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Determination of Carbonic Anhydrase and Level of Zn^{2+} in the Yarrow *(Achillea Millefolium)*

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ABSTRACT

The carbonic anhydrase (CA: Carbonate hydrolyase: E.C.4.2.1.1) content from the leaves and flowers of mature yarrow *(Achillea millefolium),* a plant believed to possess healing qualities, was purified and characterized. The purification levels were 35.58 fold in the leaves and 40.21 fold in the flowers. The optimum temperatures were 25°C and 40°C for the leaves and flowers, respectively, and optimal pH was 12.5 in the leaves and in flowers this pH varied between 3.5 and 10. In gel filtration chromatography, the molecular weights of leaf carbonic anhydrase were determined to be 29, 36, and 40 kDa. The molecular weights of the flowers were 29, 34, 36and 40 kDa, and these proteins exhibited activity. The level of Zn^{2+} in the leaves and flowers of the yarrow, the amounts of total Zn^{2+}, and Zn^{2+} found in carbonic anhydrase as a cofactor were calculated.

Key Words: Yarrow *(Achillea Millefolium)*; Plant Carbonic Anhydrase; Zn^{2+}

INTRODUCTION

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) isozymes are a family of zinc metalloenzymes that catalyze the interconversion of CO_2 and HCO_3^- (Hewett-Emmet & Tashian, 1996; Lindskog, 1997).

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

At present, approximately 14 isoenzymes (I-XIV) have been reported from living organisms (Sly & Hu, 1995; Mori *et al*., 1999). Some of these isoenzymes are cytosolic (CA I, CA II, CA III, CA VII), and others are membrane-bound (CA IV, CA IX, CA XII and CA XIV), while CA V is mitochondrial and CA VI is secreted in the saliva (Smith *et al.*, 1999).

The structure, enzymatic function and physiological role of mammalian carbonic anhydrases from various sources have been extensively studied (Smith *et al.*, 1999). The molecular weights of the carbonic anhydrases of vertebrates were 29-30 kDa, and each enzyme molecule contains one atom of zinc that is necessary for activity. Furthermore, carbonic anhydrase in plants was firs observed in 1939 (Neish, 1939) and the enzyme was partially purified and characterized from various plants such as parsley (Tobin, 1970), *Nicotiana tabacum* (Demir & Demir, 1997), *Camelia sinensis* (tea) (Demir *et al.*, 1997a), *Daucus carote* (Demir *et al.*, 1997b), *Visia canansis* (Demir *et al.*, 1999).

Yarrow *(Achillea millefolium)* has medicinal importance in that it is used in healing ointments applied to wounds. It is known that Zn^{2+} affects the rate of wound healing (Sampson, 1997). It is expected that the amount of Zn^{2+} is much higher in plants known for their wound-healing properties than in other plants (Sampson, 1997). As carbonic anhydrase has Zn^{2+} as a cofactor. We decided to examine how much carbonic anhydrase was responsible for total Zn^{2+}. The research project was divided into two parts. In the first part, the carbonic anhydrase was purified and characterized from leaves and flowers. In the second part of this study, the amount of Zn^{2+} was determined with atomic absorption in the extract and pure enzymes, and the amount of Zn^{2+} responsible for enzyme activity was obtained.

MATERIAL AND METHODS

Plant Material. Yarrow was collected from the East Anatolia region of Turkey and the flowers and leaves were separated mechanically.

Enzyme Extraction and Purification. For preparing the crude extract, yarrow leaves were cut and 500 g yarrow leaves of was homogenized in 1 L of 0.05 M phosphate buffer (pH: 7.0) containing 0.01 M 2-mercaptoethanol by using Warning blender for 2 min. The crude extract was filtered and the filtrate was centrifuged at 10.000 rpm for 30 min at 5°C. The supernatant was brought to 25% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$ and then centrifuged for 5 min at 10.000 rpm. The supernatant was further cleared by filtration through a filter pad. The filtrate was centrifuged, precipitate discarded and the supernatant brought to 60% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The precipitate CA was separated by centrifugation at 10.000 rpm for 5 min. The precipitate was dissolved in a small amount of 0.05 M phosphate buffer (pH: 7.0) and dialyzed for 3 h against 2 L of the buffer (0.1 M Tris-acetate, 0.01 M β-mercaptoethanol, pH 7.0) at 4°C. Insoluble material in the end solution was cleared by ammonium sulfate, and then the solution was centrifuged for 20 min (8.000 rpm) at room temperature; it was later centrifuged at 8.000 rpm for 5 min

at 0°C. Protein concentrations and activities were determined at each step.

