ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science **2013** Vol. 23. No. 12. 1557~1561 DOI : http://dx.doi.org/10.5352/JLS.2013.23.12.1557

- Note -

Detection of Carbonic Anhydrase in the Gills of Rainbow Trout (*Oncorhynchus mykiss*)

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

[1]*Department of Biotechnology, College of Engineering, Chonnam National University, Yeosu 550-749, Korea*
[2]*Department of Food Science and Technology, Sunchon National University, Sunchon 540-950, Korea*
[3]*Department of Anatomy, College of Medicine, Seonam University, Namwon 590-711, Korea*
[4]*Department of Aquatic Biology, College of Fisheries and Ocean Sciences, Chonnam National University, Yeosu 550-749, Korea*

Received November 5, 2013 / Revised November 29, 2013 / Accepted December 2, 2013

Carbonic anhydrase isozymes are a widespread, zinc-containing metalloenzyme family. The enzyme catalyzes the reversible inter-conversion of CO_2 and HCO_3. This reaction is the main role played by CA enzymes in physiological conditions. This enzyme has been found in virtually all organisms, and at least 16 isozymes have been isolated in mammals. Unlike mammals, there is little information available regarding CA isozymes in the tissues of non-mammalian groups, such as fish. Carbonic anhydrase is very important in the osmotic and acid-base regulation in fish. It is well-known that the gills of fish play the most important role in acid-base relevant ion transfer, the transfer of H^+ and/or HCO_3^- , for the maintenance of systemic pH. Rainbow trout, *Oncorhynchus mykiss*, is the most important freshwater fish species in the aquaculture industry of Korea, with annual production increasing each year. In addition, environmental toxicology research has shown that rainbow trout is known to be the species that is most susceptible to environmental toxins. Consequently, carbonic anhydrase was detected in rainbow trout, *Oncorhynchus mykiss*. The isolated protein showed the specific band with a molecular weight of 30 kDa and pI of 7.0, and it was identified as being carbonic anhydrase. The immunohistochemical result demonstrated that the carbonic anhydrase was located in the epithelial cells of the gills.

Key words : Carbonic anhydrase, gill, immunohistochemistry, rainbow trout

Introduction

Carbonic anhydrase (CA) was first discovered by Muldrum and Roughton in 1933 [19]. CA is a peptide with a zinc atom, and several isoenzymes in the CA family have been isolated and characterized in various animal species [1, 13, 15, 19]. In 1952, the function of CA was first revealed by Janowitz. They found that CA is associated with HCl excretion in gastric juice in mammals [14]. The parietal cells located between epithelial cells of the gastric mucosa contain a high CA concentration, which catalyze the bio-synthesis of dihydrogen oxide and carbon dioxide to carbonic acid. The CA has been found widely in mammals, birds, chondrichthyes, plants, and bacteria. However, studies on the CA family in fishes have rarely been reported.

CA is a ubiquitous metalloenzyme that catalyzes the reversible hydration of CO_2 to produce H^+ and HCO_3^-. In fishes, carbonic anhydrase also exhibits a fundamental role in a number of physiological processes such as physiological pH control and gas balance, calcification, osmoregulation, ion regulation and clearance of waste products from nitrogenous metabolism [12, 18].

In contrast to the large amount of information about the CA in mammals, much less is known about CA in non-mammalian vertebrates. Rainbow trout, *Oncorhynchus mykiss*, have been extensively used as an experimental model for environmental toxicology research. The aims of the present study are to identify and detect a CA in the rainbow trout gill by immunoblot and immunohistochemistry.

***Corresponding author**
Tel : +82-61-659-7169, Fax : +82-61-750-3257
E-mail : kkh@chonnam.ac.kr

Materials and Methods

Preparation of cytosolic protein

Rainbow trout were provided by a fish farm in Gwangyang, Chonnam province, Korea. The gills 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°C (×3). The supernatant was centrifuged at 45,000× *g* for 90 min at 4°C, and the cytosolic extract was collected.

Antibody production

Purified bovine erythrocyte CA was prepared as described by Chai *et al* [2]. The purified CA (100 µg) was injected subcutaneously as a 1:1 mix with Freund's complete adjuvant (0.5 ml) into one 6-month-old New Zealand male rabbit, and a 100-µg booster injection as a 1:1 mix with incomplete Freund's adjuvant (0.5 ml) was administered subcutaneously 2 weeks after the first injection. Antiserum was collected via biweekly bleeding from the ear vein beginning 1 month after the initial antigen injection. The antibody titer was determined by dot immunoassay [11], and a dilution of 1:10,000 was optimal for detecting CA.

