The correlation between some metal concentrations and carbonic anhydrase activity in Tuna (Thunnus Thynnus Linnaeus, 1758) Gill

Orkinos (Thunnus Thynnus Linnaeus, 1758) solungacında karbonik anhidraz enzim aktivitesi ve bazı metal derişimleri arasındaki ilişki

Research Article

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ABSTRACT

Today metal pollution is rapidly increasing with advances in technology and industry. Due to their bioaccumulative and nonbiodegradable properties, heavy metals are important contaminant of aquatic organism all over the world. Metal toxicity causes oxidative damage such as DNA damage, enhanced lipid peroxidation, the oxidation of protein sulfydryl groups and enzyme inactivation in the metabolism. In this study, we investigated *in vitro* effects of some metals on carbonic anhydrase enzyme which has vital role in fish metabolism. For this aim, The enzyme, was purified from gills of tuna with a specific activity of 1062 EU/mg proteins and 31% yield using Sepharose 4B-aniline-sulfanilamide affinity chromatography method. SDS-polyacrylamide gel electrophoresis showed a single band corresponding to a molecular weight of approximately 29 kDa. Inhibitory effects of metals (Ag^{+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Co^{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+}> Co^{2+}. From these results, we showed that Ag^{+} is the most potent inhibitor of CA enzyme.

Key words

Carbonic anhydrase, metal toxicity, inhibition

ÖZET

Bugün teknoloji ve sanayideki gelişmelerle birlikte metal kirliliği hızla artmaktadır. Ağır metaller biyobirikimleri ve doğada çözülmeyen özelliklerinden dolayı tüm dünyada sucul organizma için önemli kirliliktir. Metal toksisitesi, metabolizmada DNA hasarı, lipid peroksidasyonunun artması, protein sulfidril gruplarının oksidasyonu ve enzim inaktivasyonu gibi oksidatif hasara neden olur. Bu çalışmada, balık metabolizmasında hayati rol oynayan karbonik anhidraz enzimi üzerine bazı metallerin in vitro etkilerini inceledik. Bu amaçla enzim orkinos solungaçlarından Sepharose 4B-anilin-sulfanilamid afinite kromatografisi yöntemi kullanılarak 1062 EÜ/mg protein spesifik aktivite ve %31 verimle saflaştırıldı. SDS-PAGE jel elektroforezinde yaklaşık 29 kDa'da tek bant görüldü. İn vitro koşullar altında hidrataz yöntemi kullanılarak metallerin (Ag^{+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}) farklı derişimlerinde inhibisyon etkileri belirlendi. İn vitro inhibisyon derecesi Ag^{+}> Cu^{2+}> Pb^{2+}> Zn^{2+} > Cd^{2+}> Co^{2+} olarak belirlendi. Bu sonuçlardan Ag^{+}'nin güçlü bir CA enzim inhibitörü olduğu görüldü.

Anahtar Kelimeler
Karbonik anhidraz, metal toksisitesi, inhibisyon

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INTRODUCTION

Impact of metals to the environment is increasing problem worldwide. Metals are introduced in aquatic systems as a result of the weathering of soils and rocks, from volcanic eruptions, and from a variety of human activities involving the mining, processing, or use of metals and/or substances that contain metal pollutants. Metal toxicity can cause oxidative stress in almost all organisms including fish. This situation is also a potential risk factor for humans and other living feeding on contaminated fish [1]. It may be hazardous for living systems, including specific enzymes, directly or indirectly. Generaly, heavy metals affect the tertiary structure of enzymes by catalyzing protein destroying reactions or interfering with sulphur-sulphur cross-bridges [2]. It is well-known that enzymes catalyze almost all chemical reactions in the metabolism [3,4].

