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# Purification, characterization and determination of kinetic features of carbonic anhydrase from turbot (*Psetta maxima) muscle* tissue

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**Abstract:** In this study, carbonic anhydrase purification from turbot (*Psetta maxima*) muscle tissue, together with analysis of the kinetic behavior and some enzyme properties, is described. The purification steps comprised hemolysate preparation, Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography, and dialyzing. The yield was 69.05%, and the enzyme was found to have a specific activity of 755.2 EU/mg protein. The overall purification was about 50.65-fold. A temperature of +4 °C was maintained during the purification process. The molecular mass of the subunit was determined to be 29.7 kDa by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme had an optimal pH of 8.0, a stable pH of 8.0, and an optimal temperature of 30 °C. Km and Vmax for p-nitrophenylacetate as a substrate were also determined.

**Keywords**: Fish, carbonic anhydrase, muscle, turbot

## INTRODUCTION

Turbot, a member of the flatfish order, consists of 14 families and 716 subspecies (Munreo, 2005). As a habitat, they spread to the region, extending from the Icelandic and Baltic coasts in the north to the Scandinavian coasts and the Moroccan coasts in the south, to the Black Sea and the Mediterranean. The length of the turbot fish living on the bottom and in muddy and sandy areas at depths of 70-100 meters reach 1 meter in places. Turbot fish migrate from mid-March to mid-May in coastal waters to lay eggs (Hara, 2001).

Turbot fish have eyes on one side and are blind on the other. One of the long and simple ventral fins is on the eye side, and the other is on the blunt side. There are approximately 11-12 spine-like structures on its gills (Memis, 2010). In order to survive, they feed on small fish such as rockfish, silverfish, anchovies, fry, and mainly crustaceans (Samsun, 2004). The egg productivity of turbot fish may differ from each other.

While the average egg number of Atlantic shields is 3.5-4.2 million for adult individuals, the total egg quantity of Black Sea shields is 9 million annually (Jones, 1974; Samsun, 2004). Turbot production varies between 10100 and 14100 tons per year in Europe (Sevgili and Nezaki, 2010). In China, turbot production through aquaculture has reached levels of 50000–60000 tons in recent years (FAO, 2010).

The catalytic mechanism of the carbonic anhydrase enzyme has been tried to be clarified as a result of the studies carried out in the last sixty years. It has been understood that the CA enzyme has advantageous properties such as being extremely important in metabolism, being stable in the solution environment, and being kept for a long time without losing its activity under suitable conditions (Supuran and Scozzafava, 2001).

Today, it is known that the carbonic anhydrase (carbonate hydrolase, EC: 4.2.1.1, CA) is present in all animals, organisms with photosynthetic cells, and all organisms such as bacteria, and has 14 different isoenzymes depending on the proteins to which it is attached (Chegwitten et al., 2000, Solis et al., 1999, Clare and Supuran, 2000).

Carbonic anhydrase (CA) reversibly catalyzes the $CO_2$ hydration and the $HCO_3^-$ dehydration in organisms; it also plays a role in the H+ and $HCO_3^-$ accumulation in kidney, gastric mucosa, and eye lens tissues (Supuran et al., 2003, Şentürk et al., 2009). In addition, the important roles of this enzyme have been proven in the gills and glandular organs of fish, in some insects and bacteria, in the production of shells of crustaceans and in the formation of egg shells, in algae, and in terrestrial plant chloroplasts (Graham et al., 1984, Tsuzuki and Miyachi, 1989, Badger and Price, 1994).

The important function of the CA enzyme in the gill membrane of cartilaginous fish is related to pH and/or $CO_2$ sensitivity in the respiratory system, in addition, it ensures the continuity of $CO_2$ excretion. (Henry et al., 1988).

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Our aim is to determine the kinetic properties of the carbonic anhydrase (CA) enzyme from the muscle tissue of turbot fish for the first time.

