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Purification and characterization of the carbonic anhydrase enzyme from Black Sea trout (*Salmo trutta* Labrax Coruhensis) kidney and inhibition effects of some metal ions on enzyme activity

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ABSTRACT

In this study, the carbonic anhydrase (CA) enzyme was purified from Black Sea trout (*Salmo trutta* Labrax Coruhensis) kidney with a specific activity of 603.77 EU/mg and a yield of 35.5% using Sepharose-4B-L-tyrosine- sulphanilamide affinity column chromatography. For determining the enzyme purity and subunit molecular mass, sodiumdodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed and single band was observed. The molecular mass of subunit was found approximately 29.71 kDa. The optimum temperature, activation energy (E_a), activation enthalpy (ΔH) and Q_{10} values were obtained from Arrhenius plot. K_m and V_{max} values for *p*-nitrophenyl acetate of the purified enzyme were calculated from Lineweaver-Burk graphs. In addition, the inhibitory effects of different heavy metal ions (Fe^{2+}, Pb^{2+}, Co^{2+}, Ag^{+} and Cu^{2+}) on Black Sea trout kidney tissue CA enzyme activities were investigated by using esterase method under *in vitro* conditions. The heavy metal concentrations inhibiting 50% of enzyme activity (IC_{50}) were obtained. Finally K_i values and inhibition types were calculated from Lineweaver-Burk graphs.

1. Introduction

Carbonic anhydrase (CA, E.C.: 4.2.1.1) is a member of zinc containing metalloenzyme family that catalyses the rapid and reversible hydration of carbon dioxide (CO_2) and dehydration of bicarbonate (HCO_3^-) (Gülçin et al., 2004; Arabacı et al., 2014; Akbaba et al., 2014a,b).

$$CO_2 + H_2O \overset{CA}{\Leftrightarrow} HCO_3^- + H^+$$

This enzyme plays an important role in different processes including respiration, biosynthetic processes, controlling of physiological pH such as acid-base regulation, gas balance, bone resorption and calcification (Beydemir and Gulcin, 2004; Scozzafava et al., 2015; Akıncıoğlu et al., 2015). The CA was first isolated from mammalian erythrocytes. In the later years, the enzyme was purified from human erythrocytes, fish erythrocytes, rat erythrocytes, rat saliva, cattle bones, cattle leukocytes, some bacteria and plant sources and further that they were characterized in many sources. Molecular mass of enzyme was determined about 30 kDa in mammals (Beydemir et al., 2002). CA enzymes α-, β-, γ-, δ-, ε- and η-CA have been examined in six different classes (Göksu et al., 2014; Yıldırım et al., 2015; Boztaş et al., 2015). Among them, α-CA is found in cytoplasm of green plants, bacteria, algae and vertebrates. Furthermore, the enzyme is found in tissues of fish (Coban et al., 2009; Göçer et al., 2015). Zinc ion is present in the active site of all enzyme families. Each of these families has similar catalytic function. Zn^{+2} ions are great importance of catalysis of reactions the CA enzymes (Güney et al., 2014; Akıncıoğlu et al., 2014; Çetinkaya et al., 2014a,b). The active site of CA binds the hydroxyl group ($^-$OH) on Zn^{2+} ions. The hydration direction, first, a zinc ion simplifies the proton forming hydroxide ions exit from the water molecules. Second, OH^- attacks CO_2 and is transformed to HCO_3^- ions. As seen in Fig. 1, in the next step, the catalytic region release HCO_3^- and is restored by the binding of another water molecule (Aksu et al., 2013; Topal and Gülçin, 2014).

CA enzyme contains Zn^{2+} ion in its structure like sorbitol dehydrogenase. Zn^{2+} insufficiency to cause in many metabolic defects and results are reduced production of Zn^{2+}-containing enzymes. Decreasing the amount of CA causes metabolic imbalance and diseases (Akıncıoğlu et al., 2013). Generally heavy metals can change enzymatic activities by binding the functional

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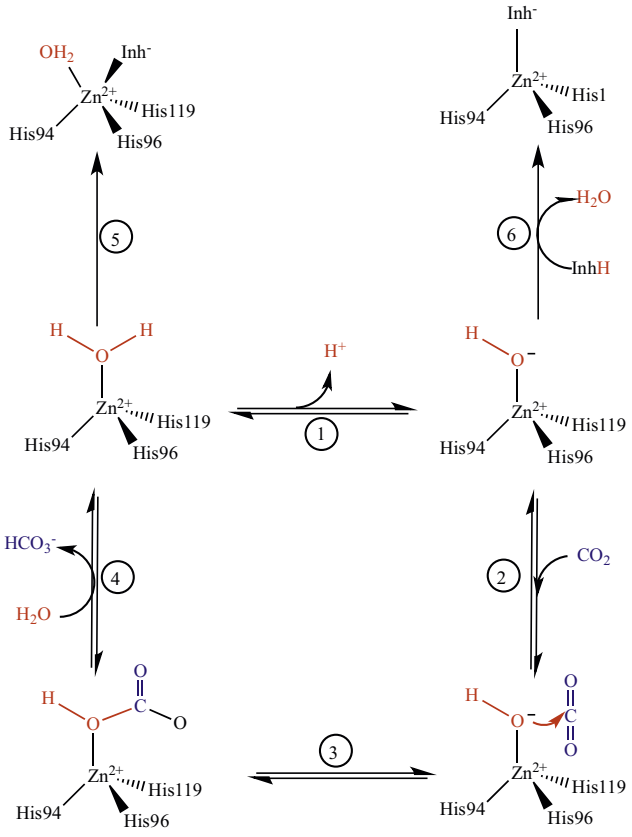