In this study, we aimed at evaluating the influences of some metals on carbonic anhydrase enzyme from gills of tuna. The carbonic anhydrases (EC 4.2.1.1., CAs) are zinc containing metalloenzyme family. CA enzyme, which exists commonly in living organisms, has various izoenzymes. Till now, 16 different CA isoenzymes have been identified in mammalian with differ in molecular weight, subcellular localization, tissue distribution, sensitivity to inhibitors [5,6]. These enzymes catalyze the reversible hydration of CO_2 and water to H^+ and HCO_3^- ion so in living organism they have vital roles different events such as physiological pH control and gas balance, calcification and photosynthesis [6,7]. In fish, carbonic anhydrase (CA), aplenty present in gill epithelial cells and it play role in respiratory gas exchange, protection of acid-base balance, ion transport, osmoregulation, nitrogen metabolism and removal of waste products [8,9]. The vertebrate gas exchange organ, the gills is essentially composed of a highly complex vasculature, surrounded by a high surface area epithelium that provides a thin barrier between a fish's blood and aquatic environment. Fish gills are the first point of contact between metals and impurities in water. Metals can bind to gill of fish and corrupt the ion-regulatory and respiratory functions of the gills [10].

Here, we purified CA enzyme from gills of tuna using Sepharose 4B–aniline–sulfanilamide affinity chromatography and examined the inhibitory effects of Ag^+, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} on CA activity were determined using the hydratase method under *in vitro* conditions.

MATERIALS AND METHODS

Materials

CNBr-activated Sepharose 4B, protein assay reagents, p-nitrophenylacetate and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). Para-aminobenzene sulfonamide and L-tyrosine were from E. Merck (Merck KGaA Frankfurter strasse 250, D-64293 Darmstadt, Germany). All other chemicals were of analytical grade and obtained from either Sigma-Aldrich or Merck.

Preparation of the homogenate

Gills samples were washed three times with 0.9% NaCl, an isotonic solution. And each of gills was homogenized by liquid nitrogen. Then, lysed sample was transferred to the buffer solution (25 mM Tris-HCl/0.1 M Na_2SO_4 [pH 8.7]) and centrifuged twice at 4°C, 20,000g for 30 min. Supernatant was used in further studies [11].

Purification of carbonic anhydrase from gills of tuna by affinity chromatograph

The enzyme was purified using Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography according to our published method [3]. The homogenate was applied to the prepared Sepharose 4B–aniline–sulfanylamide affinity column equilibrated with 25 mM Tris-HCl/0.1M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22mM Na_2SO_4 (pH 8.7). The tuna gill carbonic anhydrase was eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3). Purified CA enzyme was dialysed for 24 h against 0.05 M Tris-SO_4/1 mM 2-mercaptoethanol (pH 7.4). Protein concentrations in the column effluents were determined at 280 nm spectrophotometrically. All procedures were performed at 4 °C.

Hydratase activity determination

CA activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson (1948) [12]. CO_2-hydratase activity as an enzyme unit (EU) was calculated using the equation $(t_0\text{-}t_c/t_c)$, where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

Protein determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method [13] with bovine serum albumin being used as a standard.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The control of enzyme purity was carried out using Laemmli's procedure [14] in 3% and 8% acrylamide concentrations for running and stacking gel, respectively. SDS (10%) was added to the gel solution. The gel was stabilized in a solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. Staining was performed for about 2 h in a solution of 0.1% Coommassie Brilliant Blue R-250 + 50% methanol + 10% acetic acid. Finally, washing was carried out in a solution of 50% methanol + 10% acetic acid + 40% distilled water until the protein bands were cleared.

In vitro inhibition assays

The effects of increasing concentrations of Ag^+, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} on tuna gill CA activities were determined colorimetrically using CO_2-hydratase assay. The metals were also tested in the hydratase activity assay in triplicate at each concentration used. Different concentrations of metals were examined in preliminary assays. Enzyme activities were measured in the presence of different concentrations of Ag^+, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}. Control enzyme activity in the absence of a metal was taken as 100%. For each metal, an activity % vs. inhibitor concentration tube was drawn using conventional polynominal regression software (Microsoft Office 2007,Excel). Metal concentrations that produced 50% inhibition (IC_{50}) were calculated from graphs.

