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NOTE

Identification and intracellular localization of carbonic anhydrase I in gills, heart, muscle, and intestine of rainbow trout, *Oncorhynchus mykiss*

Kang Hee Kho[1] · Jung Woo Kim[2] · Soo Cheol Kim[3] · Myeong Rak Choi[3] · Kyeong Ho Han[1] · Won Kyo Lee[1] · Kap Seong Choi[4]

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Abstract Carbonic anhydrases (CAs) play a key role in physiological functions such as pH homeostasis, calcification, photosynthesis, and ionic regulations. Among CA isozymes, CA I is a specific marker for the cytoplasm or apical cell membranes of colonic epithelial cells, and it is related to enterocyte proliferation in mammals. The major aim of the present study was to investigate the regional, cellular, and subcellular localization of CA I in a fish, rainbow trout, *Oncorhynchus mykiss*. The CA I was identified in the gills, heart, and intestine of the rainbow trout. CA I protein detected in the tissues of the rainbow trout was similar to a CA III with a molecular weight of 29 kDa. Immunohistochemical results demonstrated that CA I was localized in the various cells of gills, heart, muscle, and intestine of the rainbow trout.

Keywords Carbonic anhydrase I · Gill · Heart · Intestine · Muscle · Rainbow trout

✉ Kap Seong Choi
chks@sunchon.ac.kr

[1] Department of Aquatic Biology, Chonnam National University, Yeosu 550-749, Republic of Korea

[2] Department of Anatomy, College of Medicine, Seonam University, Namwon 590-711, Republic of Korea

[3] Department of Biomedical and Electronic Engineering, Chonnam National University, Yeosu 550-749, Republic of Korea

[4] Department of Food Science and Technology, Sunchon National University, Suncheon 540-950, Republic of Korea

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc-containing metalloenzyme that catalyzes the reversible hydration/dehydration reactions of carbon dioxide/carbonic acid (Henry 1996). CA catalysis is a fundamental reaction in various physiological processes, suggesting that CA may have been among the earliest enzymes to appear (Tashian 1989). This has driven researchers to diversify CA isoforms, and their catalyses have implications for the regulation of various physiological processes, such as pH regulation, calcification, photosynthesis, respiration, ionic and fluid balance, metabolism, and cell growth (Lionetto et al. 2000).

CAs are divided into several distinct classes (α, β, γ, δ, and ε), and mammalian CAs belong to the α-class. The α-class has been studied to the greatest extent, but recent studies indicate the rapid advancement of knowledge of other CA classes (Fukuzawa et al. 2000; Kozliak et al. 2000; Zimmerman and Ferry 2008; Rowlett 2010). Sixteen CA genes have been identified in humans, with isozymes distributed between the cytoplasm (CA I, II, III, VII, and XIII), cell membrane (CA IV, IX, XII, and XIV), mitochondria (CA Va, and CA Vb), and the extracellular space (CA VI) (Kuo et al. 2003; Aspatwar et al. 2010). CA isozymes are involved in a number of important physiological functions, such as acid–base balance, urine formation, detoxification of metabolic waste products, cell adhesion, and modulation of neuronal transmission in mammals (Supuran and Scozzafava 2007). However, unlike in mammals, there is a dearth of information about CA isozymes in fishes (Kawk et al. 2011).

In contrast to other CA isozymes, CA I is a low-activity cytoplasmic enzyme (Tashian 1989). CA I is a specific marker for the cytoplasm or apical cell membranes of colonic epithelial cells (Lonnerholm and Wistrand 1983;

