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Carbonic Anhydrase From *Nicotiana Tabacum* Leaves

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In this study, carbonic anhydrase (CA: carbonate hydrolyase; E.C.4.2.1.1) was purified from adult *Nicotiana tabacum* leaves and studied biochemically. The enzyme was purified twice times by using $(NH_4)_2SO_4$ precipation and DEAE-cellulose column chromatography, and its activity was determined for two different substrates (CO_2 and p-nitrophenyl acetate). The enzyme obtained from the ion exchange column was purified 40.7 fold and the purity was controlled by 3%-10% discontinuous SDS-PAGE.

The pH of the purified enzyme varied between 6.0 and 7.2, the optimum being 6.9. V_{max} and K_m values were calculated with p-nitrophenyl acetate (0.1524 mM, 0.5446 mM, respectively) as substrate. The optimum temperature for the enzyme was 40 °C.

The molecular weight of the enzyme and of the subunits were found to be approximately $\approx$ 137.000 daltons and $\approx$ 22.800 daltons, respectively. The results indicate that 6 subunits are present. Changes in enzyme activity were determined in the presence of caffeine, nicotine, metal ions and some chemicals.

Key words: Carbonic anhydrase, *Nicotiana tabacum,* caffeine, nicotine

Introduction

Of the seven known isoenzymes of carbonic anhydrase in mammals, the most interest has been focused on human CA I and II (HCA I and HCA II) and bovine CA III. These are monomers with molecular weights of about 29 kDa.[1,2,3,4].

The structure, enzymatic function and physiological role of mammalian carbonic anhydrases (CA, E.C.4.2.1.1) have been extensively studied since the discovery of these enzymes by Meldrum and Roughton[5]. Work since 1960 on carbonic anhydrases from erythrocytes of cattle[6], man[7,8], rhesus monkeys[9] and horses[10] allows several general statements to be made about the structure of mammalian carbonic anhydrases.

The molecular weight of CA enzymes in spiral corded animals was about 29.000-30.000, and containing 1 atom of zinc per molecule, which is necessary for enzymatic activity[11].

Carbonic anhydrase in plants was first observed in 1939[12], and the enzyme was then partially purified from several plants and characterised[13,14,15].

The enzyme from higher plants exists in at least two electrophoretically separable types. One type, found principally in monocotyledon species, has a suggested molecular weight of 40.000[16]. The other,

isolated from dicotyledon species, is an hexameric enzyme of molecular weight 180.000 containing six tightly bound zinc ions[15,17,18]. This value was quite different from human kidney and rabbit erythrocytes, which are 66.000 and 54.000 respectively[19].

However, there are no reports on the carbonic anhydrases of *Nicotiana tabacum.* In the present study we purified carbonic anhydrases from *Nicotiana tabacum* leaves and compared its properties with carbonic anhydrases from other plants and from mammals.

Material and Methods

Extract Preparation

Nicotiana tabacum leaves collected from the eastern part of the Black Sea region of Turkey were kept in deepfreeze at -31° C. Carbonic anhydrase was purified[15] from the leaves as follows:

Step 1: Nicotiana leaves were cut and then suspended in 2 litres of the buffer (0.05 M sodium phosphate, 0.01 M 2-mercaptoethanol, pH 7.0) for each kilogram of Nicotiana leaves by using a blender. This procedure was executed in a cold room at 4° C.

Step 2: The suspension was filtered twice through a fiber glass window screen.

Step 3: The filtrate was centrifuged and the precipitate discarded.

Step 4: The supernatant fluid was added 180 g of solid ammonium sulphate per litre. The mixture was stirred for 1 hour at 4° C and then centrifuged for 5 minutes at 10.000 rpm. The supernatant was further cleared by filtration through a filter pad.

Step 5: Additional ammonium sulphate (120 g per litre) was added to the filtrate and the precipitate was recovered by filtration. The filtrate was discarded.

Step 6: The enzyme precipitated by ammonium sulphate was dialysed for 5 hours against five changes of distilled water, then against 2 litres of the buffer (0.1 M Tris-acetate, 0.01 M 2-mercaptoethanol, pH: 7.0) for 3 hours. Insoluble material in the resulting solution was then cleared off initially by centrifugation for 20 min at 8.000 rpm in a Suprafuge centrifuge, and later at 8.000 rpm for 5 minutes in a refrigerated centrifuge at 0° C, and protein concentrations and activities were determined at each step.