RESULTS AND DISCUSSIONS

In the developing world, heavy metal pollution is a significant environmental problem. Almost all living things are affected negatively from toxic substances, including heavy metals [15]. In small amounts of many metals are necessary to support life. It is well known that 99% mass of the living body consists from carbon, nitrogen, hydrogen and oxygen. Other elements including metals are 1% in the metabolism. Nevertheless, metals are crucial for vital activities. For example, many enzymes consist the metal ion in its active site, even in its structural site. For instance, carbonic anhydrase and sorbitol dehydrogenase include the Zn^{2+} in their active sites and the paraoxonase enzyme contains two Ca^{2+} ions in active site and structural. However, in larger amounts of the metals may become toxic. Heavy metals are present in the aquatic environment where they bio-accumulate along the food chain. Accumulation is toxic for fish and its high level can also be dangerous for other organisms, particularly humans through the food chain [2]. In general heavy metals produce their toxicity by forming complexes with organic compounds. For example metal complexes of sulfur, oxygen and nitrogen the most common groups. If the metals are bound to these groups, they may become inactive enzyme form. Because, metals bond with SH groups of the cysteine residues and thus, the mercaptans is formed. Enzymes are the bio-catalysts in nature which regulate the rate and direction of biochemical reactions. Inhibition of enzyme activities by toxic compounds such as metal, drugs, pesticides and gases may cause hazardous situation for living organisms [3, 16]. Therefore, toxicology studies about the effects of metals on enzyme activities are recently increasing.

The fish as most important aquatic food is an indicator organisms for the heavy metal pollution of their environment and they are a possible risk for human consumption [17]. For this reason, we investigated the effects of Ag^+, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} on gill carbonic anhydrase enzyme activity of tuna, which has very high economic value. Firstly, CA enzyme was purified from tuna

gills by using affinity chromatography with a specific activity of 1062 EUmg^{-1} and 31% yield and approximately 44,06 fold (Table 1). Sepharose 4B-aniline-sulfanilamide affinity chromatography method is more advantageous for purification carbonic anhydrase with high yield and short time. The enzyme purity and subunit molecular weight (29 kDa) were determined by SDS-PAGE electrophoresis method (Figure 2A). Up to now, CA enzyme has been purified from many different tissues and investigated effects of different drugs, metal ions and chemicals on the enzyme. These studies are very valuable in terms of CA's physiological importance in living organisms [4,18]. There are many toxicological studies about metal levels of tuna fish. Because, Tuna is considered as a predator that it can concentrate large quantities in heavy metals. For example, Voegborlo and colleagues determined the levels of mercury, cadmium and lead in samples of canned tuna fish [19]. In another study, levels of total mercury and methylmercury in the muscle tissue of albacore (Thunnus alalunga) and bluefin tuna (Thunnus thynnus) is investigated by Storelli et al. (2002) [20]. In our study, Ag^{+}, Cu^{2+}, Pb^{2+}, Zn^{2+} Cd^{2+}, Co^{2+} were chosen to investigate their inhibitory effects on tuna gill CA's hydratase activity. These metals showed inhibitory action against the enzyme. For each metal, IC_{50} parameters were determined by activity%-[Metal] graphs. Our results showed that Ag^{+} is the most potent inhibitor of CA enzyme. 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. Also, Cu^{2+}, Pb^{2+}, Zn^{2+} Cd^{2+} and Co^{2+} exhibeted inhibitory effects at low concentrations. The order of effects for all metals were determined as Ag^{+}>Cu^{2+}>Pb^{2+}>Zn^{2+}> Cd^{2+}>Co^{2+}. Our results are in good agreement with others reported literature. For example, Soyut et al. (2008) examined the inhibitory effects of cobalt, copper, zinc, silver and cadmium on carbonic anhydrase purified from brains of rainbow trout. They founded that cobalt, silver and cadmium inhibited the enzyme competitively, copper inhibited non-competitively, whereas zinc inhibited the enzyme uncompetitively [21]. In another study, Soyut and Beydemir examined the effects of some metals (cobalt, copper, zinc, silver) on the CA activity of rainbow trout liver. Their results showed that cobalt and zinc inhibited the enzyme in a competitive manner and copper and silver inhibited the enzyme in an uncompetitive manner [22].