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 using the method of Laemmli [10]. 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 (1:1,000). After washing with TBS containing Tween-20, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit antiserum (1:5,000 dilution) 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 the *Image J* program (http://rsb.info.nih.gov/ij).

Isoelectric focusing (IEF) and Western blot analysis

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, with limiting 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). The CA proteins were detected by the Western blot method.

Immunohistochemistry

After fixation with 4% paraformaldehyde, the tissues were embedded in Paraplast (McCormick, Sparks, MD, USA) using a standard procedure. Next, 5-mm-thick serial sections were cut using a RM 2155 rotary microtome (Leica Microsystems, Nussloch, Germany) and mounted on slides coated with 3-aminopropyl-tri-ethoxysilane (Sigma-Aldrich, St. Louis, MO, USA).

Immunohistochemical staining was carried out using a routine method. Briefly, tissue sections were incubated at 48°C for 24 h with primary antibody: polyclonal anti-CA. 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 captured directly using an Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan) and a C-4040Z digital camera (Olympus Corp.).

Results and Discussion

Carbonic anhydrase (CA) is a seemingly ubiquitous enzyme of profound physiological importance, which plays essential roles in the regulation of CO_2 levels in cells. Although sixteen different CA isoenzymes have been described in higher vertebrates so far, the only known physiological function of them is to facilitate the interconversion of CO_2 to HCO_3^- and H^+ [3, 4, 9, 22]. To obtain basic data for biochemical properties of CA, we investigated this protein from gill of rainbow trout by SDS-PAGE, IEF and immunohistochemistry.

The protein concentration in the gill of rainbow trout was approximately 95 mg/g (data not shown). When the sensitivity and specificity of a heterologous antiserum for CA was applied on blots of SDS-PAGE and Western blotting, a significant protein band with a molecular weight of 30 kDa was detected from the gill of rainbow trout (Fig. 1) where it plays an important role in osmoregulation, nitrogen (ammonia) excretion, acid-base balance and gas exchange [5]. This protein detected by CA antiserum was virtually the same size as those of spiny dogfish, carp, and *Cyprinus carpio*

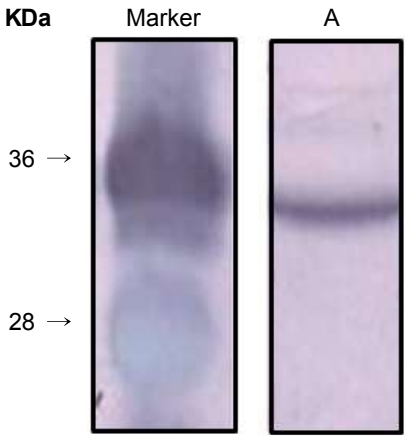

Fig. 1. SDS-PAGE and Western blot analysis of cytosolic protein from the gill of rainbow trout, *Oncorhynchus mykiss*. (A) Immunodetection of CA on a blot of PVDF membrane from gill of rainbow trout.

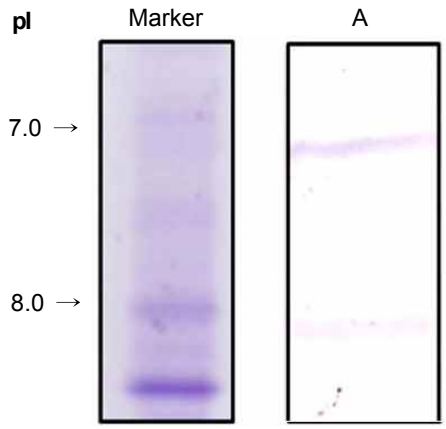

Fig. 2. Isoelectric focusing and Western blot analysis of cytosolic protein from the gill of rainbow trout, *Oncorhynchus mykiss*. Isoelectric focusing and blotting were described under Materials and Methods. (A) Approximately 20 μg of proteins was focused on isoelectric focusing gel and immunodetection of CA on a blot of PVDF membrane.

(30 kDa) [11, 17, 20]. The protein also purified from human lung membranes with the different molecular mass of 35 kDa [23].

The protein was 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). Similar finding was previously reported in the liver of flounder [8].

In the results of immunohistochemical experiments, the only CA antiserum showed immune response in the gill from rainbow trout (data not shown). Cytoplasm of gill's epithelial cell (arrow mark of Pic. B in Fig. 3) shows the strongest immune response. Also, we identified immune response at Pillar cell and nucleus and cytoplasm of chondroblast that backing the gill. Growing gill wall is the replacement bone that transformed cartilage into tibia. We observed chondro blast cell because experiment group is growing (Fig. 3).

