Research Article

Changes in carbonic anhydrase activity and gene expression of *Hsp70* in rainbow trout (*Oncorhynchus mykiss*) muscle after exposure to some metals

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Abstract: The effects of some heavy metals Co(II), Cu(II), Zn(II), and Ag(I) on carbonic anhydrase (CA) activity from rainbow trout (RT) muscle were investigated. Moreover, the effects of these metals on the expression of heat shock protein 70 (*Hsp70*) gene in muscle tissue were examined by real-time quantitative PCR (RT-PCR) in muscle tissue after exposure to the metals at the end of 6, 12, 24, and 48 h. CA was purified with a specific activity of 2300 EU mg^{-1}, a yield of 19%, and 1080-fold. The molecular weights (Mw) of subunit and native enzyme were approximately 30 and 31 kDa, respectively. Optimum pH, stable pH, optimum temperature, activation energy (E_a), activation enthalpy (ΔH), and Q_{10} (the difference in activity of enzyme caused by the increment of 10 °C) value were determined. Apparent Michaelis constant (K_m), maximum reaction rate (V_{max}), and turnover rate of the enzyme (K_{cat}) values were 1.29 mM, 0.17 µmol min^{-1}, and 28.8 s^{-1}, respectively. The catalytic efficiency (K_{cat}/K_m) was 22.3. The heavy metals decreased in vitro CA activity. Inhibition mechanisms of the metal ions were noncompetitive, except for the Co(II) ion, which was competitive. The expression of the *Hsp70* gene was increased in the presence of the metal ions. The expression level at the end of 48 h was the highest for all of the metals. Consequently, the in vitro inhibition rank order was determined as Co(II) > Zn(II) > Cu(II) > Ag(I). Interestingly, Ag(I) was the most effective metal ion on *Hsp70* gene expression.

Key words: Carbonic anhydrase, characterization, muscle, rainbow trout, heavy metals, heat shock protein 70

Introduction

The zinc-containing enzyme, carbonic anhydrase (CA) (carbonate hydrolyase, EC 4.2.1.1) is a ubiquitous enzyme in the metabolism. A total of 16 α-CA isozymes were determined from different portions of mammal cells (1). CA exists in most tissues such as muscle, salivary glands, brain, pancreas, prostate gland, uterus, and endometrium tissue. Furthermore, the enzyme is found in the gills and secretion organs of fish (2,3). The enzyme has similar molecular characteristics in the plants and

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the animal kingdom (4). In many tissues, CA enzyme has high specific activities. For instance, CAs from gills, muscle, heart, male gonads, hypodermis, and the digestive gland of marine crabs have been found to have high specific activities (5,6). CA catalyzes the reversible hydration of CO_2 to HCO_3^- and H^+ in its common physiological role (7,8).

CA has many physiological functions such as respiration, ionic transport, acid-base regulation, and calcification (5). In general, CA is involved in respiratory gas exchange in vertebrate muscle. Esbaugh and Tufts (9) reported that the first function of the enzyme is to facilitate the transport of CO_2 into the capillaries by hydrating CO_2 at the capillary wall, where its second function is to help equilibrate the post-capillary pH. In addition, CA enzyme is also found on the extracellular surface of the sarcolemma and sarcoplasmic reticulum in the vertebrate muscle (10). Aside from this, the enzyme has esterase activity for p-nitrophenyl acetate substrate under in vitro conditions (11). This is a nonphysiological reaction for the enzyme. Studies on CA enzyme have been conducted for a long time. The enzyme has been purified from various sources using different techniques (12-15).

