

Non-cytosolic Carbonic Anhydrases from Leaves and Roots of *Posidonia oceanica* (Linn.) Delile, 1813

YAŞAR DEMİR, FATMA COŞKUN, EKREM BUHAN† AND NAZAN DEMİR¹

Atatürk University Science Faculty Chemistry Department, 25240 Erzurum-Türkiye

†Bodrum Aqua Culture Research Center, Bodrum-Türkiye

¹Corresponding author's email: demim@yahoo.com, Tel: +90-442-2314439, Fax: +90-442-2360948

ABSTRACT

Carbonic anhydrases (carbonate hydrolase; E.C: 4.2.1.1) of leaves and roots of mature *Posidonia oceanica* (Linn.) were purified and characterized. The carbonic anhydrase enzymes in extracts were purified by DEAE-cellulose ion exchange chromatography. The purification levels were 115, 118 fold in the leaves and 89, 5, 101, 3 fold in the roots. V_{max} values of peripheral and integral CA enzymes purified from leaves and roots were $2.68 \cdot 10^{-2}$, $5.35 \cdot 10^{-2}$, $4.99 \cdot 10^{-2}$ and $6.31 \cdot 10^{-2}$ $\mu\text{mol/L} \cdot \text{min.}$, respectively. However, K_M values were found 1.42 and 0.26 mM for leaves and 0.09 and 0.32 mM for roots, respectively. The optimum temperatures were 30°C and 40°C for the leaves and roots, respectively and the optimal pH was 10 in the leaves and roots. This pH varied between 3.5 and 10.0. In gel filtration chromatography, molecular weights of four proteins were found 29.000 Dalton and these proteins had activity.

Key Word: Carbonic anhydrase; Non-cytosolic CA; Sea plant

INTRODUCTION

Carbonic anhydrase (CA) enzyme catalyzes the reversible hydration of CO_2 to HCO_3^- and H^+ and this system is present in all living organisms. Seven isoenzymes have been described in mammals (Sly & Hu, 1995). The structure, enzymatic function and physiological role of mammalian carbonic anhydrases from various sources have been extensively studied (Lindskog, 1960; Nyman, 1961; Nyman & Lindskog, 1964; Kandel *et al.*, 1970; Carlson *et al.*, 1980).

The molecular weights of the carbonic anhydrases of vertebrates were 29000 - 30000 Daltons, and each enzyme molecule contains one atom of zinc that is necessary for activity (Kashiwabara, 1985). Furthermore, carbonic anhydrase was partially purified and characterized in various plants (Tobin, 1970; Demir *et al.*, 1997; Demir *et al.*, 1997; Demir & Demir, 1997; Demir *et al.*, 1999). In higher plants exists at least two electrophoretically separable types. One type, found predominantly in monocotyls, has a molecular weight of 40 kD (Tobin, 1970). The other, isolated from dicotyls, is a hexameric enzyme with a molecular weight of 180 kD and contains six tightly bound Zn^{2+} ions (Tobin, 1970; Demir *et al.*, 1997; Demir *et al.*, 1997; Demir & Demir, 1997; Demir *et al.*, 1999). These values are different from other enzymes for example mammalian enzymes (Sly & Hu, 1995). This study was carried out to purify and characterize the carbonic anhydrase from *Posidonia oceanica*, a perennial sea plant.

MATERIALS AND METHODS

Extract preparation. *Posidonia oceanica* were collected from the Aegean Sea, (Bodrum) in Turkey and the leaves and roots were separated mechanically. They were stored at -31°C. Carbonic anhydrase was purified from the leaves as described earlier by Demir *et al.* (1997). In each step, protein concentrations and activities were determined.

Enzyme purification. The enzymes present in extracts were purified with ion exchange chromatography on a 3 x 50 cm column that contained DEAE-Cellulose. Elution was carried out with 0.2 M Tris-acetate, and 0.01 M 2-mercaptoethanol, at pH: 7.0.

Protein determination. Absorbency of obtained elutions was measured at 280 nm and amount of protein in elutions was determined (Bradford, 1976).

