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Determinatıon of Carbonic Anhydrase and Level of Zn^{2+} in the Hypericium (*Hyperıcum perforatum* L.)

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ABSTRACT

Hypericium (*Hypericum perforatum* L.) leaf extract is used for healing wounds. Carbonic anhydrase (CA; carbonate hydrolase: E.C.4.2.1.1) was purified and characterized from leaves and flowers of. hypericium by twice precipitation with $(NH_4)_2SO_4$, and using DEAE-Celulose ion-change chromatography. The purification level of the enzyme was 63, 8 fold and 156 fold in leaves and flowers, respectively. The optimum temperatures were 50 and 55°C, and pH optima 6 and 7 for leaves and flowers, respectively. CA from both the tissues had esterase activity. K_M and V_{max} values from were 0.00117 mM and 2.118 mM/L *min for leaves, and 0.00585 mM and 4.657 mM/L*min for flowers. In addition, the total amount of Zn^{2+} in leaves and flowers and its action as cofactor was calculated.

Key Words: *Hypericum perforatum* L.; Carbonic anhydrase; Zn^{2+}

INTRODUCTION

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) isozymes are a family of zinc metalloenzymes that catalyze the conversion of CO_2 and water to HCO_3^- and vice versa (Hewett-Emmet & Tashian 1996). There are two classes of carbonic anhyrase (CA). The β–class CA is comprised of enzymes from the chloroplasts plants. It is believed that CA has roles in the photosynthesis and respiration processes. The plant CA has been purified and characterised in parsley, *Comelia cinensis, Daucus carota, Vicia canenses* and *Nicotinia tabacum* (Demir *et al*., 1997a; Demir *et al*., 1997b, 1999; Demir, & Demir, 1997). The only γ–class CA has thus far been characterized from methanoarchaeon *Methanosarcina thermophila*.

Hypericium (*Hypericum perforatum* L.) has medicinal importance as ointment to heal wound. It is known that Zn^{2+} affects wound healing (Cott & Fung-Berman, 1998). It is expected that amount of Zn^{2+} is remarkably high in this plant due to the fact that CA has Zn^{2+} as cofactor. Therefore, CA level is correlated with the amount of total Zn^{2+} in the tissues. This research was conducted in two parts. In first part, enzyme was purified and characterized from leaves and flowers. In second part, the amount of enzyme was purified and total amount of Zn^{2+} was determined.

MATERIALS AND METHODS

Extract preparation. Leaves and flowers of Hypericium (*Hypericum perforatum* L.) were collected from West Anatolia region of Turkey. Flowers and leaves of this plant was separated mechanically to purify the carbonic anhydrase. Leaves were cut and then each 0.5 kg of leaves was suspended in 1 L of buffer (0.05 M sodium phosphate, 0.01 M 2-mercaptoethanol, pH 7.0). All this procedure was carried out at 4°C in a cold room. The suspension was filtered twice through fiberglas window screen. The filtrate was centrifuged and precipitate was discarded. The supernatant fluid was added to 120 g L^{-1} of ammonium sulphate. The mixture was stirred for 1 h at 4°C then they were centrifuged for 5 min at 10,000 rpm. The supernatant was further cleared by filtration. Additional ammonium sulphate (180 g L^{-1}) was added to the filtrate and the precipitate was recovered by filtration.

The ammonium sulphate precipitated enzyme was dialysed for 5 h against five changes of distilled water followed by 2 L of the buffer (0.1 M Tris-acetate, 0.01 M β-mercaptoethanol, pH 7.0) for 3 h. Insoluble material in the resulting solution was then cleared initially by centrifugation for 20 min at 8000 rpm in a suprafuge centrifuge, and later at 8000 rpm for 5 min in refrigerated centrifuge at 0°C. Protein concentrations and activities were determined at each step. Similar procedure was applied for flowers of Hypericium.

Enzyme purification and protein determination. Enzymes in extracts were purified with ion exchange chromatography on 3x50 cm column that contained DEAE-Cellulose. Elution was carried out with 0.2 M Tris-acetate, 0.01 M β-mercaptoethanol, pH 7.0. Eluted fractions having absorbence at 280 nm were pooled and the amount of protein was determined (Bradford, 1976).

Enzyme activity determination. Esterase activity was determined as described by Verpoorte *et al*. (1967). V_{max}, K_M and optimal pH were also determined by this method. Hydrolyse activity of purified enzymes was measured by determination of time necessary to pH changes from 8.2 to 6.3. Enzyme units were calculated according to the formula:

$$IU = \{(t_o - t_c)/t_c)$$

where t_o and t_c the time (s) needed for the pH change without enzyme and with enzyme reactions, respectively.

Effect of various chemicals on enzyme activity. The effect of sulphanilamide, KSCN, NaN_3 (inhibitors of CA) was measured on CA purified from flowers and leaves. These measures were carried out using esterase activity of enzyme (Rickli *et al*., 1964). Esterase activity of CA was detected with p-nitrophenyl acetate as substrate.