Heat shock proteins (Hsps) are a protein family defined as stress proteins. This protein family is generally argued to be responsible for many forms of biotic and abiotic stressors in fish (16). Some protein groups such as metallothioneins, which are expressed in response to heavy metal exposure, cytochrome P450 enzymes, and Hsps, can be considered as stress proteins (17). The expression of Hsps has been described from many sources such as in cell lines, primary cultures of various cells, and in the tissues of whole organisms. Studies have demonstrated that environmental contaminants, including heavy metals, affect the expression of Hsps biotic and abiotic stressors in fish cells and other tissues (17). Thus, *Hsp70* plays an important role in cytoprotection. It also has a significant role in repairing protein damage from stress (18). The main causes of environmental pollution have arisen from industrial activities with a growing population around the world. Consequently, environmental pollution is an important risk, as well as a terrorist threat, for all living things. Specifically, this environmental threat

damages the natural habitat of animals and the balance of nature (19). This is particularly the case in aquatic environments. CA also plays a critical role on the respiration and transformation mechanism of CO_2 to HCO_3^- in all living things. It is known that there is a close relationship between CA enzyme activity and oxygen consumption (20). Various stress factors such as environmental conditions affect the relationship between CA enzyme activity and oxygen consumption. Any reduction in the rate of oxygen is a vital stress factor on fish. Similarly, metal inhibition on CA activity results in a reduction in the rate of oxygen consumption and increases stress, and it also causes an increase in *Hsp70* gene expression. Hence, the expression of the *Hsp70* gene has a unique role in fish metabolism.

Inhibitions of the enzyme are vital for the metabolisms of all living things. Almost all drugs and other chemicals, including heavy metals, display their functions on enzyme interaction mechanism. Moreover, heavy metals are thought to be some of the strongest naturally occurring CA inhibitors. For instance, it is known that Ag^+ inhibits both bronchial CA and ion transport in freshwater fish (21). In addition, Söğüt and Beydemir et al. reported on the inhibition effects at low dosages of Co(II), Cu(II), Zn(II), and Ag(I) on rainbow trout (RT) liver CA activity (11). Moreover, our laboratory has studied the interactions between chemicals and different enzymes (22-27).

Consequently, we examined the in vitro inhibitory effects of Co(II), Cu(II), Zn(II), and Ag(I) on RT muscle CA activities and investigated some of its important kinetic properties. For this purpose, the enzyme was purified using only one step from the RT muscle. Furthermore, the effects of the metals on the expression of *Hsp70* in muscle tissue extracted from RT were investigated by real-time quantitative PCR (RT-PCR).

Materials and methods

Materials

Cyanogen bromide-activated Sepharose 4B, protein assay reagents, 4-nitrophenyl acetate, and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (Sigma-Aldrich, Taufkirchen, Germany). Para-aminobenzene sulfonamide and

L-tyrosine were from Merck (Merck, Darmstadt, Germany). TRIzol reagent for total RNA isolation and ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis were purchased Invitrogen Co. (Invitrogen, Darmstadt, Germany). A FastStart TaqMan Probe Master using real-time applications was purchased from Applied Biosystems. All other chemicals were analytical grade and obtained from either Sigma-Aldrich or Merck.

Fish husbandry and maintenance

Obtaining and feeding of the fish were performed as in our previous study (19).

Homogenate preparation and affinity chromatography

Fish were anaesthetized in water containing tricaine methanesulfonate (MS-222, 1/10,000) (28). Muscle homogenate preparation was carried out according to our previous study (19).

Esterase activity assay

Esterase activity measurements were performed according to our previous studies. In this method, the enzyme activity was assayed by following the change in absorbance at 348 nm of p-nitrophenyl acetate to p-nitrophenolate ion over a period of 3 min at 25 °C (29).

Protein determination

Quantitative protein during the purification steps was determined at 595 nm spectrophotometrically. Bovine serum albumin protein was used as the standard in this method (30).

Optimum pH determination

The effects of pH on the enzyme were determined in 1 M Tris-SO$_4$ buffer ranging from pH 7.0 to 9.5 and 1 M phosphate buffer ranging from pH 5.0 to 8.0 (13).

Stable pH determination

Stable pH experiments were performed using 1.0 M Tris-SO$_4$ (pH 7.0-9.5) and 1.0 M phosphate buffer (pH 5.0-7.5) (13). Enzyme activities were assayed at 6-h intervals during incubation, over 3 days, using p-nitrophenyl acetate as a substrate under optimum conditions.