Enzyme activity determination. Esterase activity was determined as described by Verpoorte *et al.* (1967). 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 cuvette and, 3 min later, the absorbance was measured (348 nm, 25°C). A blank measurement was obtained by preparing the same cuvette without the enzyme solution. V_{max} , K_M , optimal pH and optimal temperature were determined by this method. V_{max} and K_M values were determined from Lineweaver-Burk graph. The hydrolysis activities of the purified enzymes were measured by determining the amount of time necessary to change the pH from 8.2 to 6.3 (Rickli *et al.*, 1964).

Enzyme units were calculated according to the formula:

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

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

The effect of various inhibitors in enzyme activity. The hydrolyze activities of purified enzymes in the presence of sulphanylamide, KSCN, NaN₃, as inhibitors were measured (Verpoorte *et al.*, 1967).

SDS-PAGE electrophoresis. Electrophoresis was carried out 3 - 10 % SDS-PAGE gel as described by Laemmli (1970). Human CA-I was purified by affinity chromatography and used as electrophoresis standards (Demir *et al.*, 1993).

Determination of molecular weight. The molecular weights of the purified carbonic anhydrase enzymes of *Posidonia* leaves and roots were determined by using Sephadex-G 150. A mixture of standard proteins, which had a concentration of 0.2 mg/mL 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 mM dithioeritrol, pH: 7.0 buffer (Whitaker, 1963).

RESULT AND DISCUSSION

At first, the amount of carbonic anhydrase and activity were slightly high in leaves than in roots. Activity of carbonic anhydrase was, detected with p-nitro phenyl acetate as substrate. Secondly, carbonic anhydrase of leaves were purified 115 fold for peripheral and 118 fold for integral. Carbonic anhydrase of roots were purified 89 fold for peripheral and 101 fold for integral (Table Ia, Ib, Ic & Id).

V_{max} values of peripheral and integral CA enzymes purified from leaves and roots were $2.68 \cdot 10^{-2}$, $5.35 \cdot 10^{-2}$, $4.99 \cdot 10^{-2}$ and $6.31 \cdot 10^{-2}$ $\mu\text{mol/L} \cdot \text{min.}$, respectively. However, K_M values were 1.42 mM, 0.26 mM for leaves and 0.09 mM, 0.32 mM for roots, respectively.

The optimum temperatures were 30°C in the leaves and 40°C in the roots. The optimum temperatures were 30°C in the leaves and 40°C in the roots. The measured temperature range of these enzymes was from 0°C to 80°C that enzymes were active in this range (Fig. 1). The optimum temperature was lower than in land plants. Generally, the optimum temperature for land plants was nearly 70 - 80°C (Demir *et al.*, 1997). *Posidonia*, because of living in the sea, this temperature was normal for sea

Table Ia. Carbonic anhydrase from *Posidonia oceanica* (Linnaeus) Delile, 1813 leaves (peripheral)

| Enzyme Fraction | Volume mL | Activity U/mL | Total Activity | | Protein mg/mL | Specific Activity U/mg | Purification Fold |
|--|-----------|---------------|----------------|------|---------------|------------------------|-------------------|
| | | | U | % | | | |
| Crude extract | 1000 | 3,01 | 3010 | 100 | 41,5 | 0,0723 | - |
| (NH) ₂ SO ₄ 120g | 960 | 2,8 | 2688 | 89,3 | 30,3 | 0,0924 | 1,278 |
| (NH) ₂ SO ₄ 180g | 800 | 2,50 | 2000 | 74,4 | 22,5 | 0,111 | 1,2012 |
| After DEAE-Cellulose | 300 | 3,2 | 960 | 48 | 0,25 | 12,8 | 115,3 |

Table Ib. Carbonic anhydrase from *Posidonia oceanica* (Linnaeus) Delile, 1813 leaves (integral)

| Enzyme Fraction | Volume mL | Activity U/mL | Total Activity | | Protein mg/mL | Specific Activity U/mg | Purification Fold |
|--|-----------|---------------|----------------|-------|---------------|------------------------|-------------------|
| | | | U | % | | | |
| Crude extract | 950 | 4.2 | 3990 | 100 | 38.2 | 0.1099 | - |
| (NH) ₂ SO ₄ 120g | 875 | 3.7 | 3237.5 | 81.14 | 35.7 | 0.1036 | 0.942 |
| (NH) ₂ SO ₄ 180g | 750 | 2.8 | 2100 | 64.86 | 27.3 | 0.1025 | 0.9893 |
| After DEAE-Cellulose | 250 | 2.3 | 575 | 27.38 | 0.19 | 12.1 | 118.04 |

