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The *In vitro* Inhibitory Effects of Some Disinfectants on Enzyme Activity of Carbonic Anhydrase from Rainbow Trout (Oncorhynchus Mykiss) Gills

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Abstract: Traditional treatments of parasitic and bacterial disease in aquaculture are based on chemotherapeutic compounds. Although, a lot of compounds are used on fish treatment, their undesirable effects are not known in detail. In this study, the effects of some disinfectants - malachite green, methylene blue, potassium permanganate, chloramine-T, copper sulphate and formalin - on Rainbow Trout (RT) gill Carbonic Anhydrase (CA) which plays a key role in gas exchange, acid-base balance, osmoregulation and ionoregulation were investigated *in vitro*. For this purpose, CA was purified from RT gills by using sepharose-4β-L tyrosine-sulfanylamide affinity gel chromatography method at initial. CA enzyme with 55.56 (EU/mg proteins) specific activity was purified with a yield of 40 % and 104.8-fold finally. I_{50} (inhibition) values of malachite green, methylene blue, potassium permanganate, chloramine-T and copper sulphate were determined as 0.05 mM, 0.023 mM, 0.15 mM, 0.32 mM and 5.39nM by means of activity % [disinfectant] graphs, respectively. In conclusion our data showed that all the disinfectants except formalin had the *in vitro* inhibitory effects on the rainbow trout gill CA enzyme whose inhibition could be hazardous to physiological functions such as osmoregulation and acid-base balance in fish.

Key words: Disinfectants, fish, inhibition, carbonic anhydrase

INTRODUCTION

Carbonic anhydrase (CA) (carbonate hydrolyase, EC 4.2.1.1) which has been found in virtually all animals as well as plants and algae is a member of zinc metalloenzymes and for which at least 14 different isozymes have so far been isolated in higher vertebrates [1]. It is reported that distribution pattern of CA activity among gills pairs varied depending on habitat and osmoregulatory behavior of the animal [2,3]. Freshwater trout gill epithelium consists mainly of pavement, mucous and chloride cells. The only pavement cell has an apical distribution of CA that parallels the proton ATPase activity. The CA activity decreases toward the baso-lateral membrane and is unavailable to the plasma[4] .

CA, an enzyme involved in the reversible hydration of CO_2 to produce H^+ and HCO_3^-, exhibits a fundamental role in a number of physiological processes such as physiological pH control and gas balance, calcification, photosynthesis, ion movements between cell and extracellular fluids, osmoregulation and clearance of the waste products from nitrogenous metabolism [1,5,6].

It is well known that many chemicals have adverse effects on the organism when used in therapeutic or other purposes [7]. The effects can be dramatic and systemic[8]. Antibiotics, related compounds and the application of other chemicals to water as disinfectants are effective for disease control in aquaculture.

Chemicals include benzalkonium chloride[9], copper sulfate [10,11], chloramine B and T [12-14], chlorin[15], formalin [16], iodophores [17], malachite green [18] and methylene blue [19]. These organic chemical therapeutants either directly added to the water or in fish feed are used for the treatment disease out-break and/or prophylactic [13,18,20]).

Malachite green has been a very popular drug among fish culturists for years, because of the broad fungicidal and anti-parasitical spectrum and its efficacy in treating trout suffering from Proliferative Kidney Disease (PKD). However, the administration of this compound is not allowed in the European Union and USA, nowadays. Because its potential carcinogenic, genotoxic, mutagenic and teratogenic properties were demonstrated in many animal species and cell line [20].

The morphological changes associated with repeated chloramine-T exposure may have resulted in an impediment to gas exchange since hyperventilation and crowding at the water surface were reported during repeated intermittent exposure to therapeutic concentrations of chloramine-T [14].

Sahul Hameed and Balasubramanian[21]) recommended disinfecting *Artemia* nauplii with formaldehyde before introduction into the rearing systems. However, some Gram-negative bacterial strains were known to be resistant to formaldehyde disinfection because of a plasmidic

resistance gene [22]. It was therefore important to check whether the formaldehyde treatment could cause resistance spread.

