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Immunohistolocalization of Carbonic Anhydrase in Kidney and Intestine of Rainbow Trout, *Oncorhynchus mykiss*

Soo Cheol Kim, Jung Woo Kim*, Myeong Rak Choi, † Kap Seong Choi** and † Kang Hee Kho***

Dept. of Biotechnology and Chemical Engineering, College of Engineering, Chonnam National University, Yeosu 550-749, Korea
**Dept. of Anatomy, College of Medicine, Seonam University, Namwon 590-711, Korea*
***School of Food Science, Sunchon National University, Suncheon 540-950, Korea*
****Dept. of Aquatic Biology, College of Fisheries and Ocean Sciences, Chonnam National University, Yeosu 550-749, Korea*

Abstract

Carbonic anhydrase is essential for the cellular transportation of hydrogen and bicarbonate ions and plays a key role in a wide variety of physiological processes. Rainbow trout, *Oncorhynchus mykiss* is an important freshwater fish in aquaculture industry and is known to be one of the most susceptible species to environmental contamination. In this study, carbonic anhydrase was detected in the kidney and intestine of rainbow trout. Carbonic anhydrase was isolated from cytosolic proteins and identified by using SDS-PAGE, isoelectric focusing, and immunohistochemical methods. A specific protein band with molecular weight of 30 kDa and pI of 7.0 was detected by Western blotting. The immunohistochemical results showed that carbonic anhydrase was located at various cells in the kidney and intestine of rainbow trout.

Key words: carbonic anhydrase, rainbow trout, kidney, intestine, immunohistolocalization

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc-containing metalloenzyme with different catalytic activity, cellular location, chromosomal position, and tissue distribution (Supuran CT 2008a; Supuran CT 2008b). It was one of the first proteins for which a crystal structure was obtained (Fridborg et al. 1967; Kannan et al. 1971; Liljas et al. 1972). As an enzyme, CA is one of the fastest catalyst in the biological system (Chegwidden & Carter 2000). Also, the reaction that CA catalyzes is fundamental to a wide array of physiological processes, suggesting that CA may have been among the earliest enzymes to appear (Tashian RE 1989), driving the diversification of CA isoforms, and implicating the enzyme in physiological processes as varied as photosynthesis, respiration, ionic, acid-base and fluid balance, metabolism, and cell growth (Chegwidden & Carter 2000). As a result, CA appears to be expressed in almost all living organisms, with five genetically distinct enzyme families; α-, β-, ɣ-, δ-, and ζ-CAs (Hewett-Emmett & Tashian 1996; Xu et al. 2008).

It has been reported that CA is involved in a number of important physiological processes: pH homeostasis, CO_2 and bicarbonate transport, water and electrolyte balance, and biosynthetic reactions in animals (Kwak et al. 2011; Supuran CT 2008a). In fish, CA exhibits a fundamental role in various physiological processes such as pH control, ion regulation, and clearance of waste products from nitrogenous metabolism (Lionetto et al. 2000; Smith & Ferry 2000).

In contrast to the large amount of information about CA and its suggested importance in mammals, much less is known of the occurrence in non-mammalian vertebrates such as fish Rainbow

† Co-corresponding author: Kap Seong Choi, School of Food Science, Sunchon National University, Suncheon 540-950, Korea. Tel: +82-61-750-3257, Fax: +82-61-750-3058, E-mail: chks@sunchon.ac.kr
Kang Hee Kho, Dept. of Aquatic Biology, Chonnam National University, Yeosu 550-749, Korea. Tel: +82-61-659-7168, Fax: +82-61-750-3257, E-mail: kkh@chonnam.ac.kr

trout, *Oncorhynchus mykiss*, is the most important freshwater fish species in the aquaculture industry of Korea, with annual production increasing each year (Kawk et al. 2011). Moreover, this fish is known to be one of the most susceptible species to environmental contamination, and thereby has been extensively used as an experimental model for environmental toxicology research (Brack & Schirmer 2003). However, little is known about CA status under oxidative stress such as environmental contamination. Thus, we identified CA from rainbow trout tissues with particular emphasis on immunohistolocalization.

Materials and Methods

1. Preparation of cytosolic protein

Rainbow trout was provided by a fish farm in Gwangyang, Chonnam province, Korea. The tissues, kidney and intestine, were suspended in 0.5 g/10 mM Tris buffer (pH 7.2), homogenized in a glass grinder, and centrifuged at 5,000 × g for 3 min at 4℃ (×3). The supernatants were centrifuged at 45,000 × g for 90 min at 4℃, and the cytosolic extracts were collected.