## MATERIALS AND METHODS

### Chemicals

Chemicals used in our studies, p-nitrophenyl acetate, N,N,N',N'-tetramethylethylene diamine (TEMED), Sepharose-4B, a dialysis bag, standard serum albumin, and L-tyrosine from Sigma Chemical Comp. sodium hydroxide, sulfanilamide, sodium bicarbonate, sodium sulfate, trihydroxymethylaminomethane (Tris), sodium acetate, sodium perchlorate, 2-mercaptoethanol, sulfuric acid, bromine thymol blue, glycine, phosphoric acid, hydrochloric acid, sodium barbital, methanol, ethanol, isoproponal, acetone, sodium nitrogenate, acrylamide, N,N'-methylene bisacrylamide, R-250 from E. Merk AG, coomassie brillant blue G-250; carbon dioxide gas and sodium hydroxide

### Procurement of turbot muscle tissue and preparation of homogenate

Ten turbot fish (*Scophthalmus maximus*) were purchased from a fish farm in the Samsun province on the Black Sea coast of Turkey. Turbot fish were taken to the laboratory under cold-chain conditions, and muscle tissue was removed. Then, the blood and other impurities in the tissue samples were eliminated by washing with 0.9% NaCl, and this process was repeated 3 times. In order to prepare the tissue homogenate, the sample was first cut into small pieces with the help of a scalpel. It was then disintegrated in liquid nitrogen and homogenized in 10 volumes of 25 mM Tris HCl / 0.1 M $Na_2SO_4$ (pH= 8.7) buffer. The prepared suspension was centrifuged at 10,000 rpm for 30 minutes, and the supernatant was used for analysis. All experiments were performed in triplicate (Wistrand, 2002).

### Carbonic anhydrase purification

The supernatant sample was added to a buffer solution containing Tris-HCl/$Na_2SO_4$ (25 mM/0.1 M) at pH 8.7 for kinetic studies. The pH-adjusted homogenate was loaded onto the Sepharose-4B-l-tyrosine-sulphanilamide affinity column and was washed with $Na_2SO_4$ (22 mM), in Tris–HCl buffer solution (25 mM, pH8.7). At the end of this process, the carbonic anhydrase enzyme was retained in the column. Then, 0.1 M $NaCH_3COO.3H_2O$/0.5 M $NaClO_4$ (pH=5.6) solution was applied to the column to obtain the CA enzyme. The flow rate of the column was fixed at 20 ml/h using a peristaltic pump (Kucuk and Gulcin, 2016).

### Measurement of carbonic anhydrase activity

CA was detected by changes in absorbance at 348 nm of p-nitrophenyl acetate to p-nitrophenolate over a period of 3 min at 25°C using a spectrophotometer. The enzymatic reaction performed contained 0.4 mL Tris–$SO_4$ buffer solutions (0.05 M, pH 7.4), 0.36 mL of p-nitrophenyl acetate (3 mM), 0.22 mL of water, and 0.2 mL of enzyme solution in a total volume of 1 mL. An enzyme solution was not added to the control sample (Kucuk and Gulcin, 2016).

### SDS-PAGE study

After the purification of the enzymes was completed, the purity level of the purified enzymes was examined by applying 3-8% batch SDS-PAGE as described by Laemmli (1970). SDS polyacrylamide gel electrophoresis was performed to confirm the enzyme purity following the purification steps. The running and stacking gels contained 3% acrylamide, 10% acrylamide, and 0.1% SDS. A 20-µg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coommassie Brilliant Blue R-250 in 10% acetic acid and 50% methanol (Şentürk et al., 2009).

### Qualitative Protein Determination

Qualitative protein determination is achieved by determining the maximum absorbance of tryptophan and tyrosine in the structure of proteins at 280 nm (Segel, 1968). Qualitative protein determination in equal volume fractions obtained in chromatography processes was performed with the help of this method.

### Protein determination by the Bradford method

The protein amounts in the homogenate and the enzyme solution purified by affinity chromatography were determined by this method. Protein detection during purification was performed spectrophotometrically at 595 nm according to the Bradford method. Bovine serum albumin was used as a standard. (Bradford, 1976).

### Optimum pH study for the carbonic anhydrase enzyme

The activity values of the CA enzyme were measured in 20 mM potassium phosphate ($KH_2PO_4$) solutions with a pH between 7.0-9.0 and pH 5.0-8.0 and 20 mM Tris-HCl (Ceyhun et al., 2011).