High doses of copper sulfate may be acutely toxic to fish, but copper compounds quickly precipitate from water as copper oxide and toxicity can be avoided if the dose does not exceed one hundredth of the total alkalinity concentration of the water to be treated [11]. Exposure to copper results in physiological changes in fish is similar to changes induced by other physical or chemical stressors [23].

Although, so many disinfectants have been used in freshwater aquaculture for the treatment of bacterial and parasitic diseases of gills, studies at the molecular level of the various disinfectants are unfortunately rare. Moreover it can be said that the biochemical alterations on CA activity created by chemical compounds on aquatic organisms may attractive as indicators because they offer a rapid and sensitive means of monitoring the impact of chemicals[24]. Therefore, in the present study, it was aimed to determine the effects of malachite green, methylene blue, potassium permanganate, chloramine-T, copper sulphate and formalin on the branchial CA enzyme activity of rainbow trout.

MATERIALS AND METHODS

Chemicals: Sepharose 4β, protein assay reagents 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 analytical grade and obtained from either Sigma-Aldrich or Merck. Disinfectants were provided from the Department of Aquaculture at Atatürk University.

Fish Husbandry and Maintenance: The rainbow trout obtained from the Fisheries Department of Agricultural Faculty at Atatürk University in Erzurum were a year old (mean wt 200±20g). Aerated dechlorinated tap water with a constant water flow of 1.5 l/min, 9±1oC average water temperature, 9 ppm dissolved oxygen, 7.8 pH and 102 mg as CaCO3 total hardness was used in the experiment. The fish were fed a commercial pellet diet with 49.4 % protein, 18.2 % fat, 94.3 % dry matter and 9.8 % ash at a daily ration of 1 % of their wet body weight during the study. Feed was given by hand. Fish treatment protocols were conducted according to Applied Research Ethics National Association (2002).

Obtaining gill samples and preparation of the homogenate: A total of 20 rainbow trout with an average weight of 200±20 g reared intensively in the research and extension center of Fishery department of Agricultural faculty at Atatürk University were used. Before taken gill samples, fish were killed by over dose of anesthetic compound (MS-222). The gill filaments from each individual separated from the whole branchial basket were immediately excised. Afterwards, the excised gill filaments were homogenized by liquid-nitrogen and put into homogenization medium[6] and then centrifuged at 4 ?C, 10,000×g for 45 min. Supernatant was used in further experiments. Tests were carried out at 4 ?C.

Sepharose-4β-L tyrosine-sulfanylamide affinity chromatography: The pH of the supernatant was adjusted to 8.7 with solid Tris. 75 mL of supernatant was applied to the prepared Sepharose 4β-L-tyrosine-sulfanylamide affinity column (1.36x30 cm) equilibrated with 25mM Tris-HCl / 0.1M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25mM Tris-HCl / 22mM Na_2SO_4 (pH 8.7). The RT gill CA was eluted using 0.1 M CH_3COONa/ 0.5 M $NaClO_4$ (pH 5.6) (flow rate: 20 mL h^{-1}, fraction volume: 4 mL). Purified CA was dialyzed for 72 hr against 0.01 M K_2HPO_4/ 0.1 M KCl/ 5mM 2-mercaptoethanol (pH 7.4). All procedures were performed at 4°C[25].

The absorbance of the protein in the column effluents was determined at 280 nm spectrophotometrically. CO_2-hydratase activities in the eluates were determined and the active fractions were collected.

Measurement of CA activity: CA activity was assayed colorimetrically using the method of Wilbur and Anderson[26]. The activity as an enzyme unit (EU) was calculated by using the equation $[(t_0-t_c)/t_c]$ where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

Protein determination: During the purification steps, protein levels were determined spectrophotometrically (595 nm) according to the Bradford method[27], using bovine serum albumin as the standard. Protein amounts in column fractions were observed via absorbance variations at 280 nm.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE): To control of enzyme purity, using Laemmli's procedure[28] was carried out in 3 and 10 % acrylamide concentrations

Table 1: Purification scheme of CA from RT gills.