Fig. 1. Catalytic and inhibition mechanism of carbonic anhydrase.

groups, comprising the carbonyl, carboxyl and sulfhydryl or by substituting of the metal associated with the enzyme. Intense exposure to metal ions can cause different toxicological effects in fish and humans indirectly. The concentration of heavy metals in fish cause decreases the habits of the fish species (Sivaperumal and Sankar, 2006). The toxicological effects of heavy metals are usually enzyme inhibition and denaturation (Ekinci and Beydemir, 2010). Generally metal inhibition of enzyme is based on heavy metal binding to the protein. Therefore, human metabolism is affected by metal toxicity. Over consumption of fish causes a variety of diseases such as cancer, diabetes, Alzheimer and Parkinson diseases (Jomova and Valko, 2011). The CA plays a critical role on the respiration and transformation mechanism of CO_2 to HCO_3^- in living species. Also it is known that there is a close connection between CA enzyme activity and oxygen consumption (Öztürk Sarıkaya et al., 2011; Gülçin and Beydemir, 2013). Reduction in the rate of oxygen is a vital stress factor on fish. Similarly, metal inhibition on CA enzyme activity results in a reduction in the rate of oxygen consumption and increases the stress (Şentürk et al., 2011; Innocenti et al., 2010a; Beydemir et al., 2011). Enzyme inhibitions are vital for the metabolisms of all living species. Almost all drugs and most of chemicals including heavy metals display their functions on enzyme interaction mechanism (Öztürk Sarıkaya et al., 2010; Innocenti et al., 2010b). Furthermore, heavy metals are effective to be some of the strongest naturally occurring CA inhibitors. It is known that Ag^+ ions inhibit both gills CA enzyme activity and ion transport in freshwater fish (Soyut et al., 2012).

In this study, we investigated the toxicological effects of some heavy metal ions, including Fe^{2+}, Pb^{2+}, Co^{2+}, Ag^+ and Cu^{2+}, on the CA enzyme purified from the kidney of Black Sea trout (*Salmo trutta* Labrax Coruhensis) using the esterase method under *in vitro* conditions.

2. Experimental

2.1. Chemicals

$Pb(CH_3COO)_2$, $FeCl_2$, $CoCl_2$, $AgNO_3$ and $CuSO_4{\cdot}5H_2O$, *p*-nitrophenyl acetate, CNBr-activated-Sepharose-4B and protein assay reagent were obtained from Sigma-Aldrich Co. (GmbH, Germany). All other chemicals were analytical grade and purchased from Merck (Germany).

2.2. Black sea trout (Salmo trutta *Labrax Coruhensis)*

Black Sea trout (*Salmo trutta* Labrax Coruhensis) were obtained from a fish farm in the Rize province in the Black Sea cost of Turkey.

2.3. Purification of carbonic anhydrase

Kidney tissues were removed from fresh trout and were washed 3 times with 0.9% NaCI isotonic saline solution. Kidney tissues were lysed by liquid nitrogen at 10,000*g* for 30 min. Then the samples were centrifuged at medium speed (2000*g*). Precipitate and supernatant were separated from each other. Finally the supernatant sample was transferred to a buffer solution containing Tris–HCl/Na_2SO_4 (25 mM/0.1 M) at pH 8.7 for using in kinetic studies (Soyut and Beydemir, 2008; Şentürk et al., 2009). The pH-adjusted homogenate was loaded to the Sepharose-4B-L-tyrosine-sulphanilamide affinity column and was washed with Na_2SO_4 (22 mM), in Tris–HCl buffer solution (25 mM, pH 8.7). The enzyme, after binding to the column was eluted by $NaClO_4$/$NaCH_3COO$ (0.5 M/0.1 M, pH 5.6) with column flow rate 20 mL/h. All procedures were performed at 4 °C (Atasaver et al., 2013).

2.4. Determintion of CA activity

CA activity was determined by absorbance changing at 348 nm of *p*-nitrophenyl acetate to *p*-nitrophenolate over a period of 3 min at 25 °C using a spectrophotometer (Topal and Gülçin, 2014). The enzymatic reaction performed containing 0.4 mL of Tris–SO_4 buffer solutions (0.05 M, pH 7.4), 0.36 mL *p*-nitrophenyl acetate (3 mM), 0.22 mL water and 0.2 mL of enzyme solution in total volume of 1 mL. Enzyme solution was not added to the control sample.

2.5. Determintion of qualitative protein

The protein elution was recorded at 280 nm. Finally, CO_2-hydratase activity was determined according to the method previously described (Çoban et al., 2007, 2008).

2.6. SDS-PAGE study

SDS-PAGE was performed after purification of the enzyme according to Laemmle's procedure (Laemmli, 1970), which was described previously (Gülçin et al., 2005; Beydemir et al., 2005). The running and stacking gels contained 0.1% SDS and 10 and 3% acrylamide, respectively. The electrode buffer contained Tris–glycine (0.25 M/2 M, pH 8.3). The buffer solution was prepared 0.65 mL Tris–HCl (1 M pH 6.8), 3 mL SDS (%10), 1 mL glycerol, 1 mL bromophenol blue (0.1%), 0.5 mL β-mercaptoethanol and 3.85 mL water by mixing. A portion of sample (20 μg) was applied in 50 μL buffer solutions. The mixture was heated in a boiling water bath for 5 min. The purified enzyme samples were loaded into each space of stacking gel. Firstly, an electric potential of 80 V was applied until bromophenol blue reached the running gel. Then it was increased to 200 V over 3 h. Then, gel was kept in 0.1% Coomassie Brilliant blue R-