SC 11065

Zinc-free plant carbonic anhydrase; lack of inhibition by sulfonamides

The suggestion[1] that inhibition of animal carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) by sulfanilamide involves the binding of the inhibitor to the zinc moiety of the protein stimulated some interest in the effect of sulfonamides on plant carbonic anhydrase. KONDO *et al.*[2] have purified carbonic anhydrase from spinach leaves and have found Zn to be absent. This is in contrast to the work of WAYGOOD[3] and of SIBLY AND WOOD[4] who reported 0.05% and 0.056% Zn, respectively. Both KONDO *et al.*[5] and SIBLY AND WOOD have found that sulfanilamide exhibits little or no inhibitory activity toward plant carbonic anhydrase. In this investigation the findings of KONDO *et al.* with respect to the absence of Zn have been confirmed. Additionally, the effect of two potent inhibitors of animal carbonic anhydrase, acetazolamide and 2-benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide (Cl 11 366, American Cyanamid Co)[6], have been studied.

Carbonic anhydrase was purified 50-fold from the leaves of fresh parsley. All centrifugations were carried out at 0° for 20 min at 18 000 × *g*. Throughout the preparation enzyme solutions contained 5 mM cysteine (pH 7). After treatment with calcium phosphate gel these solutions contained 1 mM EDTA as well. Enzyme preparations during various stages of purification were stored frozen with no apparent loss of activity providing that cysteine was present.

Analysis for carbonic anhydrase activity was carried out according to the method of MAREN *et al.*[7] with the exception that 0.2 mM EDTA was included in the reaction mixture. Protein was determined spectrophotometrically.

Chilled parsley leaves were ground in a food grinder to a pulp which was subsequently squeezed through cotton cloth. The expressed juice was adjusted to 0.005 M cysteine and pH 7 and centrifuged. 100 ml of the supernatant fraction was stirred with 75–100 g (wet wt.) of calcium phosphate gel and centrifuged. All carbonic anhydrase activity was found to reside in the supernatant fluid. Fractionation with $(NH_4)_2SO_4$ precipitated the enzyme at 35–65% saturation. After dialysis against cysteine and EDTA, the enzyme was precipitated by the addition of ethanol to 30% concentration. The ethanol precipitate was suspended in cysteine–EDTA solution, centrifuged, and the insoluble material discarded. The procedure is summarized in Table I.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

Enzyme fraction	*mg protein/ml*	*E.U.*/mg protein*
Original juice of ground parsley leaves	56	2.6
Supernatant fraction of above	44	2.4
Calcium phosphate gel supernatant fluid	25	4.7
35–65% $(NH_4)_2SO_4$ fraction	20	78.7
0–30% ethanol fraction	0.87	128.5

* One E.U. equals that amount of enzyme which will double the uncatalyzed rate in the system described.

Analysis for Zn was performed by the dithizone method as outlined by MALMSTRÖM[9]. The original supernatant fluid of parsley juice contained 225 μg of Zn per ml or 5.1 μg per mg protein. This figure is probably somewhat high since no precautions were taken to exclude contamination by Zn during the early stages of purification. A sample of the ethanol fraction, consisting of 46 mg of protein, was dialyzed against several changes of Zn-free water and subsequently lyophilized. Analysis of this sample showed Zn to be absent. Since this method was capable of detecting as little as 0.1 μg of Zn, the ethanol fraction contained less than 0.002 μg of Zn per mg of protein.

Two powerful inhibitors of animal carbonic anhydrase, acetazolamide (K_i, $2 \cdot 10^{-8}$ M) and Cl 11 366 (K_i, $7 \cdot 10^{-9}$ M) were found to be inactive toward both the $(NH_4)_2SO_4$ and ethanol fractions of the plant enzyme. (The K_i's are the values determined experimentally under the conditions of the assay employed in this study in which the enzyme and inhibitor were not equilibrated.) The concentration of acetazolamide in the reaction mixture was $2.3 \cdot 10^{-5}$ M and that of Cl 11 366 was $1.7 \cdot 10^{-5}$ M.

The recent finding of LINDSKOG[10] that acetazolamide does not bind to metal-free inactive carbonic anhydrase but binds to the Co or Zn enzyme, corroborates the suggestion that the metal is involved in the affinity of sulfonamides for metal-containing carbonic anhydrase of animal origin. His data further support the assumption that the lack of inhibition by sulfonamides of plant carbonic anhydrase is due to the absence of Zn in this enzyme.

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Received February 25th, 1963