Enzyme purification

The Enzyme was purified from Nicotiana tabacum extract by ion exchange chromatography. DEAE-Cellulose equilibrated with 0.5 M HCl deionized water and 0.5 M NaOH was filled in a column with 0.1 M Tris-acetate buffer, 0.01 M 2-mercaptoethanol, pH: 7.0, then with 0.2 M Tris-acetate, 0.01 M 2-mercaptoethanol, pH: 7.0. The flow rate was about 150 ml per hour. Fractions were pooled as 5 ml.

Protein Determination

After scanning at 280 nm, absorbance displaying tubes were pooled, and the quantitative protein determination was made by means of coomassie brillant blue G-250[20].

Enzyme Activity Determination

Esterase activity was determined as described in Verpoorte et al.[21]. Briefly, 1.5 ml buffered enzyme (1 ml 0.1 M Tris-acetate, 0.01 M 2-mercaptoethanol, pH 7, and 0.5 ml purified enzyme of concentration 10

mg/ml), and 1.5 ml of p-nitrophenyl acetate (3 mM) were mixed, and after 3 minutes, optical density was read at 348 nm. V_{max}, K_m and optimum pH were determined. While substrate volume was increased (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.5 ml), the enzyme volume was fixed at 1 ml and buffer was added to make up a total volume of 3 ml in determining V_{max} and K_m values. V_{max} and K_m values were determined from a Lineweaver-Burk graph. Hydrolysis activity of purified enzymes was measured by determining the time necessary to change the pH from 8.2 to 6.3[22]. Enzyme units were calculated according to the formula $1U = ((t_o - t_c)/t_c)$, where t_o and t_c were the time (sec) needed for the pH change without and with enzyme and enzyme reactions, respectively.

Enzyme Activity Determination in Inhibitor Containing Media

For this purpose, caffeine, nicotine, nicotinamide, KSCN, $SbCl_3$, CuCl, sulphanilamide and acetazolamide were used as inhibitors. The Rickly method was used for determination of hydrolysis activity of the enzyme[22].

SDS-PAGE Electrophoresis

Electrophoresis was carried out on 3-10% SDS-PAGE gel as described in Laemmli[23]. Human CA-I and bovine carbonic anhydrase were purified by affinity chromatography and used as standards[24].

Determination of Molecular Weight

The molecular weight of the purified carbonic anhydrase enzyme in *Nicotiana tabacum* was determined by using Sephadex-G 150. A mixture of standard proteins, each of which having a concentration of 0.2 mg/ml, was applied on the column. Purified CA was added to the equilibrated column and eluted with 0.05 M NaPi/1 mM ditihioerythritol, buffer, pH 7.

Results and Discussion

The discovery of esterase activity of CA by p-nitrophenyl acetate substrate was of interest because it was previously reported that CA from parsley and spinach did not have esterase activity[15]. This could be due to further purificaiton of the CA enzyme of *Nicotiana tabacum* leaves. In the present study CA was purified 40.7 times (Table 1), whereas the CA of parsley was only purified 12 fold. The other factor for esterase activity might be the buffer solution; that is, the purification of CA from parsley was performed in 0.1 M N-etilmorfolin asetat acetate-0.1 M 2-mercaptoethanol, pH: 7 buffer[15], in which no esterase activity in the CA of *Nicotiana tabacum* was detected. It is probable that N-etilmorfolin asetat acetate inhibits the enzyme or CA partly hydrolyses N-etilmorfolin asetat acetate. However, in this study when 0.1 M N-etilmorfolin asetat acetate-0.1 M 2-mercapthoethanol, pH: 7 buffer was replaced with 0.1 M. This-acetate-0.01 M 2-mercaptoethanol pH: 7 buffer, esterase activity was detected. Therefore, it is suggested that this buffer solution is the most appropriate one for the elution and storage of the enzyme. The third possibility is that the amino acid sequence of CA isolated from *Nicotiana tabacum* may be different from that of the CA of spinach or parsley. As the CA of *Nicotiana tabacum* had esterase activity, enzyme V_{max} and K_m values were determined by using p-nitrophenyl acetate (0.1524 mM, 0.5446 mM, respectively).

Carbonic anhydrase of *Nicotiana tabacum* was purified by DEAE-cellulose ion exchange chromatography, and then the hydrolysis activity was measured (Fig. 1).

The enzyme was active between pH: 6.0-7.2 and the optimum pH was found to be 6.9 for the purified enzyme in tris-acetate buffer (Fig. 2).