Purification step	Activity (EU mL^{-1})	Total volume (mL)	Protein (mg/mL^{-1})	Total protein (mg)	Total activity(EU)	Specific activity (EU mg^{-1})	Yield (%)	Purification factor
Homogenate	50	75	93.8	7035	3750	0.53	100	1
Sepharose-4β-L tyrosine-sulfanylamide affinity chromatography	43.75	15	1.8	27	656.25	24.3	17.5	45.8
Dialyze	100	15	1.8	27	1500	55,56	40	104.8

Table 2: I_{50} values obtained from activity % vs [disinfectant] regression analysis graphs for RT gill CA for malachite green, methylene blue, potassium permanganate, chloramine-T, copper sulphate and formalin.

Disinfectants	malachite green(mM)	methylene blue(mM)	potassium permanganate(mM)	chloramine-T sulphate(mM)	copper (mM)	formalin
I_{50}	0.05	0.02	0.15	0.3	5.39	-

containing 10 % SDS for running and stacking gel, respectively. The gel was stabilized in the solution containing 50% propanol + 10% TCA + 40 % distilled water for 30 min. The staining was made for about 2 h in the solution of 0.1 % Coommassie Brillant Blue R-250 + 50 % methanol + 10 % acetic acid. Finally, the washing was carried out in the solution of 50 % methanol + 10 % acetic acid + 40 % distilled water until protein bands were cleared. The electrophoretic pattern was photographed (Fig.1).

***In vitro* Studies for Disinfectants:** In order to determine the effects of some disinfectants on CA, some concentrations of malachite green (0.03, 0.07, 0.08, 0.11 and 0.13 mM), Methylene blue (0.012, 0.025, 0.038, 0.05 and 0.063 mM), Potassium permanganate (0.07, 0.15, 0.225, 0.301 and 0376 mM), Chloramine-T (0.21, 0.42, 0.52, 0.63 and 0.73 mM), copper sulphate (2.9, 5.9, 8.9, 11.9 and 14.9 nM) and formalin (0.4, 0.79, 1.57, 2.36 and 3.93 mM) were added to separate tubes containing purified enzyme (with three replicates for each disinfectants). The enzyme activity was measured in these tubes taking the tubes containing no disinfectants used as control and assumed with 100 % activity. Regression analysis graphs were drawn by using mean inhibition percent values. The inhibitor concentrations causing up to 50 % inhibition (I_{50}) were determined from these graphs.

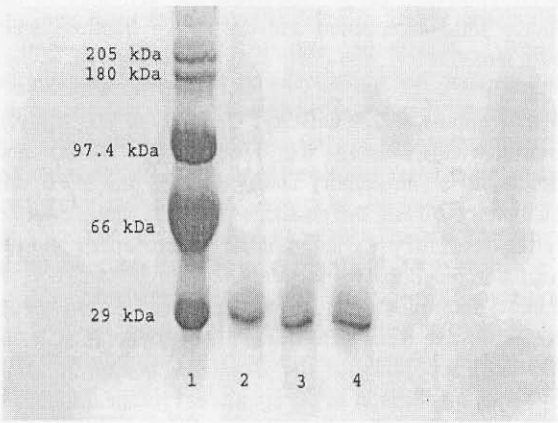

Fig. 1. SDS-polyacrylamide gel electrophoresis of CA purified by affinity gel. [(Lane 1: Standard proteins; (Rabbit muscle myosin 205,000 Da, á$_2$-Macroglobulin 180,000 Da, rabbit phosphorylase B 97,400 Da, bovine serum albumin 66,000 Da and bovine carbonic anhydrase 29,000 Da) Lanes 2: is RT erythrocyte CA; Lanes 3: is RT gill CA; Lanes 4: is RT lens CA)].