Optimum temperature, E_a, ΔH, and Q_{10} determination

Optimum temperature, activation energy (E_a), activation enthalpy (ΔH), and Q_{10} values (the difference in activity of enzyme caused by the increment of 10 °C) were determined at different temperatures, ranging from 0 to 60 °C. The experiments were performed according to our previous studies (19,31).

Molecular weight (Mw) determination

The determination of the apparent molecular weight (Mw) of the subunit and purity of the enzyme was determined by Laemmli's procedure. This method was carried out in 4% and 10% acrylamide concentrations for stacking and running gel, respectively (32). The relative migration distance (R_f) values of the standard proteins were calculated and a standard graph (Log Mw vs. R_f) was created, as in our previous studies (33). The molecular weight of the active enzyme was also determined by Sephadex G-200 gel filtration chromatography. The void volume was determined with blue dextran (2000 kDa). To prepare the standard graph, the partition coefficient (K_{av}) values of the standard proteins were calculated, and then a standard graph (Log Mw vs. K_{av}) was drawn. The Mw of the CA enzyme was extrapolated from this graph using its elution volume under identical chromatographic conditions (34).

Kinetic studies

The K_m, V_{max} and turnover rate of the enzyme (K_{cat}) values were calculated from a Lineweaver-Burk (35) plot, at room temperature and optimum pH, in 3 mL reaction mixtures as described above. The experiment was performed at 5 different cuvette concentrations of p-nitrophenyl acetate (0.1, 0.2, 0.3, 0.4, and 0.5 mM). K_{cat} was calculated from the equation V_{max}/E_t, where E_t is total enzyme (11,36).

Toxicological effects and in vitro inhibition studies

The in vitro effects of Co(II), Cu(II), Zn(II), and Ag(I) (Merck) on enzyme activity were investigated. The inhibition experiments were performed in triplicate for each concentration used. Activity assays were carried out in the following cuvette concentrations: CoCl$_2$ × 6H$_2$O (1×10^{-3}, 1×10^{-2}, 1×10^{-1}, 1.0, 10, 20, and 30 mM); CuSO$_4$ × 5H$_2$O (1.0, 10, 20, 40, and 60 mM); ZnSO$_4$ × 7H$_2$O (1×10^{-3}, 1×10^{-2}, 1×10^{-1}, 1, 10, and 20 mM), and AgNO$_3$ (80, 90, 100, 150, 200, and 250 mM). Control enzyme activity in the absence of an inhibitor was taken as 100%. For each

inhibitor, percent (%) activity vs. concentration of the inhibitor graph was plotted. Metal concentrations that produced 50% inhibition (IC_{50}) were calculated from graphs.

For the K_i values (inhibition constant), 3 different inhibitor concentrations were used; $CoCl_2 \times 6H_2O$: 0.01, 0.05, and 0.1 mM; $CuSO_4 \times 5H_2O$: 20, 40, and 60 mM; $ZnSO_4 \times 7H_2O$: 1, 10, and 20 mM; and $AgNO_3$: 150, 200, and 250 mM. As a substrate, p-nitrophenyl acetate was used at 5 different concentrations (0.60, 0.675, 0.75, 0.875, and 0.90 mM for each metal solution). Determination of K_i and the inhibitor type was carried out from Lineweaver-Burk curves.

In vivo studies

The samples were provided from the RT farm of the Department of Aquaculture, Ataturk University. Used in this study were mature, healthy, 1-year-old live fish (n = 48), with an average weight of 110-150 g. Prior to the experiment, the fish in each group were kept in 1×1.2 m (wide-deep) fiber-glass tanks for 1 month. Aeration was provided throughout the experiments. The average water temperature was 10 ± 2 °C. The water quality parameters were measured as O_2 = 8.6 ppm, pH = 7.7, SO_4^{-2} = 0.33 mg/L, PO_4^{-3} = trace, NO_3^- = 3.45 mg/L, NO_2^- = trace, and conductivity = 230 uS/cm.

