Phytochemistry, 1972, Vol 11, pp 113 to 117 Pergamon Press Printed in England

PURIFICATION AND CHARACTERIZATION OF CARBONIC ANHYDRASE FROM *PISUM SATIVUM**

W KISIEL† and G GRAF

Department of Biochemistry, North Dakota State University, Fargo, North Dakota, U S A

(*Received* 18 *May* 1971)

Abstract—A soluble enzyme with carbonic anhydrase activity was isolated from pea leaves and purified 200-fold by ammonium sulfate fractionation, gel filtration and preparative polyacrylamide gel electrophoresis. The purified enzyme behaved as a single component in analytical polyacrylamide gel disc electrophoresis at pH 8 9 Its molecular weight was 194,000 as determined by gel filtration and analytical ultracentrifugation. When the enzyme was electrophorized in the presence of sodium dodecyl sulfate, a molecular weight of 30,000 was observed The specific activity for the dehydration reaction was approximately 1000 U/mg protein at 20° and the K_m was 30 mM The enzymatic activity was inhibited by acetazolamide, *p*-chloromercuribenzoate and *o*-iodosobenzoate No esterase activity was detected with *p*-nitrophenyl acetate as the substrate The amino acid composition of the enzyme has been determined Neutron activation analysis indicated the presence of zinc in the ratio of six moles of zinc per one mole of enzyme

INTRODUCTION

CARBONIC anhydrase (Carbonate hydro-lyase, E C. 4 2.1 1) is widespread in higher plants, but molecular data of the enzyme have been reported only recently Tobin[1] purified a carbonic anhydrase from the leaves of parsley (*Petroselinum crispum* var *latifolium*) and characterized it as a zinc and cysteine containing enzyme with a hexameric structure of 29,000 molecular weight subunits. Earlier Kondo *et al*[2] and Fellner[3] were unable to detect zinc in partially purified carbonic anhydrase preparations from parsley and spinach (*Spinacia oleracea*) Contrary to the findings of Fellner,[3] Tobin[1] has demonstrated the inhibition of parsley carbonic anhydrase by acetazolamide, and Everson[4] has reported acetazolamide inhibition of partially purified spinach carbonic anhydrase This paper describes the purification and characterization of carbonic anhydrase from the leaves of pea plants (*Pisum sativum* 'Little Marvel') and relates its properties to other plant carbonic anyhdrase preparations.

RESULTS AND DISCUSSION

Purification

A study of the distribution of carbonic anhydrase activity in 21-day-old pea plants indicated that leaf tissue was the best source of the enzyme. The leaves contained ten times as much activity as the stems, but no carbonic anhydrase activity was observed in the root

* Published with the approval of the Director, North Dakota Agricultural Experimental Station, Journal Article No. 276

† Present Address Department of Biochemistry, School of Medicine, University of Arizona, Tucson, Arizona

[1] A J TOBIN, *J Biol Chem* **245**, 2656 (1970)
[2] K KONDO, T YONEZAWA and H CHIBA, *Bull Res Inst Food Sci.* **8**, 1, 17, 28 (1952).
[3] S K FELLNER, *Biochim Biophys Acta* **77**, 155 (1963)
[4] R G EVERSON, *Phytochem.* **9**, 25 (1970)

tissue The shoots and the cotyledons of 6-day-old etiolated pea seedlings exhibited only marginal activity and no activity was detected in their roots.

Quantitative data for a representative purification experiment are given in Table 1 These data indicate a 200-fold purification of the enzyme with 30% recovery. The purification steps were followed by an electrometric assay according to the method of Wilbur and Anderson [5] The Fraction IV enzyme behaved as a single component in analytical polyacrylamide gel disc electrophoresis at pH 8 9

TABLE 1 PURIFICATION OF PEA CARBONIC ANHYDRASE

Fraction	Total volume (ml)	Total protein (mg)	Total units*	Carbonic anhydrase activity (units/mg protein)	Recovery (%)	Purification (fold)
I (Crude)	520 0	1300 0	12,012	9 2	100 0	1 0
II ($[NH_4]_2SO_4$)	21 5	603 1	14,860	24 6	123 7	2 7
III (Sephadex)	149 0	169 9	8520	50 2	70 9	5 4
IV (Preparative Electrophoresis)	76 0	2 1	3840	1847 7	32 0	200 1