RESULTS

In the purification procedures, at the homogenate; total volume was 75 mL with 7035 mg total protein and a 0.53 EU/mg-protein specific activity. The yield was assumed as 100% at this stage. At Sepharose 4β-L-tyrosine-sulfanylamide affinity gel chromatography, total volume was 15 mL with 27 mg total protein, a 24.3 EU/mg^{-1}protein specific activity and 17.5 % yield. And finally at dialysis stage, total volume was 15 mL with 27 mg total protein, a 55.56 EU/mg^{-1} protein specific activity and 40 % yield. After homogenate preparation, Sepharose 4β-L-tyrosine-sulfanylamide affinity gel chromatography and dialyze, the enzyme was purified 104.8-fold (Table 1). In order to control the purity of enzyme, SDS polyacrylamide gel electrophoresis was used and its electrophoretic pattern showed a single band (Fig. 1).

All of the disinfectants except formalin inhibited the rainbow trout gills CA enzyme. I_{50} values used as inhibition indicators of malachite green, methylene blue, potassium permanganate, chloramine-T and copper sulphate were calculated as 0.05 mM and 0.023 mM 0.15 mM and 0.32 mM and 5.39 nM respectively (Fig. 2a-f).

From the I_{50} values it can be seen that copper sulphate was the strongest inhibitor and this followed by methylene blue, malachite green, potassium permanganate and chloramine-T (Table 2).

