The impacts of some metals on the activity of corb gill (Umbrina cirrosa) Carbonic Anhydrase

Bazı Metallerin Minekop (Umbrina Cirrosa) Solungacı Karbonik Anhidraz Aktivitesi Üzerine Etkileri

Research Article

Namık Kılınç[1], Zuhal Alım[1], Mehmet Mustafa İşgör[2], Şükrü Beydemir[1]*

[1]Atatürk University, Faculty of Sciences, Department of Chemistry, Biochemistry Division, Erzurum, Turkey.

[2]Mustafa Kemal University, Faculty of Veterinary Sciences, Department of Biochemistry, Hatay, Turkey.

ABSTRACT

Metal toxicity causes oxidative stress in fish. This situation is a potential risk factor for humans and other living feeding on contaminated fish. In this study, the inhibition effects of heavy metals on carbonic anhydrase enzyme from the corb fish gill were investigated. The carbonic anhydrase enzyme was purified from gill of corb fish with a specific activity of 2093,9 EUmg^{-1} and 86,51% yield and approximately 160 fold using Sepharose 4B-L-tyrosine sulfanilamide affinity chromatography method. SDS-polyacrylamide gel electrophoresis showed a single band corresponding to a molecular weight of approximately 30,8 kDa. Inhibitory effects of metals (Ag^{+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}) on CA activity were determined at different concentrations using the hydratase method under *in vitro* conditions. Consequently, *in vitro* inhibition rank order was determined as Ag^{+}> Cu^{2+}> Pb^{2+}> Zn^{2+} > Cd^{2+}> Ni^{2+}. From these results, we showed that Ag^{+} is the most potent inhibitor of CA enzyme.

Key Words

Carbonic anhydrase; Gill; Heavy metals; Inhibition

ÖZET

Metal toksisitesi balıklarda oksidatif strese sebep olur. Bu durum insanlar ve diğer balık tüketen canlılar için potansiyel bir risk faktörüdür. Bu çalışmada minekop balığı solungacından saflaştırılan karbonik anhidraz enzimi üzerine bazı ağır metallerin inhibisyon etkileri incelendi. Minekop balığı solungacından, karbonik anhidraz, Sepharose 4B-L-tyrosin sulfanilamit afinite kolonu kullanılarak 2093,9 EUmg^{-1} spesifik aktiviteyle ve 86,51% verimle yaklaşık 160 kat saflaştırıldı. SDS-poliakrilamid jel elektroforezinde tek band gözlendi ve buradan enzimin molekül kütlesi yaklaşık olarak 30,8 kDa olarak belirlendi. Bazı ağır metallerin (Ag^{+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}) CA enzimi aktivitesi üzerine etkileri hidrataz metodu kullanılarak *in vitro* olarak incelendi. Sonuç olarak, ağır metallerin inhibisyon etkileri Ag^{+}> Cu^{2+}> Pb^{2+}> Zn^{2+} > Cd^{2+}> Ni^{2+} şeklinde belirlendi. Bu sonuçlar Ag^{+}'nın en önemli potansiyel CA inhibitörü olduğunu gösterdi.

Anahtar Kelimeler

Karbonik anhidraz, solungaç, ağır metaller, inhibisyon

Article History: Received: Jul 25, 2014; Revised: Sep 27, 2014; Accepted: Oct 23, 2014; Available Online: Dec 27, 2014.

Correspondence to: Ş. Beydemir, Atatürk University, Faculty of Sciences, Department of Chemistry, Biochemistry Division, 25240, Erzurum, Turkey.