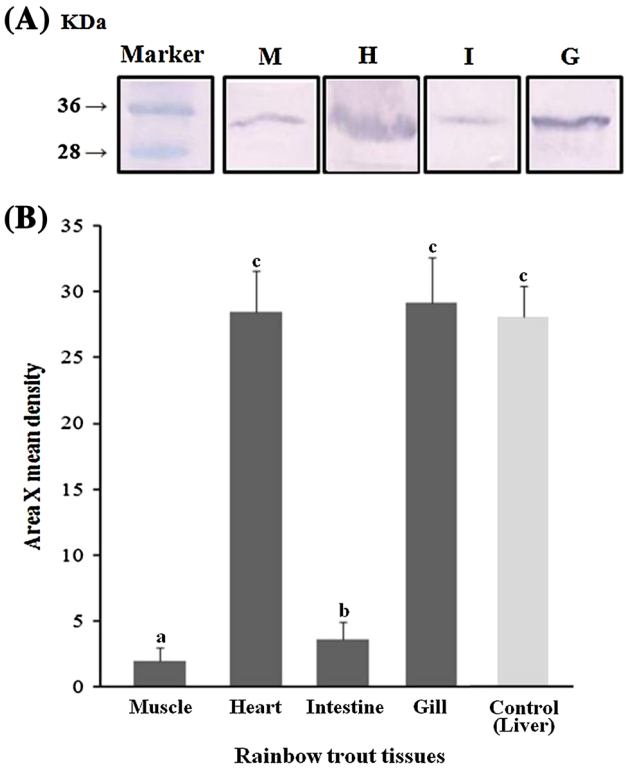

Fig. 1 Presence of carbonic anhydrase I in the tissues of rainbow trout. (**A**) SDS-PAGE and western blot analyses of cytosolic proteins from rainbow trout tissues. (**B**) Densitometric data. *Vertical bars* are the concentrations represented as the relative mean area of the band densities. Data are reported as the mean ± SD of three independent experiments. *Superscript lowercase letters* indicate significant differences at $p < 0.05$ ($a < b < c$)

Swenson 1991) and is related to enterocyte proliferation (Renes et al. 2002). CA I is also involved in electroneutral NaCl reabsorption and short-chain fatty acid uptake (Swenson 1991). However, no studies have described the distribution or intracellular localization of CA I in the tissues of the rainbow trout (*Oncorhynchus mykiss*). In the present study, we aimed to assess the distribution and localization of CA I in various tissues of the rainbow trout, which has been extensively used as an experimental model for environmental toxicology research.

Materials and methods

Materials

Rainbow trout specimens were provided by a fish farm in Korea. A polyclonal antiserum against CA I from human erythrocytes was raised in a rabbit (Accurate Chemical & Scientific Corporation, USA).

Sample preparations

Briefly, tissue samples (gill, heart, and intestine) were individually collected and suspended in 0.5 g/10 mM Tris buffer (pH 7.2), homogenized in a glass grinder, and centrifuged at 5000×*g* for 3 min at 4 °C (×3). The supernatants were centrifuged at 45,000×*g* for 90 min at 4 °C, and cytosolic proteins were prepared as described previously (Kho and Choi 2005; Kim et al. 2013).

Western blotting

Protein samples were appropriately diluted to obtain 1 mg/mL and were loaded on 12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, and the separated proteins were transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5 % skimmed milk in Tris-buffered saline (TBS) (10 mM Tris–