### Ionic strength study for the carbonic anhydrase enzyme

The pH of Tris-HCl and potassium phosphate buffers was taken at 8.0 from turbot muscle tissue, where the activity of the CA enzyme is at its optimum level. Esterase activity measurement for CA enzyme was performed in Tris-HCl buffer with ionic strength between 10 mM and 1000 mM, and then the results were brought in graphic form (Ceyhun et al., 2011).

### Studies related to finding the stable pH of the carbonic anhydrase enzyme

$SO_4$-Tris buffer with a pH of 7.0-9.0 was used to detect the pH at which enzymes are stable. After mixing 500 µl of buffer solutions with 500 µl of enzyme solution in the pH range shown, it was kept at 4 °C. Activity measurements were made with an interval of 2 days, and the pH value at which the enzyme was stable was detected. The values of the activity

values corresponding to the incubation time were plotted (Ceyhun et al., 2011).

**Investigation of the effect of temperature on muscle tissue carbonic anhydrase enzyme**

In order to determine the effect of temperature on CA enzyme activity, enzyme activities were measured between 20 ºC and 70 ºC, and the optimum operating temperature was determined at its optimum pH and optimum ionic strength value (Ceyhun et al., 2011).

**Studies on finding KM and Vmax values for p-nitrophenyl acetate substrate**

Using five different concentrations of p-nitrophenyl acetate, the activity of CA enzymes purified from turbot muscle tissue was evaluated under different optimum conditions. Then the Lineveawer-Burk graph was drawn, and calculations were made by looking at the KM and Vmax values from this graph (Ceyhun et al., 2011).

**RESULTS and DISCUSSION**

There are many studies carried out in the form of carbonic anhydrase enzyme purification and characterization in many living tissue species in order to determine how and where the enzyme is located in a living organism and how it functions (Söyüt et al., 2008; Göçer et al., 2015; Göksu et al., 2014; Maheshwari et al., 2019; Jo and Hwang, 2019; Del Prete et al., 2016). However, no study has been found on the purification and kinetic properties of the CA enzyme in turbot muscle tissue. Carbonic anhydrase (CA) reversibly catalyzes $CO_2$ hydration and $HCO_3^-$ dehydration in organisms; it also plays a role in H+ accumulation and $HCO_3^-$ in tissues (Supuran et al., 2003; Şentürk et al., 2009). The important roles of this enzyme have been proven in the gills and glandular organs of fish, in some insects and bacteria, in the production of shells of crustaceans and in the formation of eggshells, in algae, and in terrestrial plant chloroplasts (Graham, et al., 1984, Tsuzuki and Miyachi, 1989, Badger and Price, 1994). The main function of the CA enzyme in the gill membrane of cartilaginous fish is related to pH and/or $CO_2$ sensitivity in the respiratory system; in addition, it ensures the continuity of $CO_2$ excretion. (Henry et al., 1988). Many studies have been conducted on the purification of CA enzymes in different organisms. In our study, we purified and characterized the CA enzyme from turbot muscle tissue using a sepharose-4B-l-tyrosine-sulphanilamide affinity column. With the help of the preparation of CA enzyme homogenate from turbot muscle tissue and Sepharose 4B-tyrosine-sulfanilamide affinity chromatography methods, the enzyme with a specific activity of 755.2 EU/mg protein was purified 69.05 times with a yield of 50.65% (Table 1).

**Table 1.** Enzyme unit, specific activity and purification values of CA enzyme purified from turbot muscle tissue from affinity column

| Purification steps | Total volume (mL) | Protein (mg/mL) | Total protein (mg) | Total activity (EU) | Specific activity (EU/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|---|
| Homogenate | 8 | 17.6 | 140.8 | 2100 | 14.91 | 100 | 1 |
| Sepharose-4B-tyrosine-sulfanilamide affinity column chromatography | 6 | 0.32 | 1.92 | 1450 | 755.20 | 69.05 | 50.65 |

SDS-polyacrylamide gel electrophoresis was performed to examine the purity of the CA enzyme obtained from turbot muscle tissue. Standard protein markers with known molecular weights were used (29 kDa bovine carbonic anhydrase; 16.5 kDa egg white lysozyme), and isoenzymes obtained by purification from humans were applied to SDS-polyacrylamide gel electrophoresis. Photographed after the proteins became apparent. A purified enzyme was obtained, showing a single band in SDS-PAGE (Figure 1).