Table Ic. Carbonic anhydrase from *Posidonia oceanica* (Linnaeus) Delile, 1813 roots (peripheral)

| Enzyme Fraction | Volume mL | Activity U/mL | Total Activity | | Protein mg/mL | Specific Activity U/mg | Purification Fold |
|--|-----------|---------------|----------------|-------|---------------|------------------------|-------------------|
| | | | U | % | | | |
| Crude extract | 980 | 3.8 | 3724 | 100 | 21.5 | 0.1767 | - |
| (NH) ₂ SO ₄ 120g | 900 | 3.3 | 2970 | 79.75 | 20.13 | 0.1639 | 0.9275 |
| (NH) ₂ SO ₄ 180g | 800 | 2.05 | 1640 | 55.21 | 18.45 | 0.111 | 0.6772 |
| After DEAE-Cellulose | 240 | 3.48 | 835.2 | 50.92 | 0.35 | 9.94 | 89.54 |

Table Id. Carbonic anhydrase from *Posidonia oceanica* (Linnaeus) Delile, 1813 roots (integral)

| Enzyme Fraction | Volume mL | Activity U/mL | Total Activity | | Protein mg/mL | Specific Activity U/mg | Purification Fold |
|--|-----------|---------------|----------------|-------|---------------|------------------------|-------------------|
| | | | U | % | | | |
| Crude extract | 940 | 2.7 | 2538 | 100 | 18.1 | 0.0143 | - |
| (NH) ₂ SO ₄ 120g | 860 | 1.8 | 1548 | 60.99 | 10.3 | 0.1747 | 12.22 |
| (NH) ₂ SO ₄ 180g | 710 | 1.52 | 1221.2 | 78.88 | 8.4 | 0.1809 | 1.035 |
| After DEAE-Cellulose | 160 | 2.2 | 352 | 28.8 | 0.12 | 18.33 | 101.3 |

Fig. 1. Effect of temperature on the activity of purified carbonic anhydrase enzyme from *Posidonia oceanica* (*Linnaeus*) *Delile*, 1813 leaves and roots

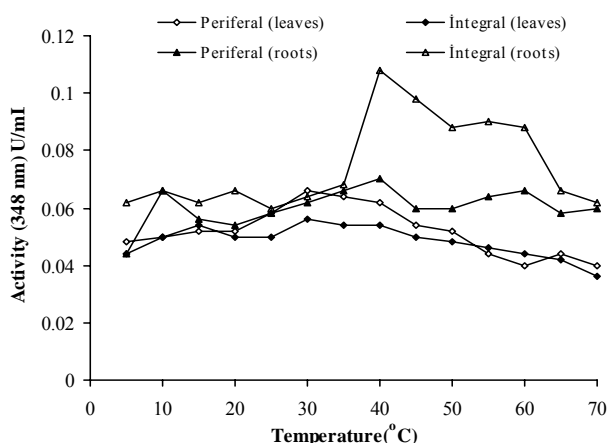


Fig. 2. The effect of pH on activity of carbonic anhydrase enzyme from *Posidonia oceanica* (*Linnaeus*) *Delile*, 1813 leaves and roots.