The results indicated that the CA was present in the gill of rainbow trout, *Oncorhynchus mykiss*. Further studies are needed to elucidate biochemical functions in the rainbow trout gill.

References

1. Asari, M., Kimura, H., Ichihara, N., Kasuya, T. and Nishita, T. 2000. Immunohistochemistry of carbonic anhydrase isozymes (CA-I,II and III) in canine salivary glands : a distributional and comparative assessment. *Anat Histol Embryol* **29**, 9-12.
2. Chai, Y. C., Jung, C. H., Lii, C. K., Asharf, S. S., Hendrich,

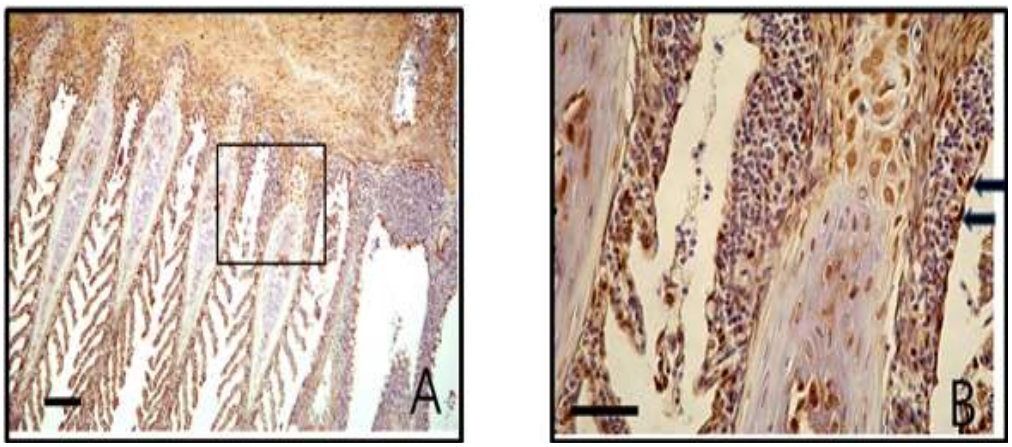

Fig. 3. Gill photography of CA immunohistochemical staining. B is enlarged pictures of the square box of A. Arrows indicate cytoplasm of gill's epithelial cell. Scale bar=50 μm.