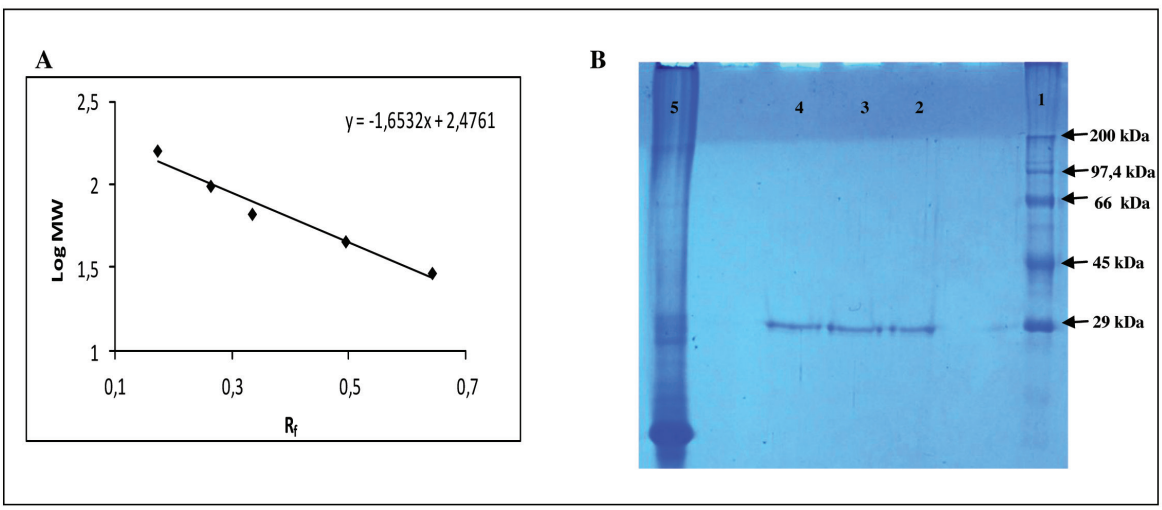

Figure 1. (A) Standard R_f –logMW graph of carbonic anhydrase (CA) using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) results. The subunit molecular weight of tuna gills CA was calculated as 29 kDa. (B)Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified carbonic anhydrase. Lane 1: the molecular mass markers (kDa): Escherichia coli β-galactosidase (116), rabbit phosphorylase B (97.4), bovine serum albumin (66), chicken ovalbumin (45), and bovine carbonic anhydrase (29) were used as standards (Sigma: Mw-SDS-200). Lane 2, 3, and 4: Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography results for gill carbonic anhydrase. Lane 5: Homogenate.

Consequently, today, the pollution levels with related metals are increasing in the marine environment and other parts of the world. This is the very big risk factor for all livings including fish and humans. We investigated in vitro inhibitory effects of metals on CA enzyme activity from tuna gills. The CA enzyme was purified from the tuna gills for the first time by using fast and simple method at one step. Carbonic anhydrase enzyme assay may be considered as a biomarker for the identification of pollution in marine environment.