Tel: +90 442 2314388 Fax:+90 442 2360948 E-Mail: beydemir@atauni.edu.tr

INTRODUCTION

Heavy metals can be intensively accumulated in natural water systems and lead to contamination because of industrial, domestic and other man-made wastes. Accumulation of contaminants in the food chain in aquatic systems leads to side effects and death in the organism. Heavy metals arisen from these contaminations can have detrimental effects on the ecological balance and diversity of aquatic organisms. Fish are the most abundant living creatures and important food resources in aquatic systems. They are vulnerable to all environmental pollution especially by heavy metals [1-3]. They are mainly used aquatic organisms for the assessment of the health of aquatic systems. For that reason, investigations of chemical accumulation in sea organisms, especially determination of heavy metal content have great importance in order the to evaluate the impact and possible risk of fish consumption on human health [4,5,9].

Metals, such as iron, zinc, copper, manganese, chromium, molybdenum and selenium, are essential metals for the biological systems because in trace amounts, they play an important role in the metabolism. In fact, some of the enzymes in living organisms need one or more metal atoms like Fe^{2+}, Mg^{2+} and Mn^{2+} called as cofactors in their structures to show activity. However, these metals can also be toxic if certain amounts of them are exceeded. On the other hand, mercury, lead and cadmium are non-essential metals and even at very low doses of these metals have hazardous effects in the living organisms [10]. Heavy metals, due to certain environmental conditions, can build up of toxic concentrations and cause ecological damage [11, 12]. Integrity of the physiological and biochemical mechanisms of fish which are an important component of the ecosystem can be disturbed by these heavy metals [13,14].

It is reported that exposure of fish species to metal ions can increase the amount of reactive oxygen species (ROS) like hydrogen peroxide, superoxide, hydroxyl radical that are main causes of oxidative stress. Morever this situation can cause tissue damage and osmoregulatory disorders by inhibiting the activity of some important enzymes in the metabolism [15-20]. One of these enzymes in fish species is carbonic anhydrase (CA), responsible for the reversible reaction of convertion of CO_2 produced in fish tissues into bicarbonate. This vital enzyme is present in great quantities in gill epithelial cells, and assumed to take part in the functions of respiratory gas exchange, ion transport, and acid-base regulation [21].

Enzyme activities are regarded as biochemical indicators in the measurement of the presence of toxic substances in fish and they are also useful parameters in the study of harmful effects of toxicants [22,23]. Due to these facts, studies related about levels and effects of some heavy metals on fish species have recently been performed by many scientists in the literature and they have an increasing popularity in this field. Our group has performed many studies related about the purification of some crucial enzymes including carbonic ahydrase from human erythrocytes, different fish tissues, fish erythrocytes etc. Besides the effects of some drugs, heavy metals and compounds on the enzyme activities have been studied many times so far [24-26]. Consequently, in this study we purified and characterized carbonic anhydrase from the gill of corb fish (Umbrina cirrosa) in a single step by using Sepharose 4B L-tyrosine sulfanilamide affinity chromatography and investigated the effects of some heavy metals Ag^{+}, Cu^{+2}, Pb^{+2}, Zn^{+2}, Cd^{+2} and Ni^{+2} on the enzyme activity *in vitro*.

MATERIALS AND METHODS

Chemicals

$Ag(NO)_3$, $CuCl_2$, $Pb(NO_3)_2$, $Zn(NO_3)_2$, $Cd(NO_3)_2$, $Ni(Cl)_2$, Sepharose 4B, protein assay reagents, 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from Merck.

Sample homogenate preparation

Fish were obtained from commercial fish farm. Gill samples were removed from each fish. The gills were washed 3 times with 0.9% NaCl, an isotonic saline solution. The gill cells were lysed by immersion in liquid nitrogen. The lysed sample was then transferred to a buffer solution containing

25 mM Tris-HCl (pH 8.7) and centrifuged 100000 x g for 60 min at 4°C. The supernatant was centrifuged again and the second supernatant was used in the subsequent studies [27,28].