2. Antibody

A polyclonal rabbit antiserum to CA from bovine erythrocytes was raised in a rabbit by Accurate Chemical & Scientific Corporation (NY, USA), and used to detect CA in rainbow trout tissues.

3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses

The cytosolic protein was mixed with 2× sample buffer, boiled for 3 min, and subjected to SDS-PAGE on 12% gels, by the method of Laemmli (1970). Four μg of protein in each sample was loaded. The separated proteins were transferred to a PVDF membrane, and the membrane was blocked with 7.5% skimmed milk in 10 mM Tris－HCl (pH 7.6) containing 0.15 M NaCl (TBS). The membrane was immersed overnight in primary antibody (1:1000). After washing with TBS containing Tween-20, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit antiserum (Sigma product A3687, 1:5000) for 2 h at room temperature. Bands were visualized with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. Western blot bands were quantified by scanning densitometry and Image J program (http://rsb.info.nih.gov/ij).

4. Isoelectric focusing (IEF) and Western blot analyses

The cytosolic protein was treated with 10 mM dithiothreitol for 30 min, and focused on a horizontal slab gel (4.5% acrylamide/1% ampholyte, pH 3.5－9.5) at 1,500 V and 2.75 mA/cm, within the power of 1.125 W/cm gel for 50 min. Isoelectric focusing gels containing Netfix (Serva, Biochemicals Inc. Paramus, NJ, USA) were used for analyzing a CA protein. The gel was transferred onto Immobilon-P (PVDF, Millipore Inc., Bedford, MA, USA) membranes, using a Semi-Dry Transfer Cell (Bio-Rad Lab., Hercules, CA, USA). A CA protein was detected by the Western blot method described previously (Kawk et al. 2011).

5. Immunohistochemical analysis

After fixing tissues with 4% paraformaldehyde, the tissues were embedded in Paraplast (McCormick, Sparks, MD, USA), according to a standard procedure. Five-mm-thick serial sections were cut, using an RM 2155 rotary microtome (Leica Microsystems, Nussloch, Germany), and mounted on slides coated with 3-aminopropyl-tri-ethoxysilane (Sigma-Aldrich Co., St. Louis, MO, USA). Immunohistochemical staining was carried out by a routine method. Briefly, tissue sections were incubated at 48℃ for 24 h with primary antibody; polyclonal anti-CA. The antibody binding was visualized, using an ImmPRESSTM avidin-biotin-peroxidase kit (Vector Laboratories Inc., Burlingame, CA, USA), according to the manufacturer's instructions. Omission of primary or secondary antibody was used, to control for false-positives. The tissue sections were stained with hematoxylin, dehydrated through a graded alcohol series, and mounted on coverslips. Images were directly captured by using an Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan) and a C-4040Z digital camera (Olympus Corp., Tokyo, Japan).

Results and Discussion

Before determining the relative density of CA in kidney and intestine of rainbow trout, the protein concentrations of cytosolic extracts from each tissue were checked in order to load equal amount of protein on SDS-PAGE. The protein concentration in the intestine was approximately 2.8 mg/g, which was slightly higher than that in the kidney. When the sensitivity and specificity of a heterologous antiserum for CA were tested on the blot of SDS-PAGE gel, a specific protein band with a molecular weight of 30 kDa was detected from both the kidney and

intestine of rainbow trout (Fig. 1A). A higher density of CA band was detected in the intestine compared with the kidney (Fig. 1B). This protein detected using the CA antiserum was virtually the same size (30 kDa) as those of spiny dogfish, carp, and *Cyprinus carpio* (Rahim et al. 1988; Linser P 1991; Wilson et al. 2000). The cytosolic proteins were also resolved by IEF and Western blotting. A major band of CA with pI 7.0 was detected on the blot of IEF gel (Fig. 2).