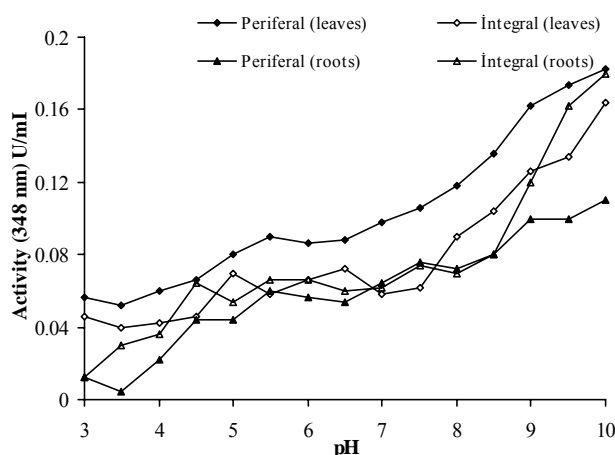
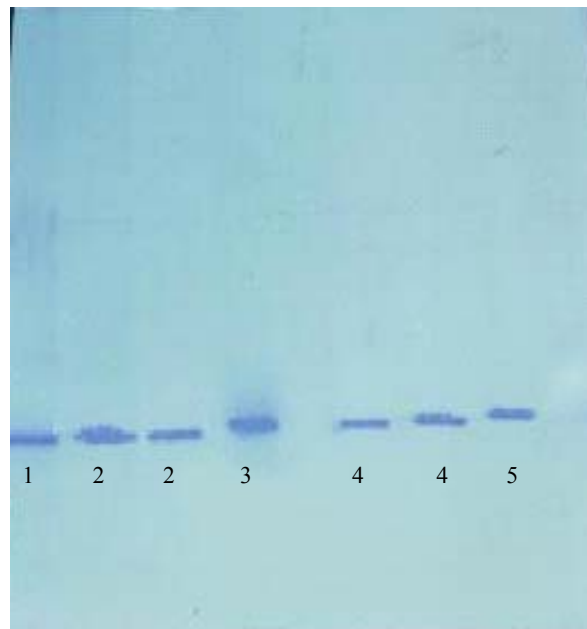


Fig. 3. Electrophoretic pattern of *Posidonia oceanica* (*Linnaeus*) *Delile*, 1813 carbonic anhydases: leaves peripheral (1), integral (2), roots peripheral (4), integral (5) and carbonic anhydrase-I (standard) (3).



plants. Always heat of the seawater was lower than lands heat.

Ion exchange chromatography was used for purification of enzyme. This technique has been applied successfully for purifying carbonic anhydrase from plants (Demir *et al.*, 1997; Demir *et al.*, 1997; Demir & Demir, 1997). Affinity column is formed from Sepharose-4B-L-tyrozin-sulphanilamide has been used but it has not been achieved.

Optimal pH was 10 in the leaves and in roots of the *Posidonia oceanica* (Fig. 2). Found value was suited to the value of *Beta vulgaris* that was an example for land plants. That value was 9 and 10 for *Beta vulgaris* leaves and roots. Furthermore, in *Vicia canencens* it was 9.2 (Demir *et al.*, 1999).

Table II. The values of I_{50} in terms of molarity of the test chemical causing a 50 % reduction of Carbonic anhydrase from *Posidonia oceanica* (*Linnaeus*) *Delile*, 1813 leaves and roots

| Enzymes | Inhibitors | I_{50} (M) 10^{-2} | I_{50} (M) 10^{-4} | I_{50} (M) 10^{-6} |
|------------------------|----------------|------------------------|------------------------|------------------------|
| Peripheral CA (leaves) | Sulphanylamide | 1.42×10^{-3} | 1.26×10^{-5} | 1.42×10^{-7} |
| | KSCN | 1.49×10^{-3} | 1.33×10^{-5} | 1.35×10^{-7} |
| | NaN_3 | - | 1.068×10^{-5} | 1.35×10^{-7} |
| | Sulphanylamide | 1.66×10^{-3} | 1.23×10^{-5} | 1.46×10^{-7} |
| Integral CA (leaves) | KSCN | 9.25×10^{-4} | 1.53×10^{-5} | 1.18×10^{-7} |
| | NaN_3 | - | 8.4×10^{-6} | 1.39×10^{-7} |
| | Sulphanylamide | 1.03×10^{-3} | 8.12×10^{-6} | 1.01×10^{-7} |
| | KSCN | 1.17×10^{-3} | 1.074×10^{-5} | 1.04×10^{-7} |
| Peripheral CA (roots) | NaN_3 | - | 1.044×10^{-5} | 1.01×10^{-7} |
| | Sulphanylamide | 1.39×10^{-3} | 1.13×10^{-5} | 1.08×10^{-7} |
| | KSCN | 1.06×10^{-3} | 1.02×10^{-5} | 1×10^{-7} |
| | NaN_3 | - | 1.02×10^{-5} | 9.8×10^{-8} |

Fig. 4. Effects of Sulphanilamide, NaN_3 and KSCN on the purified peripheral carbonic anhydrase from leaves of *Posidonia oceanica* (Linnaeus) Delile, 1813.