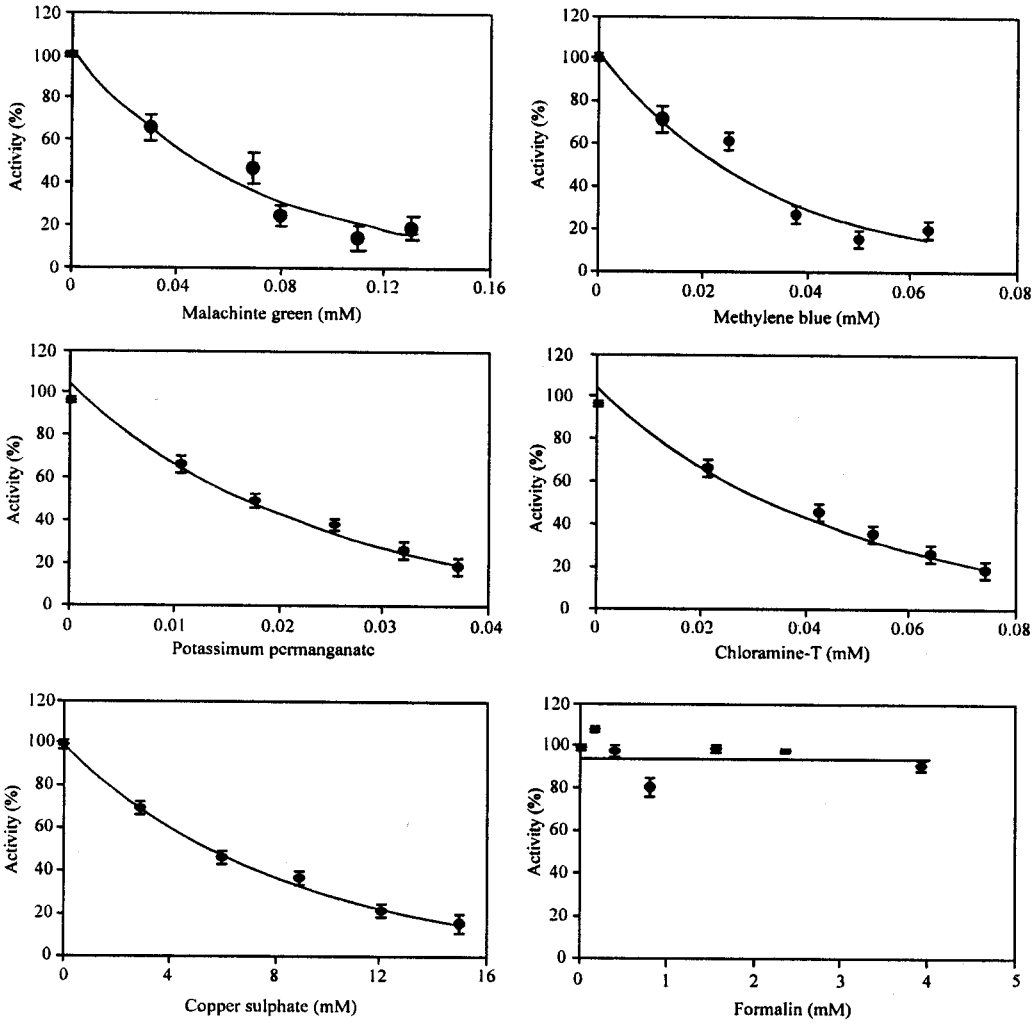

Fig. 2: Activity % *vs* [disinfectant] regression analysis graphs for rainbow trout gills CA enzymes in the presence of malachite green (mM) (a), methylene blue (mM) (b) potassium permanganate (mM) (c) chloramine-T (mM) (d), copper sulphate (nM) (e) and formalin (mM) (f) for five different concentrations.

DISCUSSION

CA was purified from many species of animals, plants, yeasts and bacteria[1,29]. In fish, the enzyme was purified by using different chromatography methods[30]. However, these methods such as sephadex G-75 gel filtration and DEAE Bio Gel anion exchange chromatography take a long time to produce results of determining enzyme activity. Therefore, sepharose 4β-L tyrosine-sulfanylamide affinity chromatography method which takes less time and obtains more purification-fold than the other methods[25] was used in this study.

Purification experiment with the method of sepharose 4β-L-tyrosine-sulfanylamide affinity chromatography showed that after purification a single with high specific activity and approximately a 29,000 Da molecular weight CA isozyme was present in erythrocytes of rainbow trout. This result was similar to the findings (28,300 Da) reported by Hall and Schraer[30].

Many chemicals at relatively low dosages affect the metabolism of biota by altering normal enzyme activity [7]. With some of these interactions there is a high reactivity involving a high degree of inhibition of a specific enzyme that accounts for the effect on the whole animal or plant[8].

The present *in vitro* experiment showed that all disinfectants except formalin inhibited the rainbow trout gill CA enzyme at different degrees either by binding to the functional group of CA or bounded to different part of

the enzyme (a site that out of the active side of enzyme). In some other studies, it was also reported that inhibitors showed their effects on CA enzyme activity by binding to the functional group or displacing the metal associated with the enzyme [31-33].

From the result of the effects of the inhibition of CA enzyme activity, it can be argued that important physiological changes may occur in the body of the organisms as reported in the following studies. It was reported that CO_2 and NH_3 excretion were linked by the action of CA in trout white muscle[34] It has also been reported that respiratory acidosis in the blood of trout occurred by the inhibition of red cell CA activity[35]. Because, inhibition of red cell CA activity caused a large increase in both, arterial and muscle P-CO_2 indicating retention of CO_2[36,37]. Moreover it was reported that creatine phosphate resynthesis was delayed by CA inhibition in exercised white muscle of trout[38].

The inhibition of ion-transporting enzyme being involved in osmoregulatory mechanisms in the fish gill may also cause physiological disorders i.e. osmotic imbalance occurred in fish. Especially, as the function of CA is considered to be the transport of H^+ and CO_2 into the surrounding medium, this enzyme activity is associated with the changes of blood pH and PO_2. Therefore these tested disinfectants except formalin in presents study might interfere with a number of physiological functions in which gill CA is involved as gas exchanges, acid-base balance, osmoregulation and clearance of the waste products from nitrogenous metabolism.

In vitro studies have also shown that copper sulphate has strongest inhibition effect but formalin has no effect on gill CA activity in the used disinfectants. From these findings; the use of malachite green, methylene blue, potassium permanganate, chloramine-T and especially copper sulphate as a disinfectant may cause serious side effects. For that reason these disinfectants must be carefully used and their dosages should be very well ordered to decrease the side-effects. In addition, because of that formalin is not any effect on this enzyme; it appears to be a good candidate as a disinfectant in salmonid rearing systems. But, effects of this therapeutants are necessary to investigate on the other enzymes in body metabolism.

CONCLUSION

It was determined that some disinfectants had the *in vitro* inhibitory effects on the activity of the CA enzyme which carried out key physiological roles. In addition,because of the lack of toxicological data on CA in freshwater fish, the sensitivity to disinfectants of this enzyme reported here could be the starting point for further studies.

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