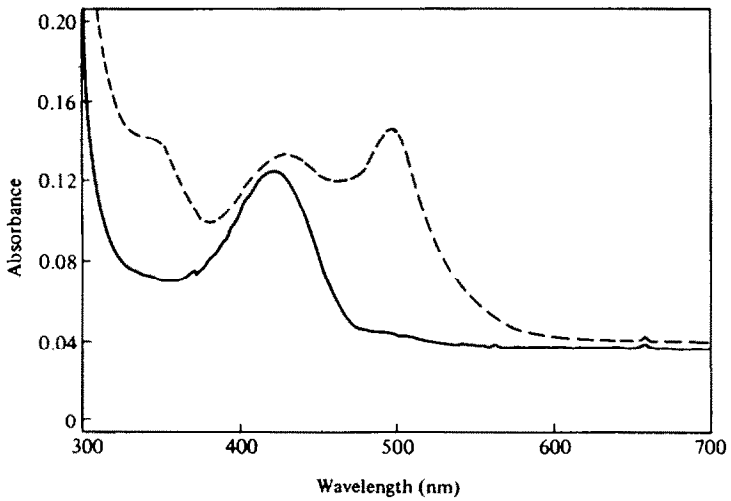

Fig. 1. Absorbance spectra of fresh hypocotyl enzyme (——) and acetone powder enzyme (- - - -) carried out as described in the Experimental. The fresh enzyme concentration was 0.75 mg/ml with a specific activity of 1.1 μkat/mg. The acetone powder concentration was 1.22 mg/ml and had a specific activity of 0.4 μkat/mg.

a marked influence on the nature of the enzyme as reflected in its appearance, cofactor binding and kinetic properties.

The fact that V_{max} is affected and that K_m is not can have several possible explanations. One is that the enzyme conformation may be modified by the acetone treatment, but the binding site for substrate is unchanged. The spectral studies certainly show that the endogenous pyridoxal phosphate is removed or altered to a considerable degree in the acetone powder. The removal of bound cofactor could result in an irreversible loss of enzyme activity producing a population of active and inactive molecules of enzyme, leading to a decrease in activity with no effect on K_m. The absorbance peak in the acetone powder enzyme spectrum at 340 nm is consistent with the conversion of bound pyridoxal phosphate to an inactive substituted aldimine by reaction with a side chain of an amino acid at the active site [7]. A third explanation could be that the acetone treatment produces a population consisting of both inactive enzyme molecules and conformationally altered enzyme molecules.

The relative ease of isolation in which a 12-fold purification gives an almost homogeneous enzyme suggests that the enzyme is a major soluble protein in the hypocotyl homogenate. The physiological reason for such a high concentration of enzyme in this tissue was suggested previously [3] as being responsible for defence against soil pathogens during germination by production of inhibitory volatile sulphur compounds.

The results reported previously [3] must be modified in the light of the present findings. The enzyme is composed of only one type of subunit rather than two dissimilar subunits. The native enzyme binds at least twice as much pyridoxal phosphate per mole as had been previously reported. Finally, the peaks observed previously in the absorbance spectrum at 340 and 498 nm appear to be an artefact of isolation from the acetone powder starting material.

EXPERIMENTAL

Purification of enzyme. The seeds were obtained from trees in the University arboretum. Seedlings were germinated and Me_2CO powders prepared as before [3]. The purification of the Me_2CO powder enzyme followed the same protocol as previously [3] except for the following modifcations: (a) the extracting buffer contained 2% (w/v) insoluble polyvinylpolypyrrolidone (PVP); (b) Sephadex G-200 was replaced with Biogel A 0.5 m in the gel filtration step; (c) elution was from a DEAE-Biogel column with a salt gradient from 0 to 0.6 M. The fresh enzyme was prepared from seedlings after removal of their cotyledons. The hypocotyls and roots were frozen at −20° and then homogenized in a chilled blendor using 50 mM K-Pi buffer, pH 7.2, containing 2% (w/v) insoluble PVP. After filtration through cheesecloth, the filtrate was centrifuged for 20 min at 10000 *g* at 4°. The resulting supernatant soln was then decanted and the enzyme isolation carried out as above. Protein was estimated by the method of ref. [8] except where specified otherwise.

Standard enzyme assay. The standard assay mixture contained the following 175 mM Tricine buffer, pH 7.8; 25 μM pyridoxal phosphate; 10 mM L-djenkolate; enzyme. The final vol. was usually 1 ml. Incubation at room temp. for the desired time, usually 3–5 min, was ended by the addition of 1 ml 10% (w/v) TCA. After clarification by centrifugation, an aliquot of the acidified reaction mixture was assayed for pyruvate colorimetrically [9].

Pyridoxal phosphate in the purified enzyme was determined by the method of ref. [10] on a sample of 1–2 mg of enzyme as estimated by the protein assay method of ref. [11]. The absorbance spectra of the purified enzymes were carried out by a Hewlett–Packard model 8450A recording spectrophotometer.

Gel electrophoresis. Sodium dodecylsulphate gel electrophoresis (SDS-PAGE) was performed according to the method of ref. [12]. The gels and gel buffers contained 0.1% SDS. The protein samples were denatured by the addition of SDS to a final concn of 1% and subsequent heating at 100° for 5 min.

Chemicals. Biogel A-0.5 m and DEAE Biogel A were obtained from BioRad. L-Djenkolic acid, pyridoxal-5′-phosphate and Tricine were purchased from Sigma. $(NH_4)_2SO_4$ was enzyme grade quality obtained from Schwarz-Mann.

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