Purification of carbonic anhydrase from the gill of corb fish (Umbrina cirrosa) by affinity chromatography

The pH of the homogenate was adjusted to 8.7 using solid Tris. 25 ml aliquot of the supernatant was applied to an affinity column (1.36x30 cm) Sepharose-4B-L tyrosine-sulfanilamide affinity gel prepared according to the published method [29]. The purified enzyme was dialyzed for against 0.05 M Tris-SO_4 and 1 mM 2-mercaptoethanol (pH 7.4) for 24 hr. The protein concentrations in the column effluents were determined spectrophotometrically at 280 nm. All procedures were performed at 4°C.

Hydratase activity assay

Activity of the carbonic anhydrase enzyme was measured by following the hydration of CO_2 according to the method described by Wilbur and Anderson [30]. CO_2-hydratase activity was calculated as enzyme unit (EU) by using the equation $(t_0\text{-}t_c/t_c)$ where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

Protein determination

Quantitative protein analyses were performed according to the Bradford method spectrophotometrically at 595 nm using bovine serum albumin as the standard [31].

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Enzyme samples were applied to SDS polyacrylamide gel electrophoresis to verify purity of the enzymes. For this purpose, enzyme samples were carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure. They were transferred into the electrophoretic medium as 20 mg samples. Gels were stained for 1.5 hour in 0.1% Coommassie Brilliant Blue R-250 in 50% methanol, 40% distilled water and 10% acetic acid. Then, it was washed with the same solvent without the dye [32].

***In vitro* studies**

In vitro effects of the metal ions (Ag^{+}, Cu^{+2}, Pb^{+2}, Zn^{+2}, Cd^{+2} and Ni^{+2}) on carbonic anhydrase from the gill of corb fish were determined by adding different metal ions concentrations to the reaction medium. The enzyme activity measured in the absence of inhibitor was used as control (100% activity). The IC_{50} values of the metals were obtained from activity (%) vs. metal ion concentration plots.

RESULTS AND DISCUSSION

Carbonic anhydrase (CA) is one of the most important enzyme in fish and it is abundantly present in fish gill, a complex organ known to be involved in so many processes like respiratory gas exchange, ion transport, and acid–base regulation [33,34]. In the present study, carbonic anhydrase enzyme from corb fish gill purified by means of a chromatographical method called Sepharose 4B L-tyrosine sulfanilamide affinity chromatography, which is one of the most efficient method for the purificaiton of this enzyme. In a single step, enzyme purified about 160 fold with a specific activity of 2093.9 EUxmg^{-1} and a yield of 86,51 percent (Table 1) The enzyme purity and subunit molecular weight (30.8 kDa) were determined by SDS-PAGE electrophoresis method (Figure 1). Our results are in good agreement with others reported literature [27,28].

Table 1. Summary of purification procedure for corb gill carbonic anhydrase by Sepharose-4B-L-tyrosine sulfanilamide affinity column chromatography.

Purification steps	Activity (EU/mL)	Protein (mg/mL)	Volume (mL)	Total Activity (EU)	Total Protein (mg)	Specific Activity (EU/mg)	Purification Fold	Yield %
Homogenate	185.7	14.176	25	4642.5	354.4	13.1	1	100
Affinity Chromatography	502.01	0.2397	8	4016.1	1.918	2093.9	160	86.51