Immunohistochemical method was used to confirm intracellular localization of CA in tissues from rainbow trout. In the immunohistochemical methods, hematoxylin counterstaining was applied with immunostaining to differentiate cell types in the tissues. As a result of immunohistochemical staining with tissue slices, intensive immune responses were shown both on intestine and kidney tissues from rainbow trout (Fig. 3 & Fig. 4). Some epithelial cells in the intestine and kidney segments of rainbow trout reacted to CA antiserum. The immunopositive cell was

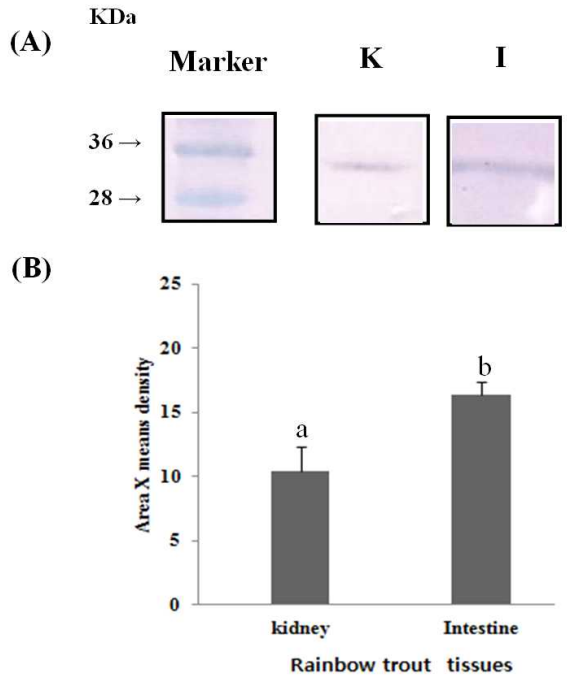

Fig. 1. Relative concentration of carbonic anhydrase in the kidney(K) and intestine(I) of rainbow trout. (A) SDS-PAGE and Western blot analysis of cytosolic protein from various tissues of rainbow trout. (B) Densitometric data. Vertical bars are the concentrations represented as relative mean area of the band densities. Data are reported as means± SE from replicate experiments (n=3). Means with different letters are significantly different according to Duncan's multiple range test (p<0.05).

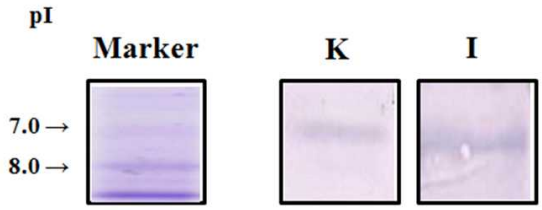

Fig. 2. Isoelectric focusing and Western blot analysis of cytosolic protein in the kidney(K) and intestine(I) of rainbow trout. Isoelectric focusing and blotting were described under Materials and Methods. Approximately 20 µg of proteins was focused on isoelectric focusing gel and was immunostained with anti-CA IgG.

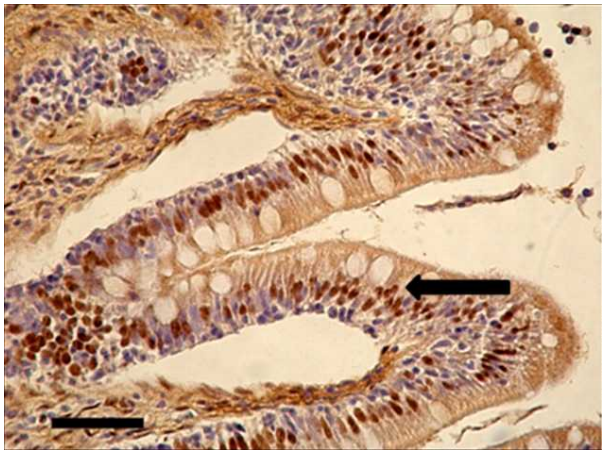

Fig. 3. Immunohistochemical staining for carbonic anhydrase in intestine. The brown dots indicate immunopositive reactions. A cell with the high density of immunohistochemical staining is marked as arrow. Scale bar = 50 µm.