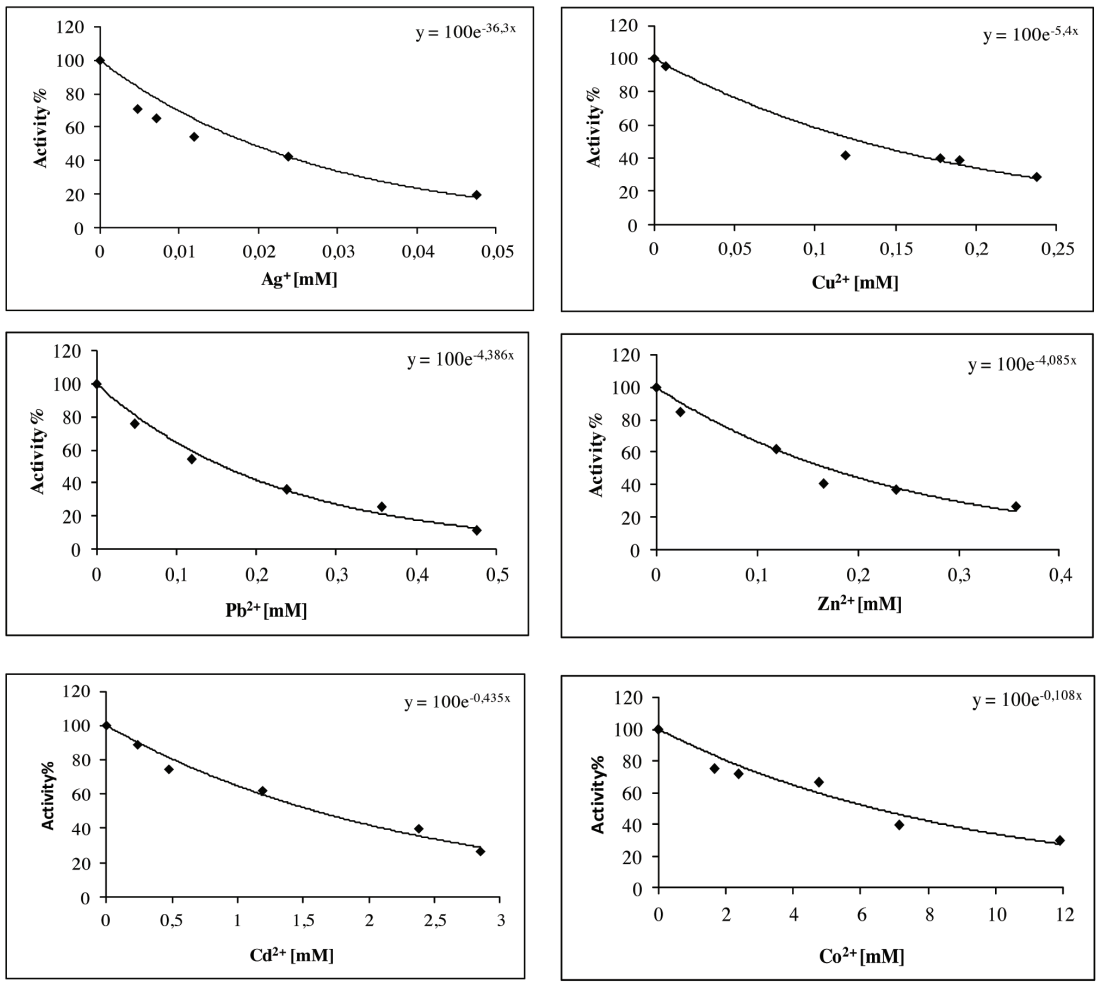

Figure 2. Activity %-[Metal] regression analysis graphs for fish gill CA in the presence of five different metal concentrations.

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

Purification steps	Activity (EU/ml)	Protein (mg/ml)	Volume (ml)	Total Activity (EU)	Total Protein (mg)	Specific Activity	Purification Fold	Yield%
Homogenate	165,57	6,866	25	413,25	171,65	24,1	1	100
Affinity Chromatography	214	0,215	6	1284	1,209	1062	44,06	31

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

Metal ions	IC_{50} [mM]
Ag^{+}	0,019
Cu^{2+}	0,12
Pb^{2+}	0,16
Zn^{2+}	0,17
Cd^{2+}	1,62
Co^{2+}	7,0

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