Table 1. Carbonic anhydrase from *Nicotiana tabacum* leaves

Enzyme Fraction	Volume (ml)	Activity EU/ml	Total activity EU	Total activity %	Protein (mg/ml)	Spesific activity EU/mg	Purification (fold)
Crude extract	1000	12.05	12050	100	25.55	0.471	-
$(NH)_2SO_4$ 120 g	970	9.87	9574	79.4	22.23	0.445	0.945
$(NH)_2SO_4$ 180 g	920	8.67	7976	83.3	18.75	0.462	1.039
DEAE-Cellulose column	215	6.59	1417	17.8	0.35	18.82	40.70

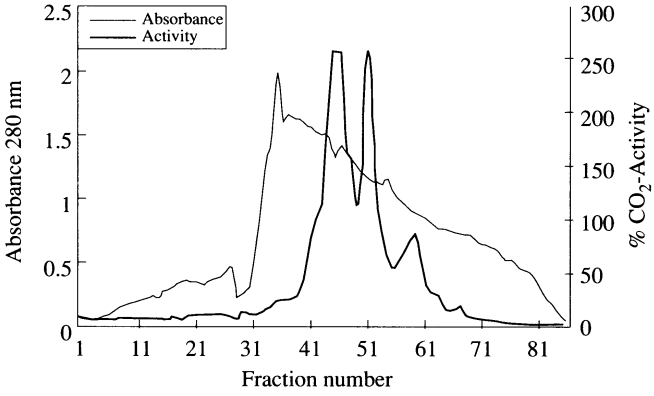

Figure 1. DEAE-cellulose ion-exchange chromatography of carbonic anhydrase from *Nicotiana tabacum* leaves in the presence of 0.2 M Tris-acetate buffer pH 7, 0.01 M 2-mercaptoethanol.

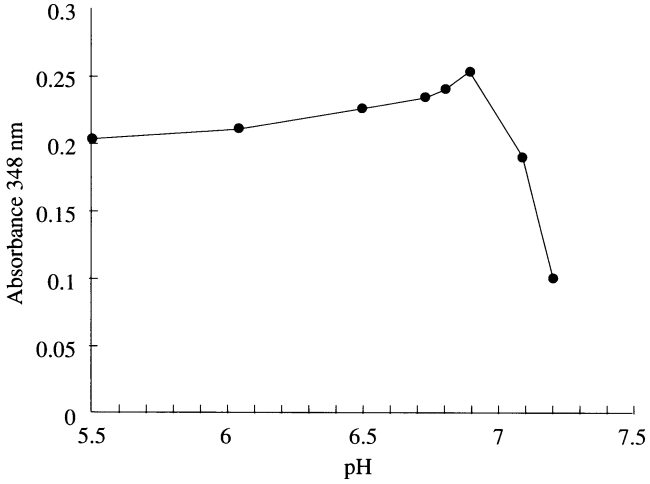

Figure 2. Activity of carbonic anhydrase from Nicotiana leaves in Tris-Acetate buffer of pH 5.5-7.5

The optimum temperature of *Nicotiana tabacum* CA was found to be 40° C (Fig. 3). However, the optimum temperature of the CA of parsley was previously reported to be between 40° C and 60° C[15].

The molecular weight of *Nicotiana tabacum* carbonic anhydrase was found to be approximately ≈137.000 by gel filtration method (Fig. 4), and approximately ≈22.800 by SDS-PAGE (Fig. 5).

SCN^-, Sb^{+3} and Cu^+ ions (Fig. 6), and caffeine, nicotine and nicotinamide (Fig. 7) increased the activity of *Nicotiana tabacum* carbonic anhydrase.

It has been reported that sulphanilamide and acetaozolamide strongly inhibited the activity of mammalian carbonic anhydrase[25]. However, in the present study, the activity of *Nicotiana tabacum* carbonic anhydrase was increased by sulphanilamide and acetazolamide (Fig. 8).

As a conclusion, new data were obtained about *Nicotiana tabacum* carbonic anhydrase and these data were compared to those on other carbonic anhydrases of different origins.