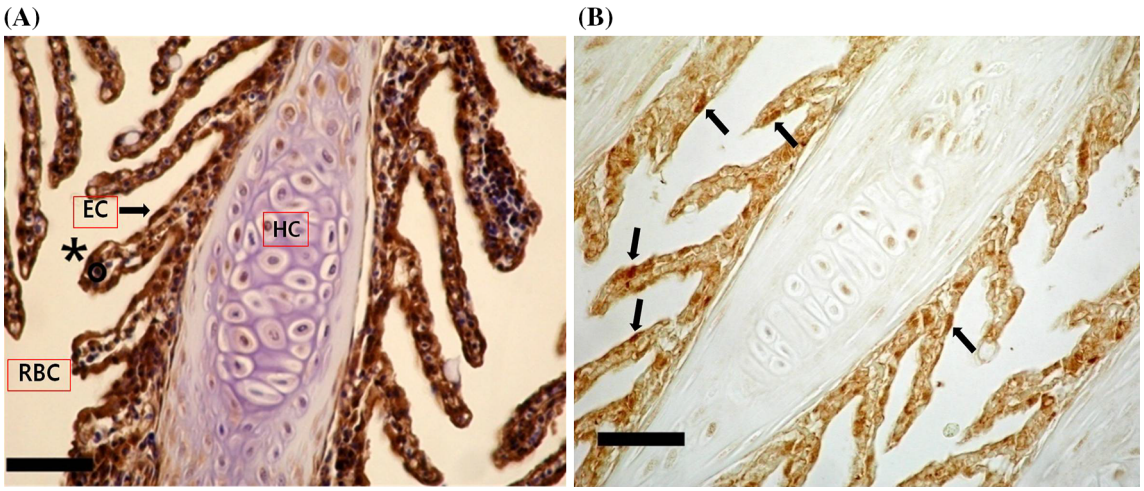

Fig. 2 Immunohistochemical staining for CA I in the gill. The *brown dots* indicate immunopositive reactions. *Arrows* demonstrate strong reactivity in epithelial cells. Mark (*asterisk*) represents nuclei. (**A**) Immunostaining and hematoxylin counterstaining of the tissue. *HC* hyaline cartilage, *EC* epithelial cell, *RBC* red blood cell. (**B**) Immunostained tissue. *Scale bar* = 100 um

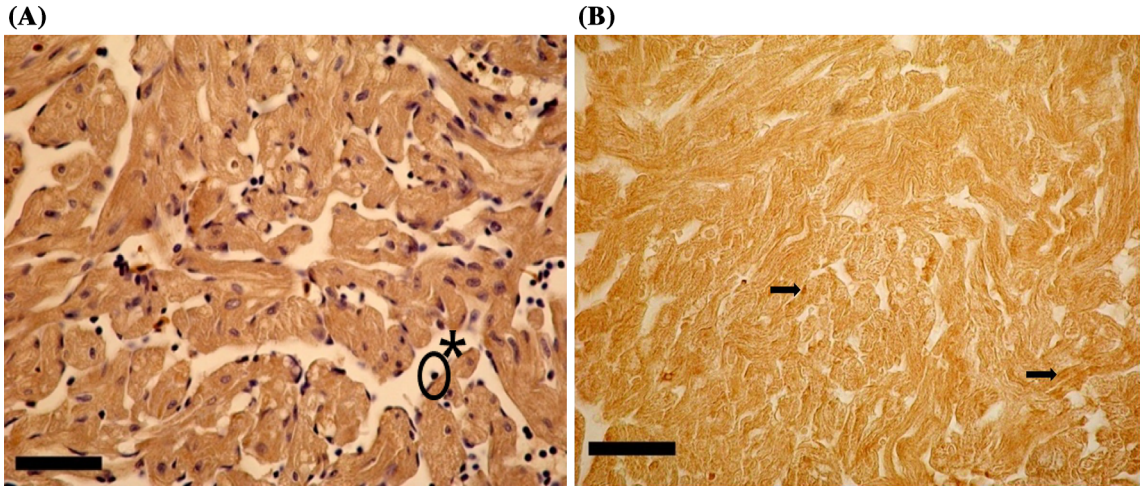

Fig. 3 Immunohistochemical staining for CA I in the heart. The *brown dots* indicate immunopositive reactions. (**A**) Immunostaining and hematoxylin counterstaining of the tissue. Mark (*asterisk*) represents nuclei. (**B**) Immunostained tissue. *Arrow* represents cardiac muscle. *Scale bar* = 100 μm