Throughout the experiments, 4 fiber-glass tanks, each of which contained 15 fish in 600 cm^3 water, were used. One tank was used as the control, and did not contain any heavy metal, while the others each had 1 of the following added: 10.000 mg $CoCl_2.6H_2O$/L, 1.000 mg $ZnSO_4.7H_2O$/L, 100 mg $CuSO_4.5H_2O$/L, and 10 mg $AgNO_3$/L. The muscle samples were collected after the heavy metal treatment from each fish group at 6, 12, 24, and 48 h.

Ribonucleic acid (RNA) isolation and complementary DNA (cDNA) synthesis

Total ribonucleic acid (RNA) was isolated from frozen muscle tissues of the treated heavy metal and control RT using TRIzol reagent. RNA concentrations and quality were verified using a spectrophotometer (OD at 260 nm) and RNA gel electrophoresis, respectively. After isolation, complementary DNA (cDNA) synthesis was performed using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. All of the cDNA was stored at −20 °C.

Real-time PCR (RT-PCR)

Quantification of gene expression by real-time PCR (RT-PCR) analysis was performed using a thermal cycler Stratagene MxPro3005. The primers and TaqMan probe were designed in Primer3 software (v. 0.4.0) (http://frodo.wi.mit.edu/) using RT *Hsp70* (GenBank accession number AB062281) and BLASTed to ensure correct messenger RNA (mRNA) sequences. *β-actin* primers and probe were taken from the report by Johansen and Overturf (GenBank accession number AF254414) (37). For each target gene, 2 primers and an internal, fluorescent labeled TaqMan probe [5'-end, reporter dye FAM (6-carboxyflourescein), 3'-end, quencher dye TAMRA (6-carboxytetramethylr-hodamine)] were used. The sequences of the primers and probes can be found in Table 1.

The PCR experiment was carried out in a reaction volume of 50 μL, containing template DNA, 900 nM of forward and reverse primer, 250 nM TaqMan probe, and 25 μL FastStart TaqMan Probe Master (Applied Biosystems), which consisted of AmpliTaq Gold DNA Polymerase, AmpErase uracil N-glycosylase

Table 1. Sequence of primers and probes used for RT-PCR.

Primers/probe	Sequence 5'-3'
Hsp70 forward	TCAAGAGGAAACACAAGAAGGA
Hsp70 reverse	TGGTGATGGAGGTGTAGAAGTC
Hsp70 TaqMan	FAM-TCAGCCAGAACAAGCGGGCT-TAMRA
β-actin forward	TGGCCGTACCACCGGTAT
β-actin reverse	GCAGAGCGTAGTCCTCGTAGATG
β-actin TaqMan	FAM-CTCCGGTGACGGCGTGACCC-TAMRA

(UNG), dNTP with dUTP, and optimized buffer component. Amplification and detection of the samples and the standards were performed using the following thermal cycling conditions: 50 °C for 2 min for activation of optical AmpErase UNG enzyme; 95 °C for 10 min as a hot start to activate AmpliTaq Gold DNA polymerase, followed by 45 cycles of denaturation at 95 °C for 15 s; and annealing and extension at 60 °C for 1 min. RT-PCR data were analyzed using the efficiency (e)$^{(-\Delta\Delta Ct)}$ method (e: amplification efficiency) and equation

$$\left[\text{Ratio} = \frac{(E_{t\,arg\,et})^{\Delta C_{T},t\,arg\,et\,(control-sample)}}{(E_{ref})^{\Delta C_{T},ref\,(control-sample)}}\right]$$

(38) which was used to determine mean fold changes in the gene expression against the control group and housekeeping gene *β-actin*. Analytical sensitivity was confirmed by running standard curves. Amplification efficiency (e) was calculated based on the slopes of the curves using the formula e = 10 $^{(-1/slope)}$ (e: amplification efficiency, s: slope of standard curve) (38), and the slope value via Stratagene MxPro3000 software.

Results

We purified the enzyme from RT muscle with a simple, 1-step method. The enzyme was obtained with a specific activity of 2300 U/mg proteins and muscle, approximately 1080-fold, with a yield of 19% (Table 2).