SDS-PAGE. SDS-PAGE was carried using 3-10% SDS-PAGE as described previously (Arslan *et al*., 1996). Bovine carbonic anhydrase was purified by affinity chromatography and was used as electrophoresis standards (Laemmli, 1970).

Determination of molecular weight. Molecular weights of purified carbonic anhydrase enzymes of flowers and leaves of Hypericium were determined by using Sephadex-G 150. Mixture of standard proteins having a concentration of 0.2 mg mL^{-1}, was applied on the column. Then, purified carbonic anhydrase enzymes were added on to the equilibrated columns and eluted with 0.05 M sodium phosphate, 1 mM ditihioeritritol, pH 7.0 buffer.

Determination of amount of Zn^{2+}. The amount of Zn^{2+} was determined in the homogenates prepared from leaves and flowers of Hypericium and enzymes extracts purified by using the DEAE-Celulose ion-change chromatography (Lajunen, 1992). Atomic absorption spectrophotometer was used for this determination.

RESULTS AND DISCUSSION

The level of enzyme was 68, 3 fold and 156 fold in leaves and flowers, respectively (Table IA & B). CA had esterase activity (Fig. 1A & B). For each enzyme, K_M and V_{max} values were determined which were 0.00117 mM and 2.118 mM/L*min for leaves, and 0.00585 mM and 4,657 mM/L*min for flowers. pH optima were 6 and 7 for leaves and flowers, respectively (Fig. 2).

This value was not much different from mammalian CA. The optimum temperatures were 50 and 55°C in leaves and flowers, respectively (Fig. 3). Range of temperature

Table IA. Carbonic anhydrase from Hypericium (*Hypericum perforatum* L.) Leaves

Enzyme Fraction	Volume ml	Activity EU/ml	Total Activity EU	%	Protein Mg/ml	Specific ActivityEU/mg	Purification Fold
Crude extract	1000	0.026	26	100	4.11	0.006	-
$(NH)_2SO_4$ 120 g	980	0.0055	5.39	20.7	2.3	0.0021	3,5
$(NH)_2SO_4$ 180 g	970	0.0078	7.85	30.1	1.9	0.0041	6,8
After DEAE-Cellulose column	260	0.0383	9.95	38.2	0.10	0.383	68,3

Table 1B. Carbonic anhydrase from Hypericium (*Hypericum perforatum* L.) Flowers

Enzyme Fraction	Volume (ml)	Activity EU/ml	Total Activity EU	%	Protein Mg/ml	Specific ActivityEU/mg	Purification Fold
Crude extract	1000	0.034	34	100	0.4	0,085	-
$(NH)_2SO_4$ 120 g	970	0.057	55.2	56	0,35	0.16	1,8
$(NH)_2SO_4$ 180 g	960	0.091	87.3	75.2	0,23	0.38	4,4
After DEAE-Cellulose column	200	0.16	94.1	22.1	0.012	13,33	156

Fig. 1A. DEAE-cellulose iyon-echange chromatography of carbonic anhydrase from Hypericium (*Hypericum perforatum* L.) flowers in the presence of 0.2 M Tris-acetate buffer pH: 7.0, 0.01 M 2-Mercaptoethanol

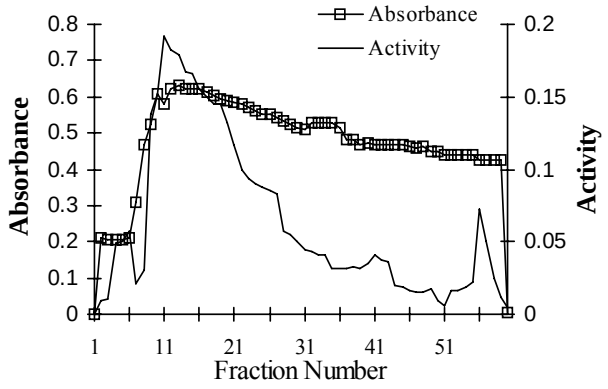


Fig 1B. DEAE-cellulose iyon-echange chromatography of carbonic anhydrase from Hypericium (*Hypericum perforatum* L.)leaves in the presence of 0.2 M Tris-acetate buffer pH: 7.0, 0.01 M 2-Mercaptoethanol

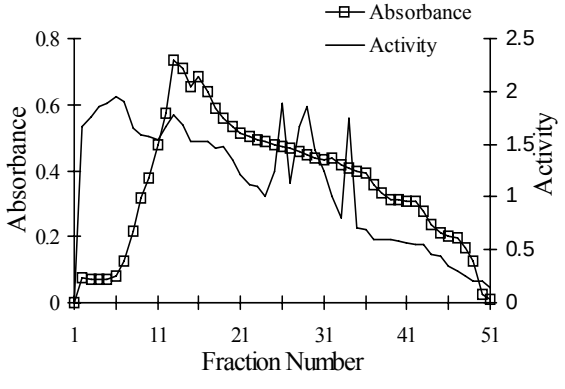


Fig. 2. Effect of temperature on the activity of purified carbonic anhydrase enzyme from Hypericium (*Hypericum perforatum* L.) flowers and leaves