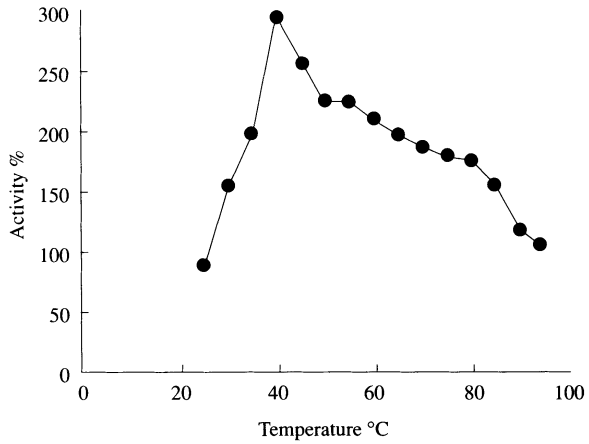

Figure 3. Effect of temperature on purified carbonic anhydrase from Nicotiana leaves.

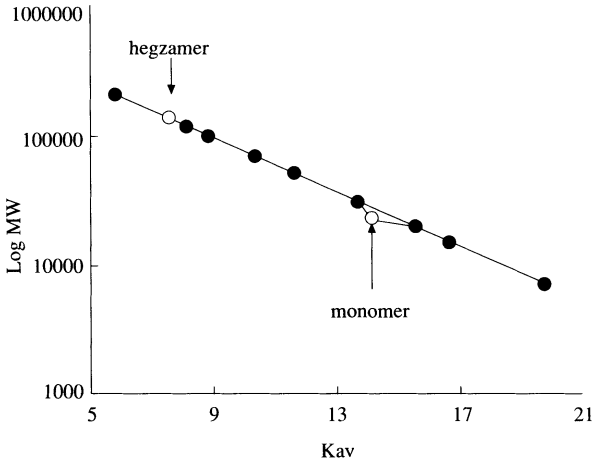

Figure 4. Gel-filtration analysis of *Nicotiana tabacum* leaves carbonic anhydrases. The chromatography was performed on a Sephadex G-150 column in 0.05 M sodium phosphate, 1 mM ditihioeritritol, pH: 7.0, $K_{av} = (V_e - V_o / V_t - V_o)$; V_e: elution volume; V_o: column void volume; V_t: total bed volume.

1. Aprotinin ..... 6500 Dalton
2. α-Lactoglobilin .... 14200 Dalton
3. β-Lactoglobilin .... 18400 Dalton
4. Carbonic anhydrase 29000 Dalton
5. Fumarase ..... 48500 Dalton
6. Serum albumin .... 66000 Dalton
7. Phosphorilase-B.... 97000 Dalton
8. β-Galactosidase... 116000 Dalton
9. α_2-Macroglobilin . 205000 Dalton

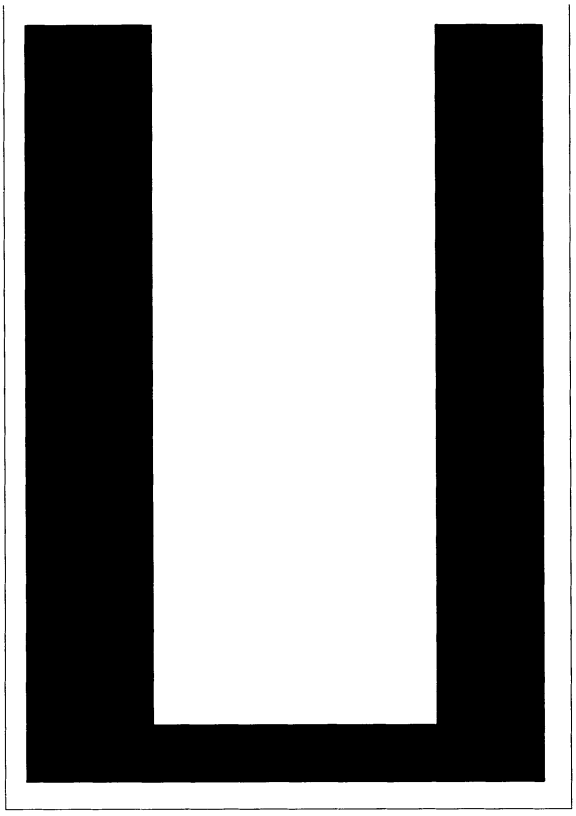

Figure 5. Electrophoretic pattern *Nicotiana tabacum* carbonic anhydrases (*on the left*), of human carbonic anhydrases (*in the middle*) and of bovine carbonic anhydrases (*on the right*).