The purity and subunit Mw of the enzyme were determined as 30 kDa using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method. The SDS-PAGE photograph was presented in a previous study (Figure 1A). Active form Mw of the enzyme was obtained as 31 kDa by Sephadex G-200 gel filtration chromatography (Figure 1B)

Both optimum pH and stable pH were found as 9.0 in 1 M Tris-SO$_4$ for p-nitrophenyl acetate substrate (Table 3). Optimum temperature, E_α of the enzymatic reaction, ΔH, and Q_{10} values were obtained as 40 °C, 6.36 kcal/mol, 5.77 kcal/mol, and 1.56, respectively (Table 3).

Kinetic constants, K_m, V_{max}, and K_{cat} of the enzyme for hydrolysis of p-nitrophenyl acetate were determined as 1.29 mM, 0.17 µmol min^{-1}, and 28.8 s^{-1} based on Lineweaver-Burk plots (Table 3). The catalytic efficiency (K_{cat}/K_m) was 22.3.

IC_{50} values for CoCl$_2$ × 6H$_2$O, CuSO$_4$ × 5H$_2$O, ZnSO$_4$ × 7H$_2$O, and AgNO$_3$ were calculated from Activity % – (Heavy metal) graphs in vitro conditions. K_i values and inhibition types of the metals were determined from Lineweaver-Burk graphs and are given in Table 4.

Figure 2 shows that metal inhibition on the CA activity caused an increase of the *Hsp70* gene expression, because inhibition of CA enzyme is an important stress factor in terms of oxygen consumption for all living things, including fish. As shown in Figure 2 the expression level at 48 h was the highest for all of the metals.

Discussion

In this study, we purified the enzyme from RT muscle using only 1 step, with approximately 1080-fold (Table 2). The purification procedure is relatively fast and

Table 2. Summary of purification procedure of CA from RT liver.

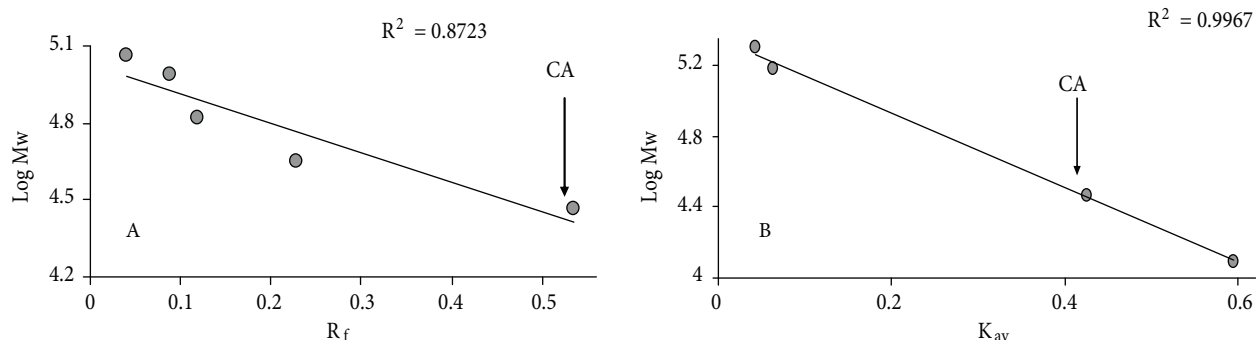| Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Lysate	30	100	14.05	1405	3000	2.13	100	1
Sepharose-4B-L tyrosine-sulfanilamide affinity chromatography and dialysis	190	3	0.083	0.25	570	2300	19	~1080

Figure 1. (A) Standard R_f vs. log Mw graph of carbonic anhydrase using SDS-PAGE [standards: *E. coli* b-galactosidase (116 kDa), rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), and bovine carbonic anhydrase (29 kDa)]. (B) Standard K_{av} vs. log Mw graph of carbonic anhydrase using Sephadex G 200 chromatography [standards: Horse heart cytochrome C (12.4 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), yeast alcohol dehydrogenase (150 kDa), and sweet potato b-amylase (200 kDa)].

takes only 4 to 5 h. A comparison of our purification procedure to that of others revealed that we have certain advantages in favor of our method, including the fact that ours both takes less time and has higher specific activity, yield, and purification fold.