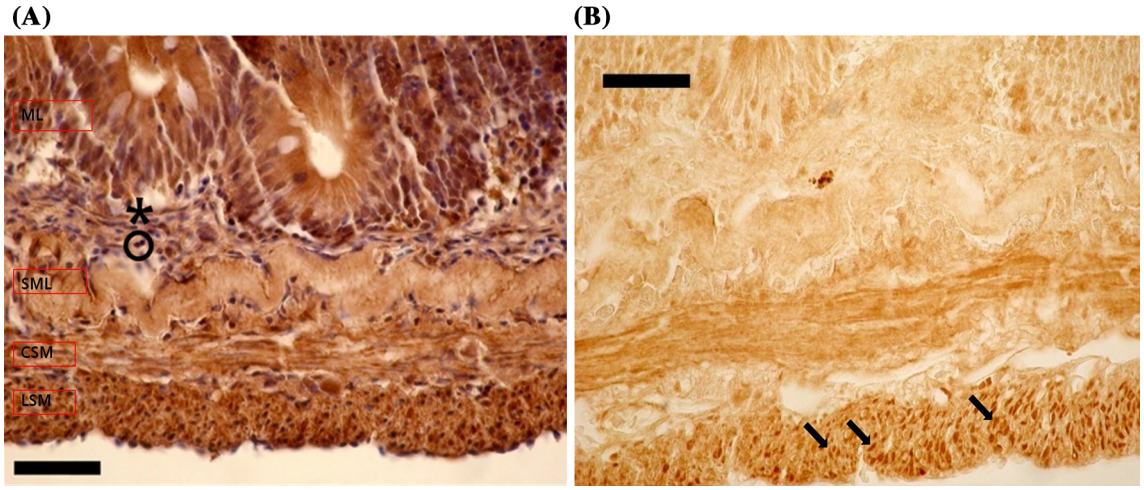

Fig. 4 Immunohistochemical staining for CA I in the intestine. The *brown dots* indicate immunopositive reactions. (**A**) Immunostaining and hematoxylin counterstaining of the tissue. *CSM* circular smooth muscle, *ML* mucous layer, *SML* submucous layer, *LSM* longitudinal smooth muscle. Mark (*asterisk*) represents the nuclei of plasma cells. (**B**) Immunostained tissue. *Arrows* represent the nuclei of LSM. *Scale bar* = 100 um

HCl (pH 7.6) containing 0.15 M NaCl) and immersed overnight in CA I antisera (1:1000). After washing with TBS containing Tween-20, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit antisera (1:5000) for 2 h at room temperature. Bands were visualized with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. A band obtained using western blotting was quantified using scanning densitometry and the Image J program (http://rsb.info.nih.gov/ij).

Immunohistochemical staining

Immunohistochemical staining was performed using the avidin–biotin complex method. Tissue slices were deparaffinized and hydrated with xylene and an ethanol series. To block endogenous peroxidase activity, 3 % H_2O_2 was applied on the tissue slices for 20 min. To avoid nonspecific antigen–antibody reactions, the tissue slices were treated with normal serum solution (10 % normal goat serum, 1 % bovine serum albumin, and 0.1 % Triton X-100 in phosphate-buffered saline (PBS)) for 1 h at room temperature and then incubated for 12 h at 4 °C with primary antibodies diluted (1:1000) in PBS. The tissue slices were then incubated with biotinylated secondary antibodies for 1 h at room temperature and visualized with streptavidin peroxidase reagent (ThemoShandon, USA) and diaminobenzidine (Sigma, USA) overnight at 4 °C. Between each process, the tissue slices were rinsed with PBS (pH 7.4) three times for 5 min. After immunolabeling, the