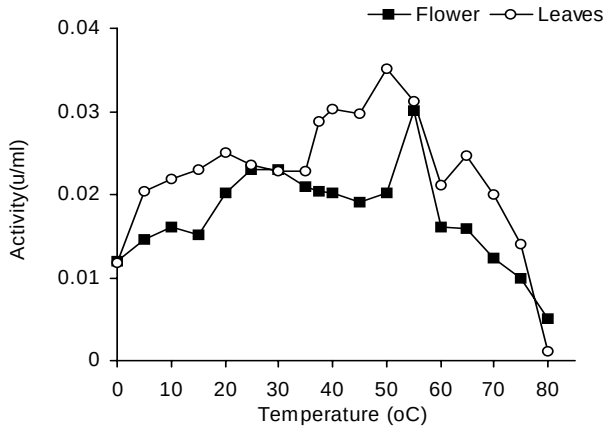


Fig. 3. Effect of pH on the activity of purified carbonic anhydrase enzyme from Hypericium (*Hypericum perforatum* L.) flowers and leaves

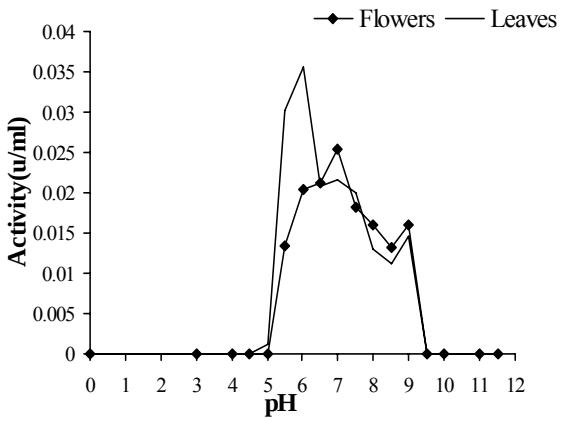
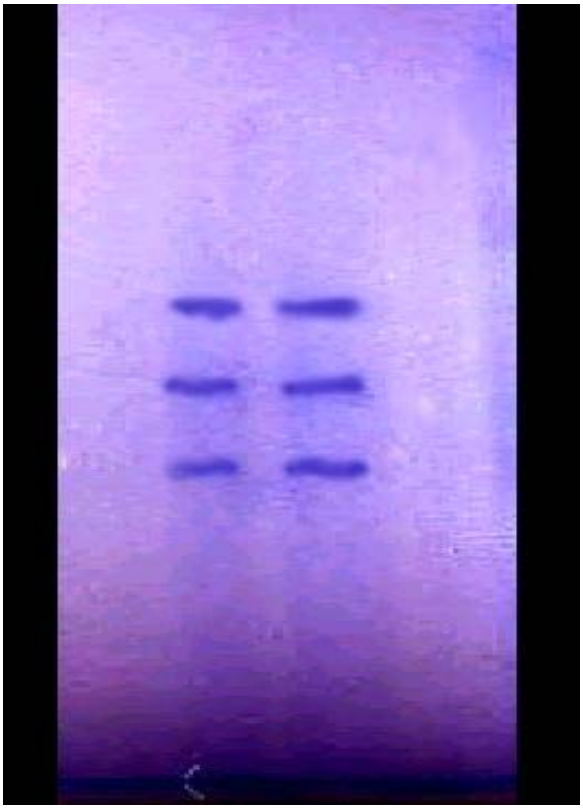


Fig. 4. Electrophoretic pattern of Hypericium (*Hypericum perforatum* L.) carbonic anhydrases: flowers'CA (1) and leaves'CA (2).



with activity has been detected as 0-80°C for this enzyme.

Molecular weights of CAs as determined by gel filtration chromatography were 55000, 67000 and 75000 Da while those from flowers were 55000, 57000 and 75000 Da. These bands are different from another plant CAs (Fig. 4).

The activities of purified CAs were determined using KSCN, NaN_3 and sulphanylamide, the inhibitors of this enzyme from mammalian. NaN_3 inhibited CA of leaves at 10^{-4} M concentration. Other inhibitors did not inhibit or activate the CAs from leaves and flowers (Fig. 5A & B).

In the homogenates and pure enzymes of leaves and flowers, amount of Zn^{2+} was determined with atomic absorption spectrophotometer (Table II). The levels of Zn^{2+} were 8 and 7 ppm, and 5 and 54 ppm in homogenates and pure enzyme of leaves; and 9 and 2 ppm, and 6 ppm in homogenate and pure enzyme of flowers, respectively. Amount of Zn^{2+} in CA as cofactor showed that 63, 78% of Zn^{2+} belong to leaves and 65, 23% of Zn^{2+} belong to flower.

Table II. Zn^{+2} amount of carbonic anhydrase from Hypericium (*Hypericum Perforatum* L.)

Fractions	Zn^{+2} (ppm/100 g)	Zn^{+2} %
Flowers Homogenate	9,2	100
Flowers' CA	6	65,23
Leaves Homogenate	8,7	100
Leaves'CA	5,54	63,78

It was shown that Zn^{2+} was included by CA to high percentage that appeared to contribute towards healing of wounds (Arsalan *et al.*, 1996).

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