The purity and molecular weight of the muscle CA enzyme were determined using the SDS-PAGE method and the Mw was calculated as 30 kDa from LogMw vs. R_f graphs (Figure 1A). The obtained Mw results were similar to CAs purified from many other tissues. For instance, subunit Mw of the CA enzyme from green alga *Chlamydomonas reinhardii* was determined as 27 kDa by Bundy and Coté (39). In a previous study, Soyut et al. found the Mw of the enzyme from RT brain to be 29.0 kDa (19). Our results

were similar to many other sources such as *Salmo gairdneri* erythrocyte, flounder gills, *Pisum sativum*, and *Plasmodium falciparum*. The Mw of the native CA was approximately 31 kDa via LogMw vs. K_{av} graphs (Figure 1B) by gel filtration chromatography in this study. The Mw results for the active enzyme were similar to other CAs from different sources (11,19). Contrary to all of the information given above, the active Mw of the enzyme was different in some tissues. For example, Bundy and Coté determined the active Mw of the green alga *Chlamydomonas reinhardii* as 165 kDa. They also calculated subunit Mw as 27 kDa and reported that the structure of the CA enzyme was hexameric (39). It is known that the active form of plant CA enzyme generally has several monomers as tetrameric, hexameric, etc. Almost all animal and

Table 3. Kinetic properties of CA from RT liver.

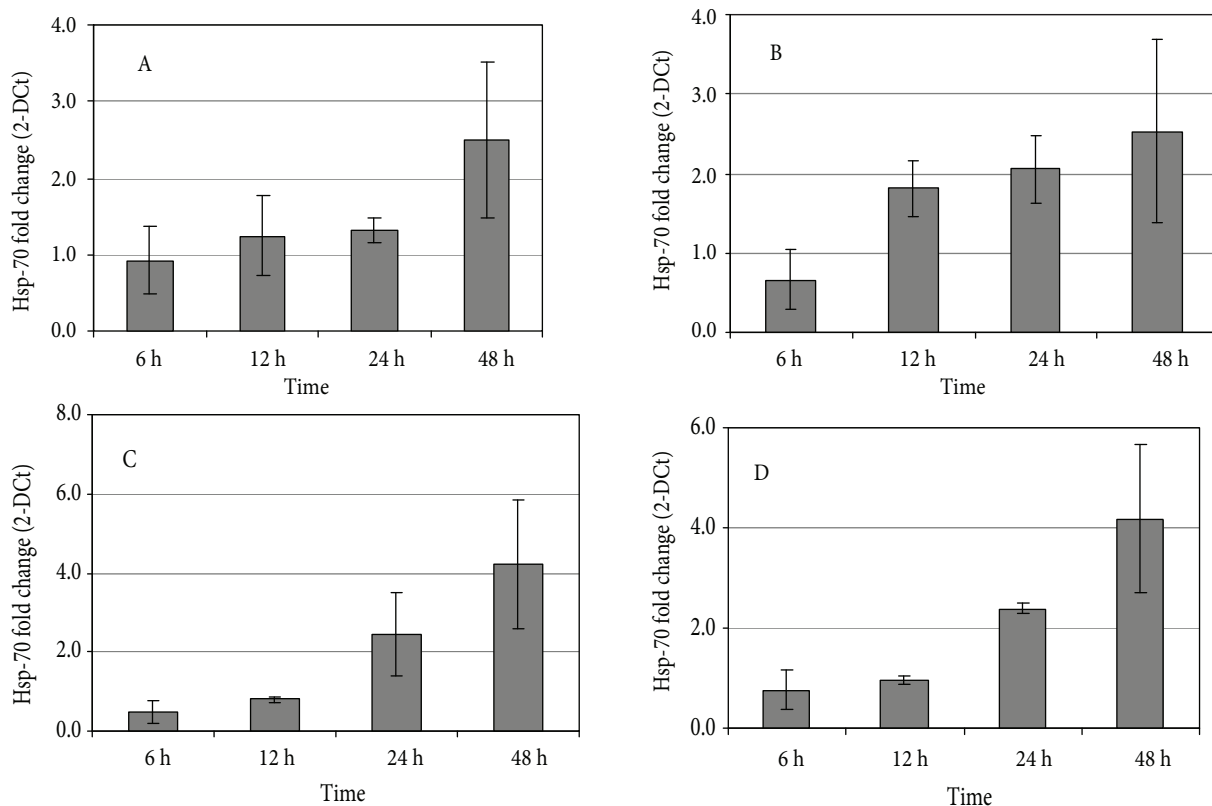| Substrate	P-nitrophenyl acetate
Optimum pH	9.00
Stable pH	9.00
Optimum temperature (°C)	40.0
E_α (kcal/mol)	6.36
ΔH (kcal/mol))	5.77
Q_{10}	1.56
K_m (mM)	1.29
V_{max} (µmol min^{-1})	0.17
K_{cat} (s^{-1})	28.8