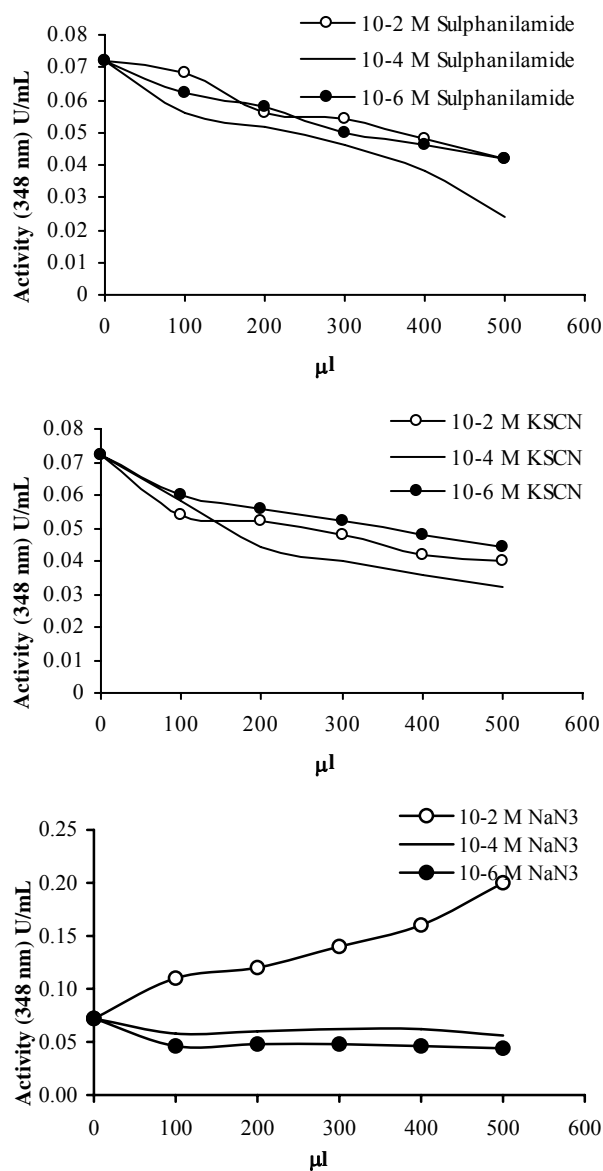
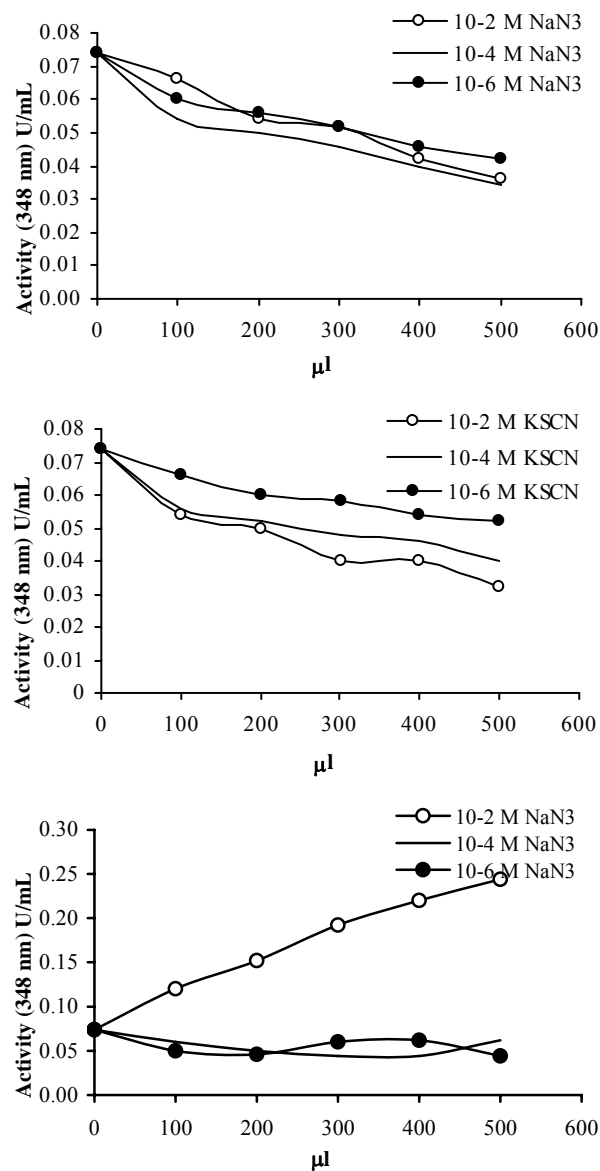