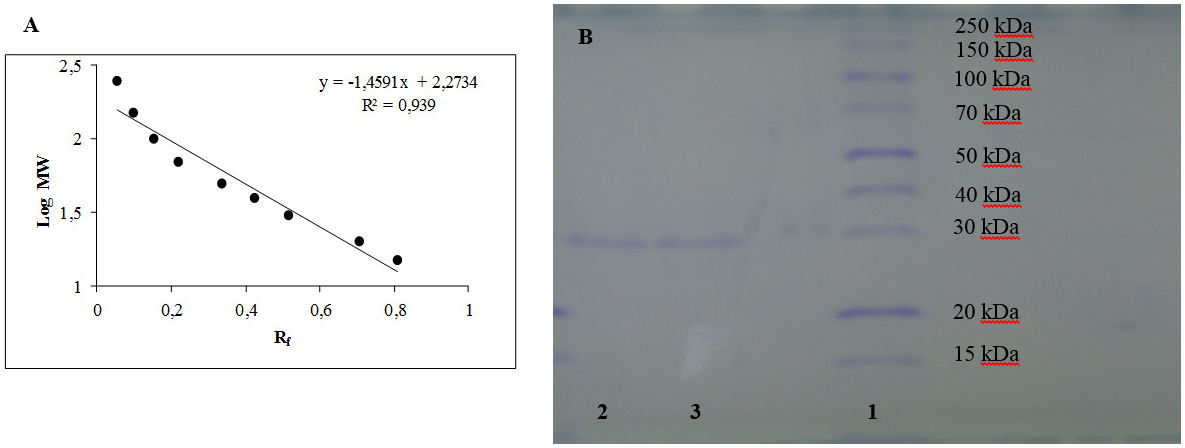

Figure 1. (A) Standard R_f–logM_W graph of carbonic anhydrase (CA) using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) results. The subunit molecular weight of corb gill CA was calculated as 30,8 kDa. (B) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified carbonic anhydrase. Lane 1: the molecular mass markers (kDa): Lane 2, 3: Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography results for gill carbonic anhydrase.

Although heavy metals in aquatic environments are often in trace amounts, they have been found in increasing concentrations in recent years because of the factors such as rapid population growth, industrialization, mining, agricultural activities, concentrated industrialization at sea coast, drainage water and wastewater of settlements [27]. Heavy metal increase in aquatic media causes accumulation of these metals in fish tissues. Besides, these metals in fish metabolism affect growth and development, various blood parameters and enzyme activities. Carbonic anhydrase is an important enzyme that catalyzes hydration of carbon dioxide and bicarbonate dehydration reaction reversibly. In this study, considering the effects of some toxic metals in fish metabolism, we examined the effects of several metals (Ag^+, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}) on the activity of purified carbonic anhydrase enzyme by using hydratase activity measurement method in *in vitro* conditions [30]. IC_{50} values (inhibitor concentration which reduces enzyme activity by half) calculated by plotting inhibitor concentration vs % activity plots (Figure 2). According to the results (Table 2) we got in the inhibition studies, we can see all of the metals inhibited purified CA enzyme *in vitro*. Two of these metals (Cd^{2+} and Ni^{2+}) had an IC_{50} value of milimolar levels that means Cd^{2+} and Ni^{2+} increase enzyme activity by half at milimolar concentrations. Cu^{2+}, Pb^{2+} and Zn^{2+} showed lower IC_{50} values than that of Cd^{2+} and Ni^{2+}, therefore, we can say that these 3 metals decrease CA enzyme functions at lower concentrations (micromolar levels). Among these 6 metals, Ag^+ was found to have the lowest IC_{50} value (8,04 μM). CA enzyme activity reduced by half in the presence of Ag^+ ion even at micromolar concentration of this metal. It is well known that, many enzymes inhibited by silver because of silver ions react with –SH groups of cysteine residues in the protein chain and this distrupts the structure of the enzyme.

Table 2. IC_{50} values obtained from regression graphs for corb gill CA in the presence of different metal ion concentrations.

Metal ions	IC_{50} [mM]
Ag^+	0.00804
Cu^{2+}	0.028
Pb^{2+}	0.056
Zn^{2+}	0.087
Cd^{2+}	1.486
Ni^{2+}	4.166

Rapid industrialization brings an increase in exposure to toxic heavy metals of organisms and human beings. Accumulation of heavy metals in the organism are taking place increasingly towards larger organisms in the food chain. When concentration of heavy metals increase in water, they accumulate in fish and ultimately in the human body. Increased metal concentrations known to affect some important enzymes activities