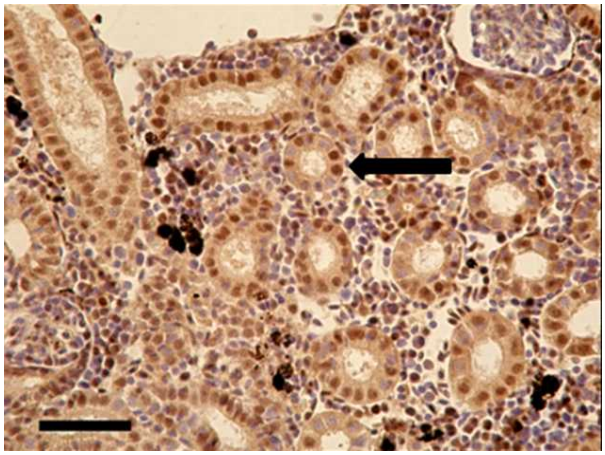

Fig. 4. Immunohistochemical staining for carbonic anhydrase in kidney. The brown dots indicate immunopositive reactions. A cell with the high density of immunohistochemical staining is marked as arrow. Scale bar = 50 µm.

localized at stalky cytoplasm, especially in the kidney (arrow mark in Fig. 4). In each part of the intestine and kidney segments of rainbow trout, the intensity of immunopositive reaction was almost the same. The results indicate that the CA protein with a molecular weight of 30 kDa and pI of 7.0 might be expressed in the other tissues, as well as the intestine and kidney of rainbow trout, *Oncorhynchus mykiss*. Further studies are needed to elucidate why the CA was distributed and localized in both intestine and kidney cells of rainbow trout.

References

Brack W, Schirmer K. 2003. Effect-directed identification of oxygen and sulfur heterocycles as major polycyclic aromatic cytochrome P 4501 A-inducers in a contaminated sediment. *Environ Sci Technol* 37:3062-3070

Chegwidden WR, Carter ND. 2000. Introduction to the carbonic anhydrase. In: Chegwidden WR, Carter ND and Edwards YH (eds) The Carbonic Anhydrases, New Horizons, Birkhäuser Verlag, Basel, pp 13-28

Fridborg K, Kannan K, Liljas A, Lundin J, Strandberg R, Tilander B, Wiren G. 1967. Crystal structure of human erythrocyte carbonic anhydrase C.3. Molecular structure of enzyme and of one enzyme-inhibitor complex at 5.5 A° resolution. *J Mol Biol* 25:505-516

Hewett-Emmett D, Tashian RE. 1996. Functional diversity, conservation, and convergence in the revolution of the α-, β-, and ɣ- carbonic anhydrase gene families. *Mol Phylogenet Evol* 5:50-77

Kannan K, Liljas A, Waara I, Bergstén PC, Lövgren S, Strandberg B, Bengtsson U, Carlbom U, Fridborg K, Järup L, Petef M. 1971. Crystal structure of human erythrocyte carbonic anhydrase C.6. 3-dimensional structure at high resolution in relation to other mammalian carbonic anhydrases. *Quant Biol* 36:221-231

Kwak DJ, Choi KS, Han KH, Kho KH. 2011. Discovery of carbonic anhydrase isozyme in sea bass and rainbow trout. *J Korean Soc Appl Biol Chem* 54:649-652

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277: 680-685

Liljas A, Kannan K, Bergstén PC, Waara I, Fridborg K, Strandberg B, Carlbom U, Järup L, Lövgren S, Petef M. 1972. Crystal structure of human carbonic anhydrase C. *Nature New Biol* 235:131-137

Linser P. 1991. Comparative immunochemistry of elasmobranch retina muller cells and horizontal cells. *J Exp Zool* 5:88-96

Lionetto MG, Giordano ME, Vilella S, Schettino T. 2000. Inhibition of eel enzymatic activities by cadmium. *Aquat Toxicol* 48:561

Rahim SM, Delaunoy JP, Laurent P. 1988. Identification and immunocytochemical localization of two different carbonic anhydrase isoenzymes in teleostean fish erythrocytes and gill epithelia. *Histochem* 89:451-459

Smith KS, Ferry JG. 2000. Prokaryotic carbonic anhydrase. *FEMS Microbiol Rev* 24:335-366

Supuran CT. 2008a. Carbonic anhydrases－an overview. *Curr Pharm Des* 14:603-614

Supuran CT. 2008b. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat Rev Drug Discov* 7:168-181

Tashian RE. 1989. The carbonic anhydrases: widening perspectives on their evolution, expression and function. *Bioessays* 10: 186-192

Wilson JM, Randall DJ, Vogl AW, Harris J, Sly WS, Iwama GK. 2000. Branchial carbonic anhydrase is present in dogfish, *Squalus acanthias*. *Fish Physiol Biochem* 22:329-336

Xu Y, Feng L, Jeffrey PD, Shi Y, Morel FM. 2008. Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. *Nature* 452:56-61

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