The enzymes present in extracts were purified with ion exchange chromatography on a 3x50 cm column that contained DEAE-Cellulose. Elution was carried out with 0.2 M Tris-acetate, and 0.01 M β-mercaptoethanol, at pH 10.0. The elution prosess was performed in the presence of a decreasing pH gradient in range of pH 10 to 4. The same procedure was applied to yarrow flowers.

Protein Determination. The absorbance at 280 nm was used to monitor the protein in the column effects. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford, with bovine serum albumine as astandart (Bradford, 1976).

Enzyme Activity Determination

Esterase activity. The principle of this determination is that the substrate of CA (p-nitrophenyl acetate) is hydrolyzed to p-nitro phenol plus acetic acid. The reaction is detected at 348 nm. For this procedure 1.5 mL of a buffered enzyme solution (0.1 mL enzyme+1.4 mL 0.05 M Tris-SO_4, (pH: 7.4) and 1.5 mL of substrate were mixed in a measurement cuvette and three minutes later the absorbance was measured (348 nm, 25°C). A blank measurement was obtained by preparing the same cuvette exception that saline was added to the cuvette instead of enzyme solution (Verpoorte, 1967).

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 0.1 mL and buffer was added to make up a total volume of 3 mL in determining V_{max} and K_M values ware determined from a Lineweaver-Burk graph.

CO_2-Hydratase activity. Two mL of veronal buffer (pH: 8.2), 0.4 mL of brome thymol blue (0.004%), 0.8 mL of diluted enzyme solution and 2 mL of a CO_2 solution (saturated at 0°C) were mixed. The time (t_C) interval was determined between addition of CO_2 solution and the occurrence of a yellow-green color. The same interval was recorded without enzyme solution (t_o). Enzyme units were calculated according to the formula (Rickli, 1964):

$$IU = \frac{(to - tc)}{tc},$$

where *to* and *tc* are the time (s) needed for the pH change without the enzyme and with the enzyme reactions, respectively.

Effects of various inhibitors on enzyme activity. The hydrolyze activities of purified enzymes in the presence of $1x10^{-4}$ M sulphanylamide, KSCN, and NaN_3 as inhibitors were measured (Rickli, 1964).

SDS-PAGE. Electrophoresis was carried out in 3-10% SDS-PAGE gel as described by Laemmli (Laemmli, 1970). Bovine carbonic anhydrase was used as an electrophoresis standard (Arslan, 1996).

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

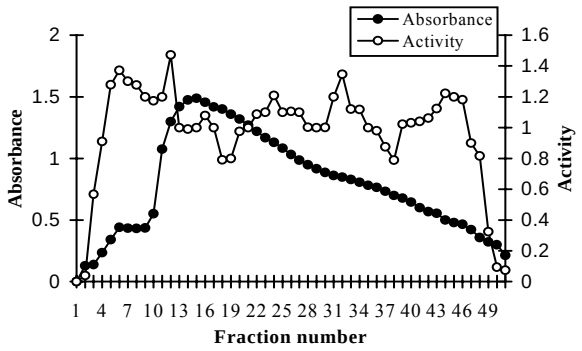


Fig. 1B. DEAE-cellulose iyon-echange chromatography of carbonic anhydrase from Yarrow (*Achillea millefolium*) leaves in the presence of 0.2 M Tris-acetate buffer pH: 7.0, 0.01 M 2-Mercaptoethanol.