Fig. 5. Effects of Sulphanilamide, NaN_3 and KSCN on the purified integral carbonic anhydrase from leaves of *Posidonia oceanica* (Linnaeus) Delile, 1813.



According to the results of gel filtration chromatography, the molecular weights of the both leaves and roots carbonic anhydrases were 29.000 Dalton. Purified peripheral and integral CA enzyme of *Posidonia oceanica* was formed of 6 subunits. These molecular weights were similar value of that of land plants. Generally, land plants had hexamer including six subunit (Demir *et al.*, 1997).

Sulphanilamide, NaN_3 and KSCN decreased in leaves and roots carbonic anhydrase activity (Fig. 4, Fig. 5, Fig. 6 & Fig. 7). Sulphanilamide, NaN_3 and KSCN had inhibitor effect for mammals' carbonic anhydrase, but it had activation effect for plant carbonic anhydrase. Sea plants were separated from land plants by this way and it had characteristics as mammals carbonic anhydrases.

Fig. 6. Effects of Sulphanilamide, NaN_3 and KSCN on the purified peripheral carbonic anhydrase from roots of *Posidonia oceanica* (Linnaeus) Delile, 1813.

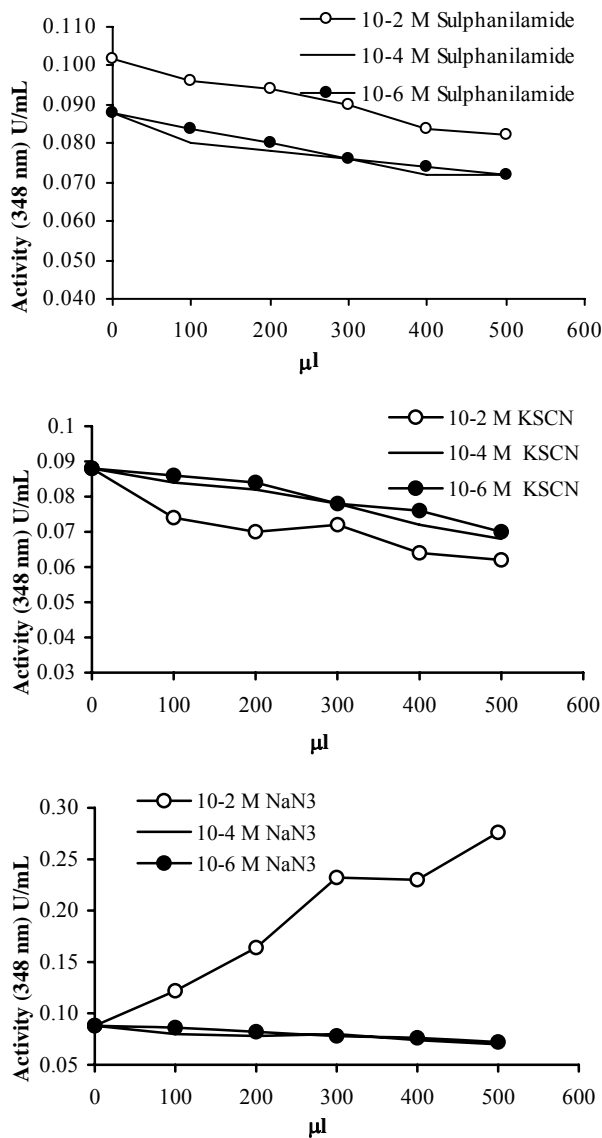
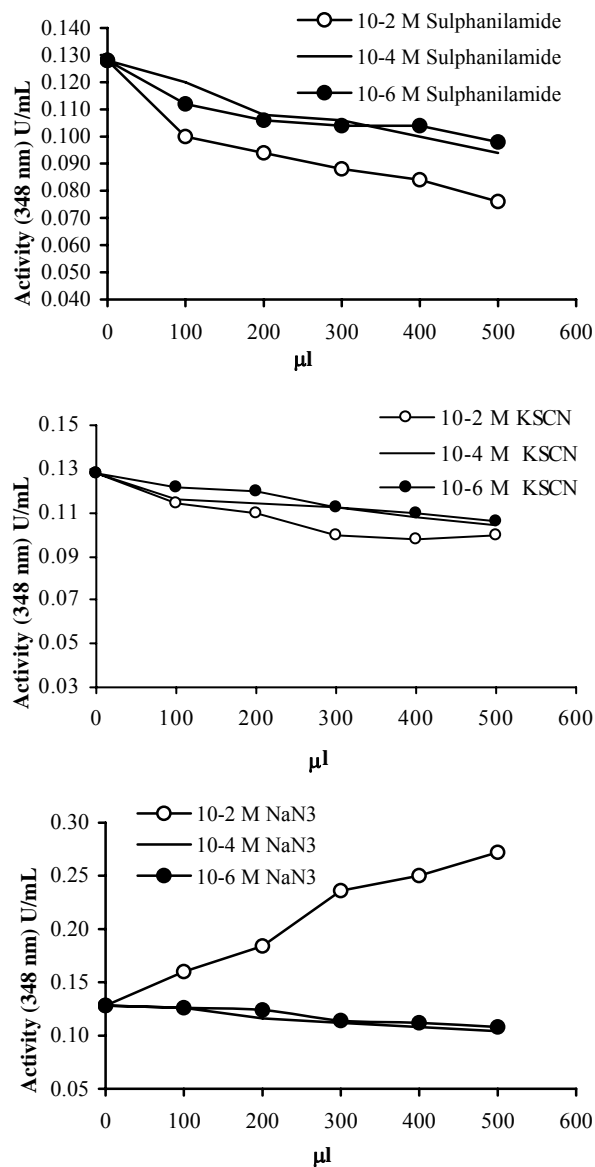