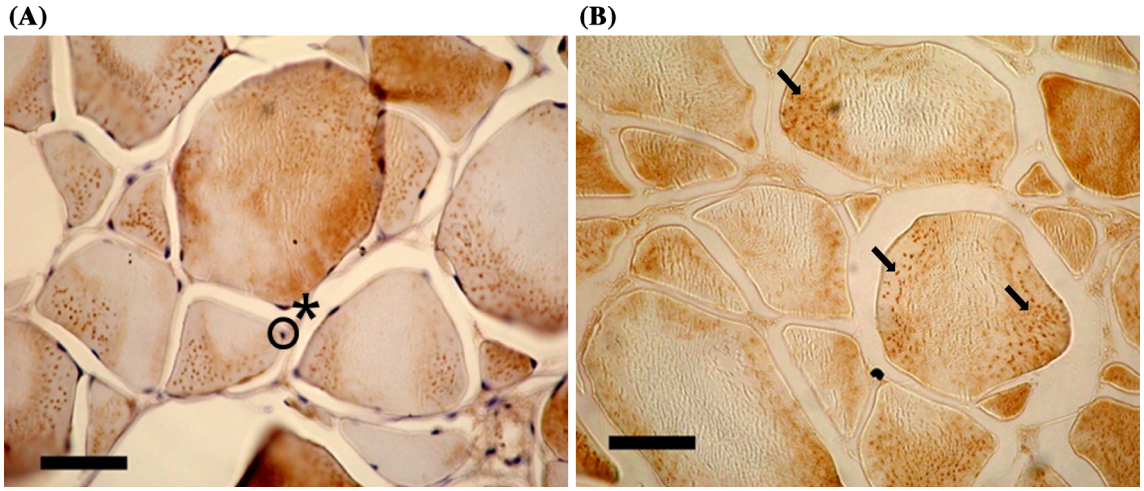

Fig. 5 Immunohistochemical staining for CA I in the skeletal muscle. The *brown dots* indicate immunopositive reactions. (**A**) Immunostaining and hematoxylin counterstaining of the tissue. Mark (*asterisk*) represents the nuclei of skeletal muscle. (**B**) Immunostained tissue. *Arrows* demonstrate the skeletal muscle fiber. *Scale bar* = 100 um

tissue slices were stained with hematoxylin and sealed using a cover glass. Images were captured directly by using an Olympus BX-50 microscope (Olympus Corp., Japan), and a C-4040Z digital camera (Olympus Corp., Japan).

Results and discussion

The soluble proteins in the tissues were separated using SDS-PAGE, and sensitivity and specificity of the heterologous antiserum for CA I were compared on SDS-PAGE blots. When the amount of cytosolic proteins in the tissues was determined before electrophoresis, protein concentration in the gills was approximately 19.5 mg/g, which was the higher than that in the kidney. CA I bands separated and visualized using SDS-PAGE and western blotting are shown in Fig. 1. A specific protein with a molecular weight of 29 kDa was detected in the gills, heart, muscle, and intestine of rainbow trout (Fig. 1A). A similar result for molecular weight was obtained in a previous study, in which the 30-kDa band represented CA III in sea bass (*Lateolabrax japonicas*) and rainbow trout (Kawk et al. 2011). The CA I concentration was higher in the gill and heart tissues than in the muscle and intestine tissues, while the control sample (liver tissue) was not significantly different from those of gill and heart (Fig. 1B).

Intracellular localization of CA I in rainbow trout tissues was studied using immunohistochemical methods. To distinguish cell types in the tissues, hematoxylin counterstaining was performed with immunostaining. When tissue slices were stained immunochemically, reactivity was observed in the gill, heart, intestine, and muscle tissues. In the gill tissue, the epithelial cells showed strong positive immunoreaction against the CA I antibody (Fig. 2). We also carried out immunostaining for CA I in the heart (Fig. 3) and found that the cells of the cardiac muscle showed a positive immune reaction. However, the reaction in these cells was weaker than that in the epithelial cells of the gill. In the intestine, positive reactivity occurred in the intestinal smooth muscle. The nucleus of the longitudinal smooth muscle showed strong reactivity (Fig. 4B). In addition, skeletal muscle fibers showed a strong positive reactivity for CA I (Fig. 5B). In fish, especially teleosts, CA activity in gills is dominantly cytoplasmic (Henry et al. 1988, 1993). The major role of cytoplasmic CA is to provide counterions for transport processes involved with ion regulation and pH balance (Henry and Swenson 2000; Claiborne et al. 2002; Marshall 2002; Evans et al. 2005). However, the muscle also has significant amounts of cytoplasmic CA. According to a previous study, the cytoplasmic CA isozymes present in mammalian muscle are CA II (white muscle) and CA III (red muscle) (Chegwidden and Carter 2000). In this experiment, we also detected CA I in the muscle of rainbow trout, and the intensity of CA I in the muscle was relatively weaker than that in the gills of the rainbow trout. The results of the western blot and immunohistochemical reactions in various tissues demonstrate that CA I, a member of the α-CA family, is possibly expressed in the gills, heart, intestine and muscle of the rainbow trout. Further studies are needed to confirm these important findings and to elucidate the function of CA I in rainbow trout tissues.

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