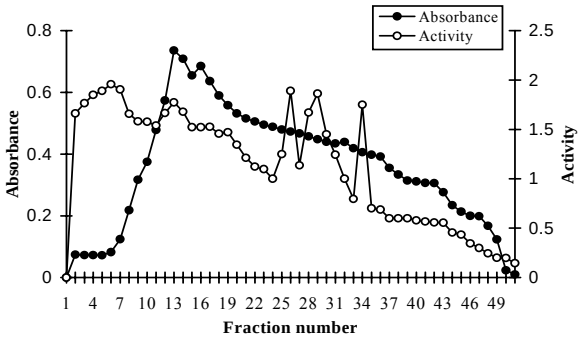


Fig. 2. Effect of temperature on the activity of purified carbonic anhydrase enzyme from Yarrow (*Achillea millefolium*) flowers and leaves.

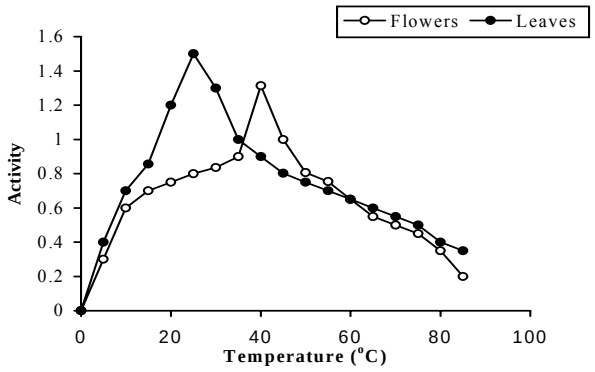


Fig. 3. Activity of carbonic anhydrase enzyme from Yarrow (*Achillea millefolium*) flowers and leaves in Tris-SO_4 buffer.

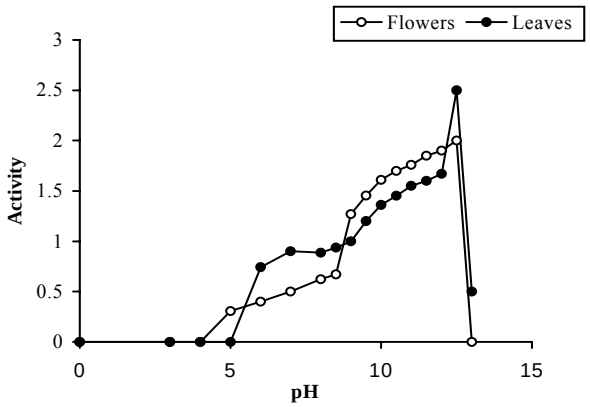
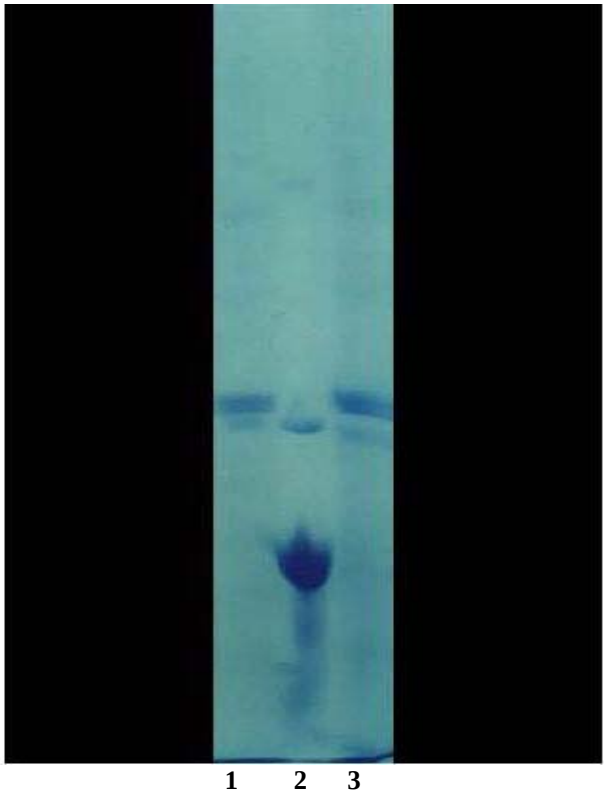


Fig. 4. Electrophoretic pattern of Yarrow (*Achillea millefolium*) carbonic anhydrases: flowers'CA (1), BCA (2) and leaves'CA (3).