The molecular mass of the subunit was determined to be 29.7 kDa by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Our findings were similar to those of previous studies (Demirdağ et al., 2013; Kucuk and Gulcin, 2016). When the studies on CA purification in different organisms and tissues are examined, CA is 30.5 kDa in the gills of sea bream, CA 29 kDa in the erythrocytes of zebrafish, 28 kDa in the gills of Antarctic icefish, and 29.4 kDa in the liver tissues of rainbow trout (Kaya et al., 2013; Rizzello et al., 2007; Peterson et al., 1997). A step affinity chromatographic technique was used (Ceyhun et al., 2011; Ekinci et al., 2010). It has been determined that the enzyme is stable at pH = 8.0, the optimum ionic strength is 20 mM Tris-HCl at pH = 8.0, and the

temperature with the highest activity is 20°C (Figs. 2, 3, 4, 5, 6).

Similar findings were found in other studies (Öztürk Sarikaya et al., 2011; Ceyhun et al., 2011; Bayram et al., 2008; Ekinci et al., 2010). With their catalytic versatility, alpha-CAs phosphatases can act as esterases and paraoxonases. (Demirdağ et al., 2013). Thus, we have researched the esterase activity of the turbot enzyme with 4-nitrophenyl acetate (NPA) as a substrate. The KM and Vmax values were calculated for NPA hydrolysis catalyzed by the fish enzyme by means of Lineweaver-Burk graphs (Fig. 7). In the present study, at pH 8.0, the Vmax value was 0.253 EU/ml and the KM value was 0.344 mM for p-nitrophenyl acetate (Fig. 7).

Consequently, we purified carbonic anhydrase from turbot muscle tissue for the first time and analyzed the properties of this enzyme.

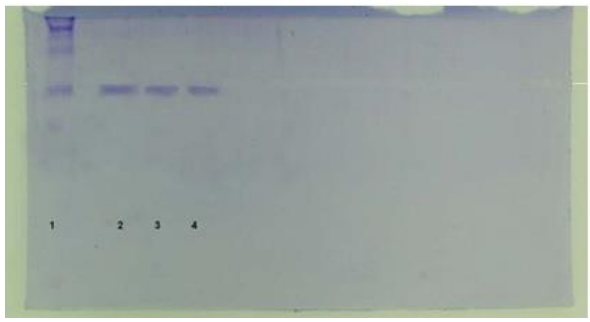


**Figure 1.** SDS-polyacrylamide gel electrophoresis photograph of turbot muscle carbonic anhydrase enzyme purified by affinity chromatography ((1) standard proteins (E. coli β-galactosidase 116 kDa, rabbit phosphorylase B 97 kDa, bovine serum albumin 66 kDa, and bovine erythrocyte CA 29 kDa), (2), (3) and (4) Muscle CA)

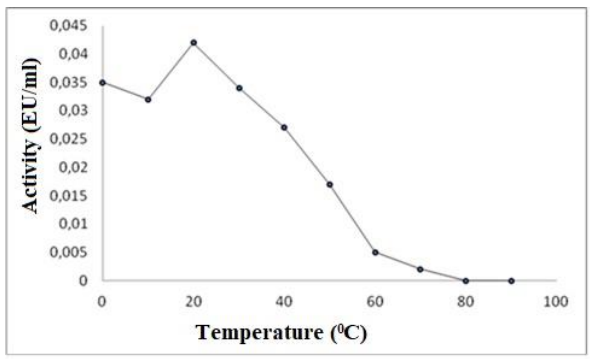


**Figure 2.** Optimum temperature measurement for turbot muscle CA enzyme and the temperature-activity graph of the values drawn