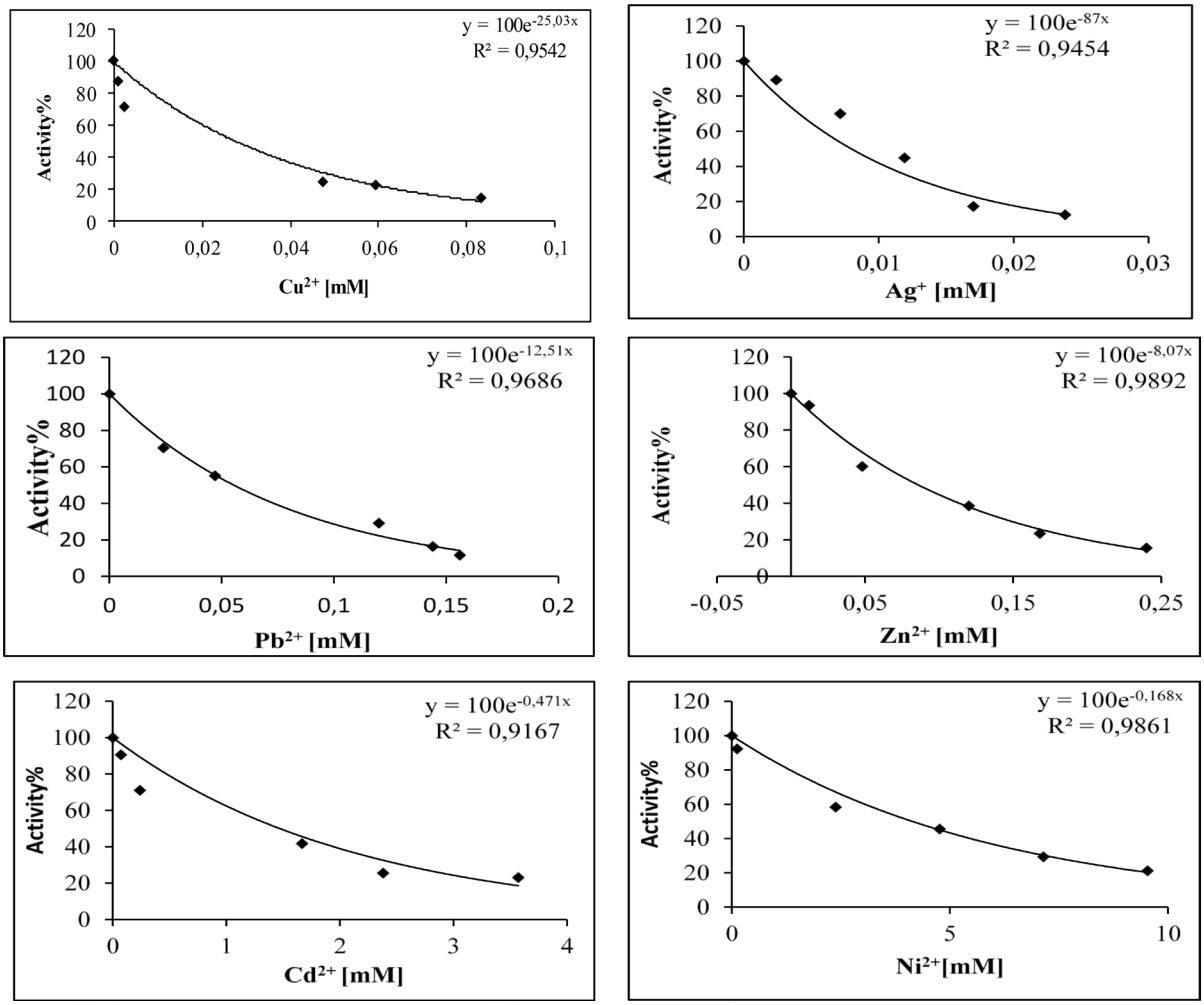

Figure 2. Activity %–[Metal] regression analysis graphs for corb gill CA in the presence of five different metal concentrations.

in metabolism. Since fish is an important food source and it is being consumed at a significant proportion in the Black Sea region, we examined inhibitiory effects of some metals on corb fish gill CA enzyme activity. Because of harmful effects of these metals on CA enzyme activity and directly to fish metabolism, water pollution by heavy metals in this area threaten the aquatic organisms and eventually human body.