Determination of Molecular Weight. The molecular weights of the purified carbonic anhydrase enzymes of yarrow flowers and leaves were determined by using Sephadex-G 150. A mixture of standard proteins, which had a concentration of 0.2 mg mL^{-1}, was applied to this column. Purified carbonic anhydrase enzymes were added to the equilibrated column and they were eluted with 0.05 M sodium phosphate 1^{-1} mM ditihioeritritol, pH 7.0 buffer (Whitaker, 1963).

Determination of Zn^{2+}. The amount of Zn^{2+} was estimated in the homogenates prepared from yarrow leaves and flowers; pure enzymes were obtained by using the DEAE-Celulose ion-change chromatography (Lajunen, 1992). Since esterase activity is observed in yarrow leaves and flowers homognate and pure enzymes, they were heated in a boiling water bath for 1 h in capped tubes and centrifuged. Then the samples were diluted (1/4) with deionized water and they were deproteinized with tricloroacetic acid (ACD) (sample/TCA: 1/1). The resulting contents were centrifuged (for 15 min. 1500xg). By this procedure the esterase activities of yarrow leaves and flowers homognate and pure enzymes were used for Zn^{2+} determination with atomic absorbtion spectroscopy (Lajunen, 1992).

Fig. 5A. Effect of 1 x 10^{-4} M Sulphanhyamide, KSCN and NaN_3 on the activity of purified carbonic anhydrase enzyme from Yarrow (*Achillea millefolium*) flowers.

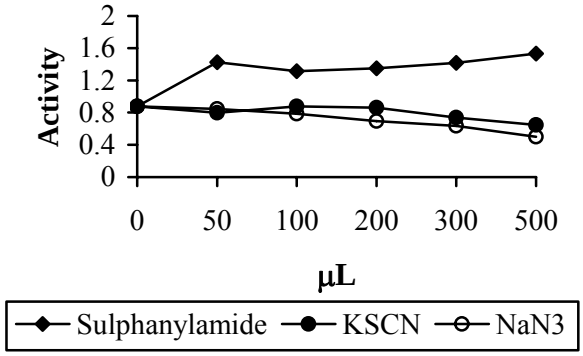


Fig. 5B. Effect of 1 x 10^{-4} M Sulphanylamide, KSCN and NaN_3 on the activity of purified carbonic anhydrase enzyme from Yarrow (*Achillea millefolium*) leaves.

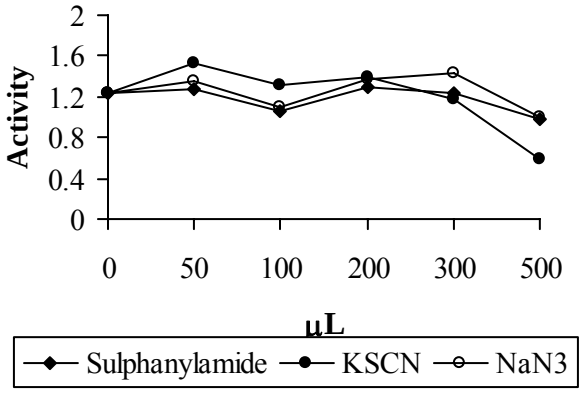


Table IA. Carbonic anhydrase from Yarrow (*Achillea millefolium*) flowers.

Enzyme Fraction	Volume (mL)	Activity (EU/mL)	Total Activity EU	Total Activity %	Protein (mg/mL)	Specific Activity (EU/mg)	Purification Fold
Crude extract	1000	6.40	6400	100	21.67	0.3	-
$(NH)_2SO_4$ 120 g	980	4.98	4880	76.3	19.4	0.26	0.87
$(NH)_2SO_4$ 180 g	970	3.54	3434	70.4	10.32	0.34	1.31
After DEAE-Cellulose column	260	1.67	434	12.6	0.138	12.10	35.58

Table IB. Carbonic anhydrase from Yarrow (*Achillea millefolium*) leaves.