* Wilbur–Anderson units of activity

Enzyme Properties

The specific activity of the electrophoretically pure enzyme for the dehydration reaction at 20°, measured by the modified Krebs-Roughton[6] assay, was approximately 1000 U/mg protein Extrapolation of the Lineweaver–Burk plot yielded a K_m of $3\,0 \times 10^{-2}$ M for HCO_3^- Significant substrate inhibition was observed with HCO_3^- concentrations greater than 0 1 M

In contrast to human and bovine carbonic anhydrase, no esterase activity of pea carbonic anhydrase was detected with *p*-nitrophenyl acetate as the substrate The heat stability of the purified enzyme was high and 40% of its activity was retained after incubation at 60° for 15 min

The molecular weight of pea carbonic anhydrase was estimated as 193,000 by gel filtration on Sephadex G-200 A molecular weight of 194,000 ± 5% was calculated from meniscus depletion sedimentation equilibrium experiments at 20° in 0 05 M phosphate–0 001 M dithioerythritol buffer, pH 7 0 No significant variation in the molecular weight was observed over the range of protein concentrations 0 25–1 0 mg/ml A partial specific volume of 0 723 was obtained from amino acid composition data by the method of Cohn and Edsall [7]

When electrophorized in the presence of sodium dodecyl sulfate, pea carbonic anhydrase exhibited a molecular weight of 30,000 The major portion of the sample migrated as a 30,000 molecular weight species, however, a few minor bands were observed with molecular weights between approximately 30,000 and 90,000 These may represent various aggregation states of the subunits

[5] K M WILBUR and N G ANDERSON, *J Biol Chem* **176,** 147 (1948)
[6] H A KREBS and F J W ROUGHTON, *Biochem J* **43,** 550 (1948)
[7] E J COHN and J T EDSALL, *Proteins, Amino Acids and Peptides* Reinhold, New York (1943)

Pea carbonic anhydrase was tested for inhibition by various heavy metal ions and sulfhydryl inhibitors *o*-Iodosobenzoate (0 5 mM) and *p*-chloromercuribenzoate (0 25 mM) completely inhibited the enzyme after 10 min incubation at 20°. Among zinc(II)-, iron(III)-, nickel(II)-, cobalt(II)-, lead(II)-, magnesium(II)-, mercury(II)- and manganese(II)-ions at 0 5 mM concentrations, only mercury(II) ion was significantly inhibitive and caused complete inhibition after 10 min incubation at 20° None of these heavy metal ions activated the enzyme. Acetazolamide, a strong inhibitor of mammalian carbonic anhydrases, inhibited the hydration reaction measured by the Wilbur–Anderson method[5] ($I_{50} = 2\,8 \times 10^{-5}$ M). In the dehydration reaction, measured by the Krebs–Roughton manometric assay,[6] higher concentrations of acetazolamide were required ($I_{50} = 58 \times 10^{-5}$ M) Since I_{50} values may be buffer-, and pH-dependent, it is difficult to evaluate the significance of this variation under the different conditions of the two assays

The neutron activation analysis of the purified enzyme indicated that pea carbonic anhydrase contained zinc in the ratio of six moles zinc per mole enzyme This result and the molecular weight determination by electrophoresis in the presence of sodium dodecyl sulfate suggested a hexameric quaternary structure for the pea carbonic anhydrase similar to that observed for parsley carbonic anhydrase.[1] The amino acid composition of pea carbonic anhydrase has been determined and is shown in Table 2 From these data and the information obtained from the analytical ultracentrifugation (mol wt 194,000), a residue molecular weight of 197,800 was calculated.

TABLE 2 AMINO ACID COMPOSITION OF PEA CARBONIC ANHYDRASE

Amino acid	Residues/ 100 g protein	Min (MW)	Moles/ 194,000	No residues/ 194,000	No residues x min MW	No residues x MW residue
Aspartic	8 57	1343	144 45	144	193,392	16,574
Threonine*	4 28	2363	82 11	82	193,766	8292
Serine*	6 23	1398	138 78	139	194,322	12,103
Glutamic	10 10	1279	151 74	152	194,408	19,628
Proline	4 57	2125	91 28	91	193,375	8839
Glycine	5 04	1132	171 33	171	193,572	9759
Alanine	5 84	1217	159 37	159	193,503	11,303
Valine†	7 59	1306	148 51	149	193,594	14,773
Cystine (1/2)	1 53	6676	29 06	29	193,605	2963
Methionine	0 78	16,822	11 53	12	201,864	1575
Isoleucine	4 04	2801	69 26	69	193,269	7809
Leucine	9 86	1148	169 02	169	194,012	19,126
Tyrosine	5 18	3150	61 58	62	195,300	10,118
Phenylalanine	8 39	1754	110 58	111	194,694	16,338
Ammonia	2 46	692	280 23	280	193,760	4768
Tryptophan‡	1 44	12,932	15 00	15	193,980	2793
Lysine	9 33	1374	141 20	141	193,734	18,075
Histidine	3 45	3976	48 79	49	194,824	6721
Arginine	3 18	4912	39 50	40	196,480	6248
					194,550 ± 1940	197,802

