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CARBONIC ANHYDRASE OF THE COTTON PLANT

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Key Word Index—*Gossypium hirsutum*; Malvaceae; carbonic anhydrase; chloroplasts; effects of aging; sulfhydryl reagents.

Abstract—Nonaqueous fractionation of leaves of the cotton plant suggested that carbonic anhydrase was associated with the chloroplasts. Activity of this enzyme in aqueous extracts prepared in media containing no reductants was stable at 4°. Response to sulfhydryl reagents varied. The results indicated that thiol groups, necessary for the activity of the enzyme, were partially protected from oxidation.

INTRODUCTION

In leaves of plants such as *Spinacea oleracea* and *Beta vulgaris*, most of the carbonic anhydrase is associated with the chloroplasts [1,2]. However, in leaves of *Tetragonia expansa* and *Tropaeolum majus*, carbonic anhydrase was reported to be cytoplasmic [3], although Everson and Slack [1] opposed this view. The intracellular locations of this enzyme thus seem to vary with the plant species. The activity of carbonic anhydrase from grapefruit was also inhibited by *p*-chloromercuribenzoic acid [4]. It is likely that carbonic anhydrase is one of those enzymes whose activities depend greatly on –SH groups. However, the different requirements of reductant for active preparations of this enzyme [1,4] suggest that the behaviors of the thiol groups toward oxidants differ with the proteins of carbonic anhydrase from various plant sources.

The distribution and stability of carbonic anhydrase, and response of this enzyme to sulfhydryl reagents have therefore been investigated in leaves of cotton.

RESULTS AND DISCUSSION

As shown in Table 1, the distributions of carbonic anhydrase, a chloroplastic marker enzyme, and a cytoplasmic marker enzyme were compared with that of chlorophyll in leaf fractions prepared in nonaqueous media. Photosynthetic pyridine nucleotide reductase (a chloroplast enzyme) and carbonic anhydrase were distributed in a manner similar to chlorophyll, while acid phosphatase (a

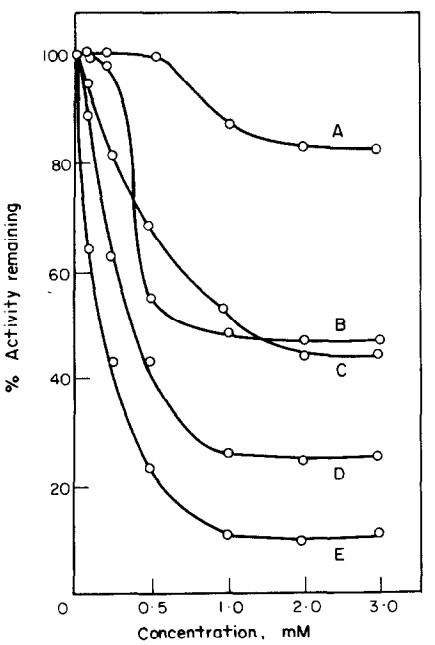

Fig. 1. Effects of various concentrations of different sulfhydryl reagents on activities of carbonic anhydrase from cotton leaves. The enzyme extracts were dialyzed at 4° for about 8 hr against 0·1 M Tris–HCl buffer at pH 8. Aliquots of this preparation were treated in 0·05 M phosphate buffer (pH 7) containing various sulfhydryl reagents by incubating at 4° for 30 min. Then activities of enzyme were determined in 0·05 M phosphate buffer at pH 7 as described in Experimental. Results were averages of three experiments. (A) Ferricyanide; (B) 5,5′-dithiobis-(2-nitrobenzoic acid); (C) oxidized glutathione; (D) *p*-chloromercuribenzoic acid; (E) iodine.

Table 1. Distribution of enzymes and chlorophyll in leaf fractions prepared in nonaqueous media

Fraction density	Chlorophyll % total	Enzyme activity (% total activity)		
		Carbonic anhydrase*	Photosynthetic pyridine nucleotide reductase	Acid phosphatase
<1·34	52	53	59	26
1·34–1·4	26	29	28	11
>1·4	22	18	13	63

* The enzyme activity was determined by the method of Wilbur and Anderson [11]. Each value represents the average result of two independent analyses.