Fig. 7. Effects of Sulphanilamide, NaN_3 and KSCN on the purified integral carbonic anhydrase from roots of *Posidonia oceanica* (Linnaeus) Delile, 1813.



I_{50} values of the enzymes from *Posidonia oceanica* was found versus Sulphanilamide, NaN_3 and KSCN (Table II).

As a result, carbonic anhydrase has been separately purified and characterized from leaves and roots of *Posidonia oceanica*. Enzyme was active even more than CA-II, which is thought to be the most active enzyme that was the most interesting point in this characterization.

In all measure has been used in dilute solution of enzyme. Another important point was that enzyme lost its activity in a short period (Fig. 8, Fig. 9). In 4°C enzyme has lost its activity in tubes, which had open edge in a short time as one week. That was the most interesting discovery for enzyme. Because on -31°C other plants carbonic anhydrases could be hid percentage 5 loss activity instead of a carbonic anhydrase.

Fig. 8. Activity-time graph of carbonic anhydrase purified from leaves of *Posidonia oceanica* (Linnaeus) Delile, 1813.

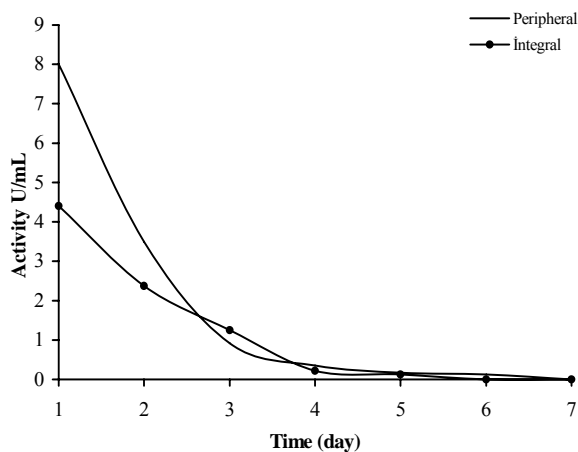
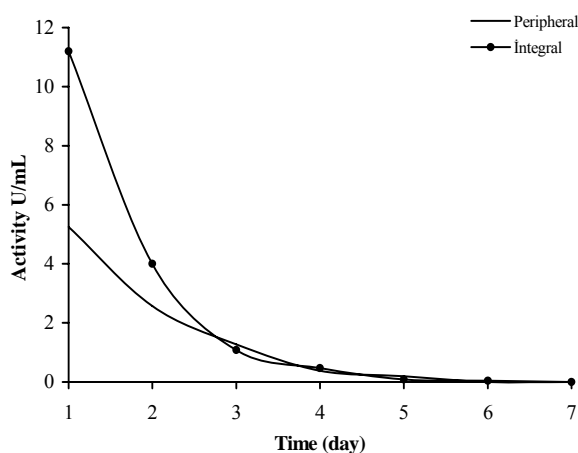