Figure 2. Relative mRNA levels of *Hsp70* genes in muscle of *Oncorhynchus mykiss* after exposure to (A) Ag$^+$, (B) Co^{2+}, (C) Zn^{2+}, and (D) Cu^{2+} was evaluated by RT-PCR, *β-actin* gene was used as control. The histogram represents the mean fold change relative to the control ± SE of the experiment.

human CA enzymes are monomeric. As a result, we found close values for both subunit and native Mws and we suggest the enzyme to be a monomer in the active form.

The optimum pH result (Table 3) was similar to our previous liver and brain CA results, but the stable pH was slightly different (11,19). The optimum temperature, E_α of the enzymatic reaction, ΔH, and Q_{10} results (Table 3) were similar to those of RT liver and brain CAs obtained in our previous study. While the E_α of liver CA enzyme was 2.88 kcal/mol, it was 6.36 kcal/mol for muscle. Moreover, the E_α of muscle and brain CAs were similar. Since we have not found any information about the E_α, (ΔH) and Q_{10} values for carbonic anhydrase in the literature, we think that these physicochemical results on the enzyme can be important. Characterizations of various enzymes are generally investigated with regard to some kinetic parameters and some genetic properties, but the physicochemical behaviors of the enzyme

have almost been ignored. However, interestingly, 3-dimensional structures of the enzymes may change under some conditions such as temperature, pH, and even laboratory conditions.

The apparent K_m, V_{max}, and K_{cat} values were determined for p-nitrophenyl acetate (Table 3). Compared to the RT liver and brain CAs in our previous studies, muscle CA revealed lower catalytic efficiency (K_{cat}/K_m = 22.3). The K_m values have a rank order of muscle > brain > liver (11,19). This result strongly suggests that p-nitrophenyl acetate is the best substrate for liver CA in esterase activity.

In recent years, toxicology studies have gained more popularity than others because they reveal important information about the living standards of humans and other organisms like animals and plants. Almost all reactions of living metabolisms are catalyzed by enzyme systems. It is well known that these systems are greatly affected by external factors such as drugs and other chemicals. Specifically,

heavy metals from various sources such as factories and other technological requirements have various toxicological effects on humans, animals, and plant metabolisms. Hence, many studies have been conducted on this subject (14,40-42). For instance, it has been reported that the binding of heavy metals to membrane transport ligands can alter their catalytic function (5,43). Additionally, our previous study showed that cobalt, copper, zinc, and silver inhibited CA activity from RT liver at low concentrations (11). Similar results were obtained for Co^{2+}, Cu^{2+}, Zn^{2+}, Ag^+, and Cd^{2+} on trout brain CA (19). In the present study, we investigated the in vitro effects of Co(II), Cu(II), Zn(II), and Ag(I), on the activity of RT muscle CA. Table 4 compares the IC_{50} and K_i values of RT liver, brain, and muscle for heavy metal concentration.

As seen in Table 4, while Ag^+ is a potential inhibitor for liver CA enzyme, Co^{2+} also has a potential inhibitory effect on both brain and muscle CA enzyme. It is clear that inhibitory potentials on the CA enzyme activity of RT muscle have the following sequence: $Co^{2+} > Zn^{2+} > Cu^{2+} > Ag^+$ (Table 4).