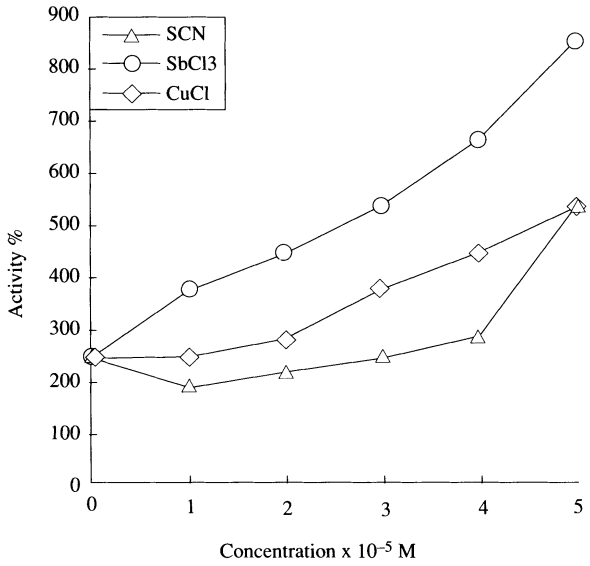

Figure 6. Effect of some chemical materials on purified carbonic anhydrase activity from Nicotiana leaves.

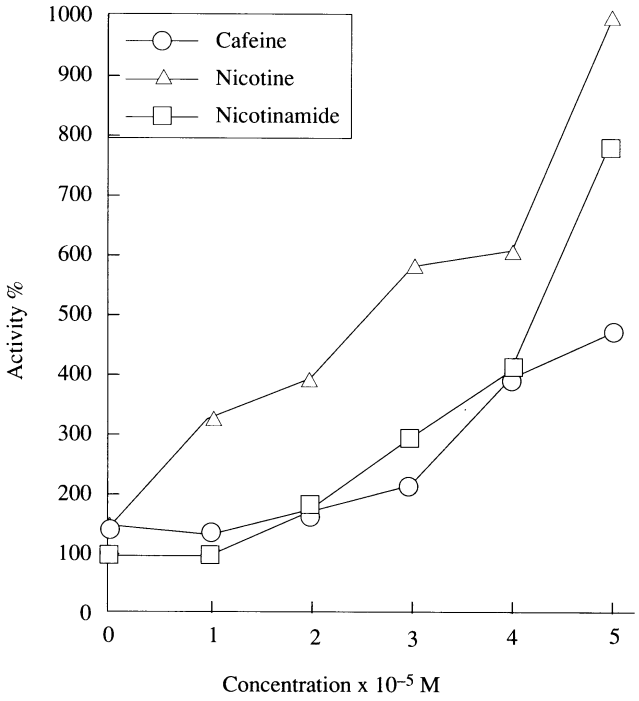

Figure 7. Effect of caffeine and nicotine on purified carbonic anhydrase activity from Nicotiana leaves.

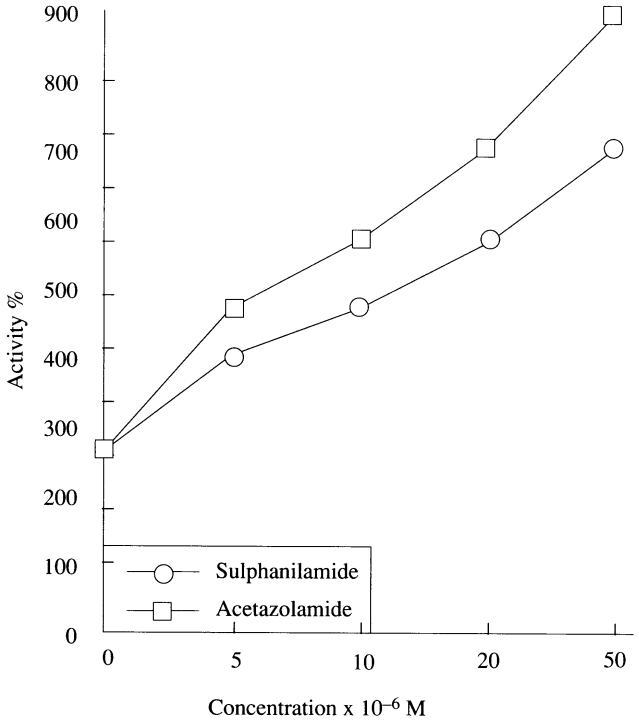

Figure 8. Effects of sulphanilamide and acetazolamide on carbonic anhydrase activity from Nicotiana leaves.

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