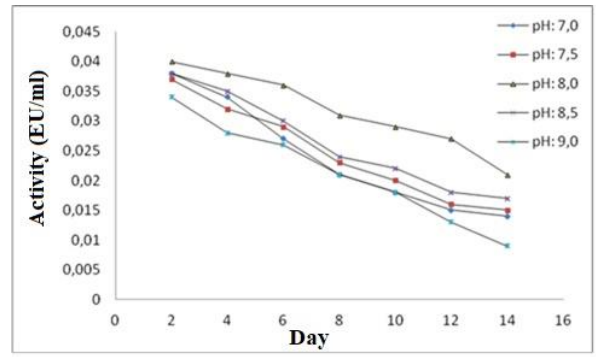


**Figure 3.** Stable pH graph for turbot muscle CA enzyme obtained using 20mM Tris HCl buffer solution at different pH

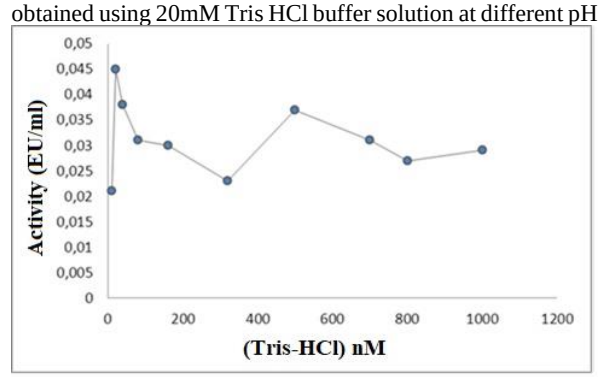


**Figure 4.** Ionic strength-activity graph for turbot muscle tissue CA enzyme obtained using Tris-HCl buffer solutions at different concentrations

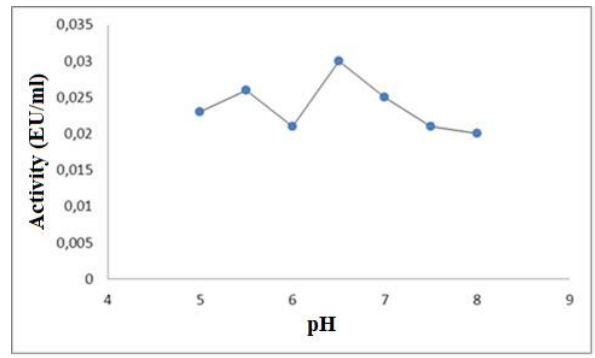


**Figure 5**. Activity measurement graph using 20mM phosphate buffer solution for optimum pH of turbot muscle CA enzyme

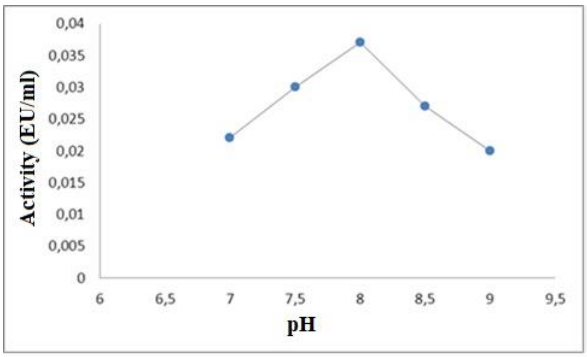


**Figure 6.** Activity measurement graph created using 20 mM Tris-HCl buffer solution for optimum pH of turbot muscle CA enzyme

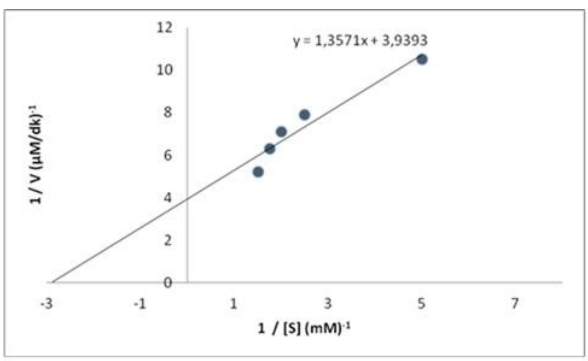


**Figure 7.** Lineweaver-Burk curves for turbot muscle CA enzyme


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## CONFICT OF INTEREST

The authors declare no competing interests

## ETHICAL APPROVAL

Ethics committee approval is not required.


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## CONSENT FOR PUBLICATION

Not applicable.

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