In addition to all of this information, some chemicals such as pesticides, drugs, organochlorines, metals, and many chemical wastes from different origins lead to stress-related tissue damage. Heavy metals affect not only different types of enzyme activity but also various gene expressions, including *Hsp70*. Hsps are well known as biotic and abiotic stressors in fish cells and other tissues and they play an important role in cytoprotection. It also has a significant part in repairing protein damage from stress (18). Thus, expression of the gene has a unique role in fish metabolism. It is known that denaturing conditions such as exposure of cells to hypoxia, ethanol, heavy metals, and sodium arsenite also induce synthesis of Hsps (44). In addition, Nath et al. (45) and Schilsky et al. (46) reported that heavy metal exposure impairs the protein synthetic machinery. Therefore, we decided to determine the effects of metals on the expression of gene encoding for heat shock protein (*Hsp70*) by using RT-PCR after exposure to metals for 6, 12, 24, and 48 h. Quantitative mRNA levels increased in all of the heavy metal treatment groups. These increases were parallel to time elevation, but were not statistically significant (P > 0.05). We also determined that the efficiency rate was 96.4%, which was equal to the 1.96 calculated via the conversion formula used to calculate the fold-chance value. Similarly, Feng et al. (47) found that $CuSO_4$ treatment (0, 25, 50, 100, and 200 mM) on RT resulted in a dose-dependent elevation in *Hsp70* expression at 24 and 48 h post-exposure. Another experiment showed that heavy

Table 4. Comparison of heavy metal concentrations that inhibited in vitro 50% of carbonic anhydrase activity (IC_{50}) and K_i values for different RT tissues.

RT tissues	Metals	IC_{50} (mM)	Average K_i (mM)	Inhibition type	Reference
Liver	Ag^+	0.01	2.19	Uncompetitive	Soyut and Beydemir, 2008
	Co^{2+}	0.032	0.05	Competitive	
	Cu^{2+}	30.0	1.95	Uncompetitive	
	Zn^{2+}	47.1	7.035	Competitive	
Brain	Co^{2+}	0.05	1.4×10^{-2}	Competitive	Soyut et al., 2008
	Zn^{2+}	0.31	2.15	Uncompetitive	
	Cu^{2+}	30.0	27.6	Noncompetitive	
	Ag^+	159	193.8	Competitive	
	Cd^{2+}	82.5	82.5	Competitive	
Muscle	Co^{2+}	0.099	0.065	Competitive	Present study
	Zn^{2+}	5.330	4.790	Noncompetitive	
	Cu^{2+}	29.90	34.15	Noncompetitive	
	Ag^+	157.4	211.0	Noncompetitive	

metal exposure resulted in higher *Hsp70* expression in fish tissues, including hepatocytes (17,48). The results of another study clearly demonstrated an elevated *Hsp70* accumulation of trout hepatocytes (3- to 6-fold) after exposure of trout hepatocytes to $CuSO_4$, $CdCl_2$, or $NaAsO_2$ (49). Our results were similar to the literature. In our study, $AgNO_3$ was the most effective heavy metal on *Hsp70* gene expression when heavy metals were evaluated in terms of treatment dose concentration. The results showed that heavy metals increased *Hsp70* gene expression in a rank order of $AgNO_3 > CuSO_4 > ZnSO_4 > CoCl_2$ (Figure 2).

Consequently, we concluded that many environmental contaminants including heavy metals can affect the activities of various enzymes, both in protein form and mRNA form. For this purpose, the hydrolyase enzyme CA was purified from RT muscle by a simple 1-step procedure. Several kinetic parameters and enzyme behaviors in some solutions, as well as temperature, were investigated. Furthermore, the IC_{50} and K_i values were calculated for Co(II), Zn(II), Cu(II) Ag(I), and $CoCl_2$, which were regarded as a potential inhibitors among all of the metal solutions used. We also investigated the expression of *Hsp70* stress gene after treatment of metal ions to RT muscle. We determined increased *Hsp70* gene expression via metal application and, in particular, Ag(I) was the most effective metal in the expression. In light of these results, further studies are planned.

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