Table 2. Restoration of enzyme activities of PCMB-inhibited carbonic anhydrase after treatment with excess 2-mercaptoethanol

Conc of PCMB (mM)	Enzyme activity	
	% Inactivation	% Restoration
0·13	12	98
0·25	37	102
0·50	58	97
1·00	74	105

Enzyme extracts were treated in 0·05 M phosphate buffer (pH 7) containing various concentrations of PCMB at 4° as described in Fig. 1. After an incubation period of 30 min, each sample mixture was dialyzed for about 7 hr and treated with 2-mercaptoethanol (about 5 mM at the final concentration). At the end of an incubation period of 120 min, the activity of enzyme was determined as described in the Experimental. Each value was an average of three replicates.

cytoplasmic enzyme) was not. Thus, the carbonic anhydrase in cotton leaves appeared to be associated with chloroplasts.

As shown in Fig. 1, the activity of carbonic anhydrase from cotton leaf was most sensitive to inhibition by iodine and *p*-chloromercuribenzoic acid (PCMB). However, enzyme inactivation was not complete, even at 3 mM concentration of these reagents. In contrast, the enzyme activity was least affected by treatment with ferricyanide. The effects of 5,5′-dithiobis-(2-nitrobenzoic acid) and oxidized glutathione on the activity of the enzyme were intermediate, as compared with those of PCMB and of ferricyanide. As shown in Table 2, enzyme inactivation by PCMB was completely reversed by treatment with excess 2-mercaptoethanol, confirming inhibition due to mercaptide formation. The kinetics of reversibility of enzyme inhibition were characterized by a prolonged lag period of 60 min followed by a gradual restoration of activity over a period of an additional 60 min. These data indicate that the geometric configuration of the enzyme protein may be such that the sulfhydryl groups are not readily accessible to oxidants. This property of the enzyme protein is consistent with observations on the stability of the enzyme during aging. The enzyme, when prepared in a medium containing no reductant, was found to be quite stable for a period of about 20 hr at 4°.

EXPERIMENTAL

Glandless cotton plants (*Gossypium hirsutum* L. cv. Coker 100) were raised in a greenhouse in a soil-vermiculite mixture. Cotton leaf laminae (4·5 g) were homogenized in 50 ml of 0·1 M Tris–HCl buffer at pH 8·3. The material was squeezed through two layers of cheesecloth and the filtrate was centrifuged at 27000 *g* for 5 min. The supernatant was decanted and saved. The residue was made into a thin paste. To this material, the supernatant solution was returned, since the level of retention of carbonic anhydrase to chloroplasts was unknown. The mixture was stirred gently to obtain a uniform enzyme suspension. The procedure of Smillie *et al.* [5] was followed for fractionation of cotton leaves in a mixture of hexane–CCl_4 prepared in various densities. Acid phosphatase and photosynthetic pyridine nucleotide reductases were assayed by the procedures described by Lowry [6] and Arnon and Jegendorf [7], respectively. The content of chlorophyll was determined by the method of Arnon [8].

The Krebs–Roughton Warburg Technique [9], with minor modifications, was used to assay enzyme activity in a Gilson differential respirometer. At 9°, 3 ml of 0·05 M phosphate buffer at pH 7·0 (with or without various concentrations of sulfhydryl reagents) plus 0·5 ml of enzyme were placed in the main compartment of a reaction vessel and 0·5 ml of 0·05 M $NaHCO_3$ was placed in the side arm. After equilibration, the 2 soln were mixed, shaken at 131 oscillations/min, and pressure changes recorded at 30-sec intervals over a period of 5 min. The pressure changes in both the control (nonenzymically catalyzed reaction) and the reaction catalyzed by enzyme were a linear function of time until about one-third of the final pressure change was attained. The micrometer readings were corrected using the formula given in Manometric and Biochemical Techniques [10].

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