* Values obtained by extrapolation to zero time
† 72 hr value
‡ Determined according to Spande and Witkop [13]

EXPERIMENTAL

Extraction and purification procedure Pea (*Pisum sativum* 'Little Marvel') plants were harvested after 21 days growth in vermiculite with half strength Hoagland's solution under controlled conditions in a growth chamber 100-g portions of excised pea leaves were homogenized at 4° in 800 ml 0 1 M phosphate buffer (0 1 M in NaCl, 0 005 M in cysteine), pH 7 5, in the presence of 100 g Polyclar AT in a Waring blender for 1 min The homogenate was filtered through cheese cloth and the filtrate centrifuged at 30,000 *g* for 30 min in a Sorvall RC-2-B refrigerated centrifuge The pellet was discarded and the supernatant (Fraction I) was brought to 30% saturation with solid $(NH_4)_2SO_4$ The precipitate was removed by centrifuging at 30,000 *g* for 10 min and the pellet discarded The supernatant was adjusted to 60% $(NH_4)_2SO_4$ saturation and the precipitate collected by centrifuging at 30,000 *g* for 10 min The supernatant was discarded and the active pellet was redissolved in 20 ml 0 1 M phosphate buffer (0 1 M in NaCl), pH 7 5, and centrifuged at 30,000 *g* for 10 min to remove any insoluble material

The supernatant (Fraction II) was subjected to gel filtration at 4° on a Sephadex G-200 column The Sephadex had been previously equilibrated with 0 1 M phosphate buffer (0 1 M in NaCl) pH 7 5 The sample was eluted from the column with the equilibrating buffer at a flow rate of 0 5–1 0 ml/min The active portion of the eluate (Fraction III) was brought to 70% $(NH_4)_2SO_4$ saturation at 0° with saturated neutralized $(NH_4)_2SO_4$ solution The precipitate was collected by centrifugation at 30,000 *g* for 10 min, redissolved in 2 ml 0 05 M tris-borate buffer (1 5 mM in EDTA), pH 8 1, and subjected to preparative polyacrylamide gel electrophoresis at 0° in a Buchler Poly-Prep apparatus For the electrophoretic separation, a dual gel system was employed which consisted of 80 ml 5% gel above 40 ml 7 5% gel Both gels were chemically polymerized with $(NH_4)_2S_2O_8$ The electrophoresis buffer in the gels and in the buffer compartments was 0 05 M tris-borate (1 5 mM in EDTA), pH 8 1 The membrane holder buffer was 0 2 M tris-borate (6 0 mM in EDTA), pH 8 1 The gels were pre-run at 300 V for 5 hr prior to sample application The sample was electrophorized at a constant voltage of 300 V for approximately 15 hr The active portion of the electrophoresis eluate (Fraction IV) was concentrated by ultrafiltration in an Amicon ultrafiltration apparatus through a PM-10 membrane and stored at 5° The characterization studies were performed on the electrophoretically pure sample

Analytical polyacrylamide gel disc electrophoresis Analytical polyacrylamide gel disc electrophoresis was carried out according to a slightly modified procedure of Ornstein and Davis[8] in a Buchler Poly-Analyst apparatus A photopolymerized 2 5% gel (0 4 ml) was cast over a chemically polymerized 7 5% gel (0 6 ml), and the electrophoresis was performed at room temp Samples of 200 μl containing 25–200 μg protein were electrophorized

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed according to Shapiro *et al*[9] with some modifications Bovine serum albumin, bovine carbonic anhydrase, ovalbumin, and lysozyme were used as molecular weight standards 50 μg portions of each protein in 10 μl vol were electrophorized in 1 0 ml 7 5% gel containing 0 01 M phosphate buffer (0 1% in sodium dodecyl sulfate), pH 7 3, The electrophoretic buffer was 0 01 M phosphate buffer (0 1% in sodium dodecyl sulfate), pH 7 3 The electrophoresis was performed at room temp in a Buchler Poly-Analyst apparatus