References

1. A. Farkas, J. Salanki, A. Specziar, Relation between growth and the heavy metal concentration in organs of bream Abramis brama L. populating lake Balaton. Arch. Environ. Contam. Toxicol., 43 (2002) 236.
2. M.H.A. Yousuf, El-Shahawi. Trace metals in Lethrinus lentjan fish from Arabian Gulf: Metal accumulation in Kidney and Heart Tissues. Bull. Environ. Contam. Toxicol., 62 (1999) 293.
3. H. Soyut, S. Beydemir, The impact of heavy metals on the activity of carbonic anhydrase from rainbow trout (Oncorhynchus mykiss) kidney. Toxicol Ind Health 2011 DOI:10.1177/0748233711410914.
4. B.P. Cid, C. Boia, L. Pombo, E. Rebelo, Determination of trace metals in fish species of the Ria de Aveiro (Portugal) by electrothermal atomic absorption spectrometry. Food Chemistry. 75 (2001) 93.
5. D. Velez, R. Montoro, Arsenic speciation in manufactured seafood products: a review. J. food. Protect. 61 (1998) 1240.
6. H.B. Conacher, B.D. Page, J.J. Ryan, Industrial chemical contamination of foods [Review]. Food Addit. Contam. 10(1993) 129.
7. E.O. Farombi, O.A. Adelowo, Y.R. Ajimoko, Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African Cat fish (Clarias gariepinus) from Nigeria ogun river. Int. J. Environ. Res. Public Health., 4 (2007) 158.
8. M.Z. Vosyliene, A. Jankaite, Effect of heavy metal model mixture on rainbow trout biological parameters. Ekologija. 4 (2006) 12.