Fig. 9. Activity-time graph of carbonic anhydrase purified from roots of *Posidonia oceanica* (Linnaeus) Delile, 1813.



REFERENCES

- Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biol Chem.*, 72: 248-54
- Carlson, U., B. Kjellström and B. Antonsson, 1980. Purification and Properties of Cyclostome Carbonic Anhydrase from Erythrocytes of Hagfish. *Biochem. Biophys. Acta.*, 612: 60-70
- Demir, N., Y. Demir and G. Açar, 1997. Carbonic Anhydrase from Camelia Sinensis (TEA) Leaves. *Preparative Biochem. and Biotechnol.*, 27: 271-8
- Demir, N., Y. Demir and A. Yıldırım, 1997. Carbonic Anhydrase from Leaves and Trunks of *Daucus Carota*. *Phytochemistry*, 44: 1247-50
- Demir, N. and Y. Demir, 1997. Carbonic Anhydrase from Nicotinia Tabacum Leaves. *Doğa, Tr. J. Chem.*, 21: 111-7
- Demir, Y., N. Demir and Ö.İ. Küfrevioğlu, 1999. Carbonic anhydrase from *Vicia Canencens* Leaves. *Preparative Biochem. and Biotechnol.*, 29: 235-44
- Demir, N., Ö.İ. Küfrevioğlu, E.E. Keha and E. Bakan, 1993. An Enzymatic Method for Zinc Determination in Serum. *Biofactors*, 4: 129-32
- Kandel, M., A.G. Gornall, S.C. Wong and S.I. Kandel, 1970. Some Characteristic of Human, Bovine and Horse Carbonic Anhydrase as Revealed by Inactivation Studies. *J. Biol. Chem.*, 245: 2444-50
- Kashiwabara, K., T. Hobo, E. Kobayashi and S. Suzuki, 1985. Flow Injection Analysis for Traces of Zinc with Immobilized Carbonic Anhydrase. *Analytica Chem. Acta.*, 178: 209
- Laemmli, U.K., 1970. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature.*, 227: 680-5
- Lindskog, S., 1960. Purification and Properties of Bovine Erythrocyte Carbonic Anhydrase. *Biochem. Biophys. Acta.*, 39: 218-26
- Nyman, P.O. and S. Lindskog, 1964. Amino Acid Compositions of the Various Forms of Bovine and Human Erythrocyte Carbonic Anhydrase. *Biochem. Biophys. Acta.*, 85: 141-51
- Nyman, P.O., 1961. Purification and Properties of Carbonic Anhydrase from Human Erythrocytes. *Biochem. Biophys. Acta.*, 52: 1-12
- Rickli, E.E., S.A.S. Ghazanfar, B.H. Gibbons and J.T. Edsall, 1964. Carbonic Anhydrases from Human Erythrocytes. *J. Biol. Chem.*, 239: 1065-78
- Sly, W.W. and P.Y. Hu, 1995. Human Carbonic Anhydrases and Carbonic Anhydrase Deficiencies. *Annu. Rev. Biochem.*, 64: 375-400
- Tobin, A.L., 1970. Carbonic Anhydrase from Parsley Leaves. *J. Biol. Chem.*, 245: 2656-66
- Verpoorte, J.A., S. Mehta and J.T. Edsall, 1967. Esterase Activities of Human Carbonic Anhydrase. *J. Biol. Chem.*, 242: 4221-9
- Whitaker, J.P., 1963. Determination of Molecular Weight of Proteins by Gel Filtration on Sephadex. *Anal. Chem.*, 35: 1950-4

(Received 28 March 2006; Accepted 11 June 2006)