Analytical ultracentrifugation Sedimentation equilibrium experiments were performed at 20° according to Yphantis[10] in a Spinco Model E analytical ultracentrifuge equipped with interference optics The enzyme was dissolved in 0 05 M phosphate–0 001 M dithioerythritol buffer, pH 7 0, and the operating speed was 11,300 rev/min The interference pattern was photographed on Kodak Spectroscopic Type II-G plates and the fringe displacements were measured with a Nikon microcomparator

Molecular weight determination by gel filtration The molecular weight of the enzyme was estimated on a Sephadex G-200 column according to Whitaker[11] The gel was equilibrated with 0 1 M phosphate buffer, pH 7 0 Bovine serum albumin, γ-globulin, ovalbumin and bovine carbonic anhydrase were used as molecular weight standards and Dextran 2000 was employed to determine the void volume 1-mg portions of each protein in 1 0 ml equilibrating buffer were applied to the column and eluted at a flow rate of 0 3 ml/min The determination was performed at room temp

Amino acid analysis Amino acid analyses were performed in a Technicon AutoAnalyzer on duplicate enzyme samples hydrolysed in 6 N HCl according to Moore and Stein[12] for 24, 48 and 72 hr at 110° Tryptophan was determined according to the method of Spande and Witkop[13] by *N*-bromosuccinimide titration in 8 M urea adjusted to pH 4 0 with HOAc

[8] L ORNSTEIN and B J DAVIS, *Ann N Y Acad Sci* **121**, 404 (1964)
[9] A C SHAPIRO, E VINUELA and J V MAIZEL, *Biochim Biophys Res Commun* **28**, 815 (1967)
[10] D A YPHANTIS, *Biochemistry* **3**, 297 (1964)
[11] J R WHITAKER, *Analyt Chem* **35**, 1950 (1963)
[12] S MOORE and S W STEIN, *Methods Enzymol* **6**, 819 (1963)
[13] T F SPANDE and B WITKOP, *Methods Enzymol* **11**, 498 (1967)

Neutron activation analysis Neutron activation analysis of the zinc content of the enzyme was performed by PPB, Inc, Research Park, Columbia, Missouri

Assay methods The distribution of protein in volumn eluates was monitored by recording the absorbance at 280 nm Protein was determined according to Lowry *et al*[14] using a calibration curve obtained with bovine serum albumin (Fraction V) All fractions obtained in the purification procedure were assayed electrometrically at 0° with a Heath recording pH meter equipped with a multi-speed chart drive and a combination electrode The reaction mixture contained 10 0 ml 0 02 M Veronal buffer, pH 8 0 and 0 2 ml enzyme solution at 0° Five ml saturated CO_2 solution at 0° was injected by means of a syringe into the mixture and the time required to reduce the pH from 8 0 to 6 2 was recorded (approx 3–15 sec) A blank determination was also performed substituting 0 2 ml buffer for the enzyme solution and the time to obtain the same pH change was recorded (approx 150 sec) Carbonic anhydrase activity for the hydration reaction was calculated from the following formula units $= (t_0 - t)/t$, where t_0 is the time of the uncatalysed reaction and t is the time of the enzyme catalysed reaction

Kinetic studies in the dehydration reaction were performed manometrically with a Gilson differential respirometer at 20° The reaction mixture contained 0 8 ml 0 1 M phosphate buffer, pH 7 0, and 0 2 ml enzyme solution in the respirometer flask, and 1 0 ml 0 2 M $NaHCO_3$ in the side arm. The volume of CO_2, produced within 15 sec after adding the substrate, was recorded The reaction rate was corrected with a blank run without enzyme to compensate for the nonenzymatic production of CO_2 The specific activity for the dehydration reaction is defined as the μmoles CO_2 produced/min/mg protein at 20°.

Esterase activity was assayed with *p*-nitrophenyl acetate as the substrate according to Pocker and Stone[15] at pH 7 45 and 25° in a Hitachi Coleman 124 recording spectrophotometer

All chemicals used were high quality commercial samples and all solutions were prepared with deionized water

Acknowledgments—This research was supported by the North Dakota State University Agricultural Experiment Station The authors wish to acknowledge the assistance of Dr H J Klosterman in the analytical ultracentrifuge experiments

[14] O H. Lowry, N J Rosebrough, A L Farr and R J Randall, *J Biol Chem*, **193**, 265 (1951)
[15] Y Pocker and J T Stone, *Biochem* **6**, 668 (1967)

Key Word Index—Pisum sativum; Leguminosae, carbonic anhydrase