Enzyme Fraction	Volume (mL)	Activity (EU/mL)	Total Activity EU	Total Activity %	Protein (mg/mL)	Specific Activity (EU/mg)	Purification Fold
Crude extract	1000	7.20	7200	100	15.30	0.47	-
$(NH)_2SO_4$ 120 g	970	4.16	4035	56	10.20	0.51	0.73
$(NH)_2SO_4$ 180 g	960	3.16	3034	75.2	8.32	0.38	1.31
After DEAE-Cellulose column	200	2.75	670	22.1	0.18	15.28	40.21

Table II. Results of the amount of Zn^{2+} in the homogenates and pure enzymes of the leaves and flowers from Yarrow (*Achillea millefolium*) flowers.

Fraction	Amount of Zn^{2+} (ppm)	% Zn^{2+}
Crude Extracte from Flower	3.019	-
Pure Enzyme from Flower	1.656	54.85
Crude Extracte from Leaves	1.871	-
Pure Enzyme from Leaves	1.601	85.57

DISCUSSION

The carbonic anhydrase enzymes of yarrow leaves and flowers were purified by DEAE-celulose ion-change chromatography (Fig. 1). The esterase activity of carbonic anhydrase was detected with p-nitrophenyl acetate as substrate. All fractions which had been activity was collected. In our research, the carbonic anhydrase of leaves was purified 35.58 fold, and 40.21 fold in flowers (Table IA & Table IB). As the carbonic anhydrase of yarrow leaves and flowers exhibited esterase activities, for each enzyme K_M and V_{max} values were determined to be 0.00117 mM and $2.118*10^{-3}$ μmol L^{-1}*min for leaves and 0.00585 mM and $4.657*10^{-3}$ μmol L^{-1}*min for flowers.

In yarrow leaves and flowers carbonic anhydrase had both esterase and hydratase activities. Graphs of activity-absorbance were drawn for the leaf and flower carbonic anhydrases (Fig. 1A & B). The optimum temperatures were 25°C and 40°C in the leaves and flowers of the yarrow, respectively (Fig. 2). The range of activity temperature was from 5 to 85°C for these enzymes. Optimal pH was 12.5 in the leaves and flowers of the yarrow (Fig. 3). This value was very different from that of mammalian carbonic anhydrase (Hewett–Emmet & Tashian, 1996; Mori *et al.,* 1999).

According to the results of gel filtration chromatography, the molecular weights of yarrow leaf carbonic anhydrases were 29, 36 and 40 kDa. The molecular weights of yarrow flower carbonic anhydrases were 29, 34, 36 and 40 kDa, as shown on gel (Fig. 4).

The activities of purified carbonic anhydrases were determined against the effect of KSCN, NaN_3 and sulphanylamide, all of which are known inhibitors of mammalian carbonic anhydrase. While sulphanylamide activated the carbonic anhydrase of flowers, other carbonic anhydrase inhibitors wasn't inhibited or activated to carbonic anhydrases of leaves and flowers (Fig. 5A & B).

As a result of our study, carbonic anhydrase was purified and characterized from the leaves and flowers of the yarrow, which is a plant thought to have healing qualities due to its high Zn^{2+} content. The characterization of carbonic anhydrase is important as it is believed to play a role in photosynthesis (Demir & Demir, 1997).

In the homogenates and pure enzymes of the leaves and flowers, the amount of Zn^{2+} was determined via atomic absorption (Table II). According to the results of our research, Zn^{2+} levels were 1.871 ppm and 1.601 ppm in the homogenate and pure enzyme of leaves, respectively. Levels of Zn^{2+} were 3.019 ppm and 1.656 ppm in the homogenate and pure enzyme of flowers, respectively. The amount of Zn^{2+} in carbonic anhydrase as a known cofactor was shown to be 85.57% of Zn^{2+} in the leaves and 54.85% of Zn^{2+} in the flowers.

We demonstrated that Zn^{2+} was present in the carbonic anhydrase of the yarrow. It is suspected that this high level of Zn^{2+} gives the yarrow its healing qualities (Sampson, 1997, Godfrey *et al.*, 1996).

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