9. W. Ashraj, Accumulation of heavy metals in kidney and heart tissues of Epinephelus microdon fish from the Arabian Gulf. Environ. Monit. Assess. 101 (2005) 311.
10. E. Somer, Toxic potential of trace metals in foods. A review. Journal of Food Science. 39 (1974) 215.
11. K. Güven, C. Özbay, E. Ünlü, A. Satar, Acute lethal toxicity and accumulation of copper in Gammarus pulex (L.) (Amphipoda). Turkish Journal of Biology. 23 (1999) 510.
12. D.J. Jefferies, P. Freestone, Chemical analysis of some coarse fish from a Suffolk River carried out as part of the preparation for the first release of captive-bred otters. J. Otter Trust, 1(8) (1984) 17.
13. C. Hogstrand, E.A. Ferguson, F. Galvez, R. Shaw, N.A. Webb, C.M. Wood, Physiology of acute silver toxicity in the starry flounder (Platichthys stellatus) in seawater. J. Comp. Physiol. B. 169 (1999) 461.
14. P.S. Basha, A.U. Rani, Cadmium-induced antioxidant defense mechanism in freshwater teleost Oreochromis mossambicus (Tilapia). Ecotoxicol. Environ. Saf. 56 (2003) 218.
15. A.A.R. Radi, B. Matkovics, Effects of metal ions on the antioxidant enzyme activities, protein contents and lipid peroxidation of carp tissues. Comp. Biochem. Physiol. C 90 (1988) 69.
16. H., Roche, G.Boge, Effects of Cu, Zn and Cr salts on antioxidant enzyme activities in vitro of red blood cells of a marine fish Dicentrarchus labrax. Toxicol. in Vitro. 7 (1993) 623.
17. M., Canli, R.M. Stagg, The effects of in vivo exposure to cadmium, copper, and zinc on the activities of gill ATPases in the Norway lobster Nephrops norvegicus. Arch. Environ. Contam. Toxicol. 31 (1996) 491.
18. C.K.C. Wong, M.H. Wong, Morphological and biochemical changes in the gills of tilapia (Oreochromis mossambicus) to ambient cadmium exposure. Aquat. Toxicol. 48 (2000) 517.
19. M.G.L. Ribeiro, A.R. Pedrenho, Hasson-Voloch, A. Electrocyte (Na^+–K^+)-ATPase inhibition induced by zinc is reverted by dithiothreitol. The Int. J. Biochem. Cell Biol. 34 (2002) 516.
20. C. Dautremepuits, S. Paris-Palacios, S. Betoulle, G. Vernet, Modulation in hepatic and head kidney parameters of carp (Cyprinus carpio L.) induced by copper and chitosan. Comp. Biochem. Physiol. C, 137 (2004) 325.
21. K.M. Gilmour, S.F. Perry, Carbonic anhydrase and acid–base regulation in fish. The Journal of Experimental Biology 212 (2009) 1647.
22. F.M. El-Demerdash, E.I. Elagamy, Biological effects in Tilapia nilotica fish as indicators of pollution by cadmium and mercury. Int. J. Environ. Health Res. 9 (1999) 173.
23. Ş. Gül, E. Belge-Kurutaş, E. Yıldız, A. Şahan, F. Doran, Pollution correlated modifications of liver antioxidant systems and histopathology of fish (Cyprinidae) living in Seyhan Dam Lake, Turkey. Environ. Int. 30 (2004) 605.
24. D. Ekinci, S. Beydemir, Z. Alim, Some drugs inhibit in vitro hydratase and esterase activities of human carbonic anhydrase-I and II. Pharmacological Reports 59 (2007a) 580.
25. D. Ekinci, S. Beydemir, O.I. Kufrevioglu, In vitro inhibitory effects of some heavy metals on human erythrocyte carbonic anhydrases. Journal of Enzyme Inhibition and Medicinal Chemistry 22 (2007b) 745.
26. D. Ekinci, S. Beydemir, Risk assessment of pesticides and fungicides for acid–base regulation and salt transport in rainbow trout tissues. Pesticide Biochemistry and Physiology. 97 (2010) 66.
27. H. Soyut, S. Beydemir, O. Hisar, Effects of some metals on carbonic anhydrase from brains of rainbow trout. Biol. Trace Elem. Res., 123 (2008) 179.
28. H. Soyut, S. Beydemir, Purification and some kinetic properties of carbonic anhydrase from rainbow trout (Oncorhynchus mykiss) liver and metal inhibition. Protein Peptide Lett. 15 (2008) 528.
29. E.D. Kaya, H. Söyüt, S. Beydemir, Carbonic anhydrase activity from the gilthead sea bream (Sparus aurata) liver: the toxicological effects of heavy metals. Environ Toxicol Pharmacol. 36(2) (2013) 514.
30. K.M Wilbur, N.G. Anderson, Electrometric and colorimetric determination of carbonic anhydrase. J. Biol. Chem. 176 (1948) 147.
31. M.M. Bradford, A rapid and sensitive method for the quantition of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1976) 248.
32. D.K. Laemmli, Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227 (1970) 680.
33. S. Sender, K. Böttcher, Y. Cetin, G. Gros, Carbonic Anhydrase in the Gills of Seawaterand Freshwater-acclimated Flounders Platichthys flesus: Purification, Characterization, and Immunohistochemical Localization. The Journal of Histochemistry & Cytochemistry 47 (1999) 43.
34. S.F. Perry, P. Laurent, The role of carbonic anhydrase in carbon dioxide excretion, acid-base balance and ionic regulation in aquatic gill breathers. In Truchot J-P, Lahlou B, eds. Animal Nutrition and Transport Processes. 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects. 6 (1990) 39.