S., Wolf, B., Sies, H. and Thomas, J. A. 1991. Identification of an abundant S-thiolated rat liver protein as carbonic anhydrase III; characterization of S-thiolated and dethiolation reactions. *Arch Biochem Biophys* **284**, 270-278.
3. Gilmour, K. M. and Perry, S. F. 2009. Carbonic anhydrase and acid-base regulation in fish. *J Exp Biol* **212**, 1647-1661.
4. Hewett-Emmett, D. and Tashian, R. E. 1996. Functional diversity, conservation, and convergenc in the evolution of the α-, β-, r- Carbonic anhydrase gene families. *Mol Phylogenet Evol* **5**, 50-77.
5. Henry, R. P. and Heming, T. A. 1998. Carbonic anhydrase and respiratory gas exchange. *Fish Physiol* **17**, 75-111.
6. Hilvo, M., Innocenti, A., Monti, S. M., De Simone, G., Supuran, C. T. and Parkkila, S. 2008. Recent advances in research on the most novel carbonic anhydrase, CA XIII and XV. *Curr Pharm Des* **14**, 672-678.
7. Inaba, K., Akazome, Y. and Morisawa, M. 1993. Purification of proteasomes from salmonid fish sperm and their localization along sperm flagella. *J Cell Sci* **104**, 907-915.
8. Kho, K. H. and Choi, K. S. 2005. Presence of carbonic anhydrase III in liver of Flounder, *Limanda yokohamae*. *Food Sci Biotechnol* **14**, 551-553.
9. Krishnamurthy, V. M., Kaufman, G. K., Urbach, A. R., Gitlin, I., Gudiksen, K. L., Weibel, D. B. and Whitesides, G. M. 2008. Carbonic anhydrase as a model for biophysical and physical-organic studies for proteins and protein-ligand binding. *Chem Rev* **108**, 946-1051.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277**, 680-685.
12. Lionetto, M. G., Giordano, M. E., Vilella, S. and Schettino, T. 2000. Inhibition of eel enzymatic activities by cadmium. *Aquat Toxicol* **48**, 561.
13. Moyle, S., Jeffery, S. and Carter, N. D. 1984. Localization of human muscle carbonic anhydrase isozymes using immunofluorescence. *J Histochem* **32**, 1262-1264.
14. Nishita, T., Kondo, H., Ishida, S., Ochiai, H. and Asari, M. 2000. Isolation and measurement of carbonic anhydrase isoenzymes in erythrocytes of dogs. *Am J Vet Res* **61**, 387-392.
15. Nishita, T., Matsuura, K., Ichihara, N. and Asari, M. 2002. Isolation and measurement of carbonic anhydrase isoenzyme III in plasma, sera and tissues of dogs. *Am J Vet Res* **63**, 229-235.
16. Purkerson, J. M. and Schwartz, G. J. 2007. The role of carbonic anhydrase in renal physiology. *Kindey Int* **71**, 103-115.
17. Rahim, S. M., Delaunoy, J. P. and Laurent, P. 1988. Identification and immunocytochemical localization of two different carbonic anhydrase isoenzymes in teleostean fish erythrocytes and gill epithelia. *Histochem* **89**, 451-459.
18. Smith, K. S. and Ferry, J. G. 2000. Prokaryotic carbonic anhydrase. *FEMS Microbiol Rev* **24**, 335-366.
19. Spicer, S. S., Ge, Z. H., Tashian, R. E., Hazen-Martin, D. J. and Schulte, B. A. 1990. Comparative distribution of carbonic anhydrase isozymes III and II in rodent tissues. *Am J Anat* **187**, 55-64.
20. Wilson, J. M., Randall, D. J., Vogl, A. W., Harris, J., Sly, W. S. and Iwama, G. K. 2000. Branchial carbonic anhydrase is present in dogfish, *Squalus acanthias*. *Fish Physiol Biochem* **22**, 329-336.
21. Wistrand, P. J. 2002. Carbonic anhydrase III in liver and muscle of male rat purification and properties. *Upsala J Med Sci* **107**, 77-88.
22. Zhang, J. L. and Zheng, Q. C. 2011. Theoretical improvement of the specific inhibitor of human carbonic anhydrase VII. *Comput Biol Chem* **35**, 50-56.
23. Zhu, X. L. and Sly, W. S. 1990. Carbonic anhydrase IV from human lung. *J Biol Chem* **265**, 8795-8801.

초록 : 무지개 송어 rainbow trout, *Oncorhynchus mykiss*의 아가미에서의 carbonic anhydrase의 존재

김수철[1] · 최갑성[2] · 김정우[3] · 최명락[1] · 한경호[4] · 이원교[4] · 고강희[4]*

([1]전남대학교 공학대학 생명산업공학과, [2]순천대학교 생명산업과학대학 식품공학과, [3]서남대학교 의과대학 발생학전공, [4]전남대학교 수산해양대학 양식생물학전공)

Carbonic anhydrase (CA)는 생물체 내에 널리 존재하는 아연(Zinc)을 함유한 금속성효소(metalloenzyme)이다. 이는 생리학적 조건에서 주로 CO_2의 hydration과 bicarbonate의 dehydration의 반응을 촉매하는 기능을 한다. 이러한 CA는 거의 모든 생물체 내에서 발견되고 16개 이상의 동질효소들이 포유류에서 분리되었다. 반면 포유류와 달리 포유류가 아닌 생물체, 특히 어류와 해양생물에 대한 CA와 그에 대한 동질효소에 관한 자료는 매우 제한적이다. 어류 내에서 CA는 삼투압과 산-염기 평형을 조절하는 매우 중요한 효소로 알려져 있으며, 또한 어류 내 조직 중의 하나인 아가미는 산-염기 조절, 이온 교환, 생체 내 pH 조절 등을 수행하는 부위로 알려져 있다. 실험생물인 무지개송어는 국내 해양 양식 산업 분야에 있어서 매년마다 그 생산량이 증가하는 매우 중요한 해양자원이다. 게다가 환경 독성 연구 분야에 있어서 그 실험적인 가치가 인정되어 국내·외에서 실험동물로 널리 이용되고 있는 어류이다. 아가미 조직에서 분리한 단백질에서 분자량 30 kDa, 등전점 7.0의 위치에 해당하는 특이적인 band가 형성된 모습을 관찰할 수 있었고 이는 확인 결과 CA인 것으로 판명되었다. 또한 CA의 존재여부가 확인된 아가미 조직 내에서 세부적인 발현 위치를 파악하기 위해 진행한 면역조직화학 실험 결과 CA가 아가미의 상피세포 내에 존재하는 것을 파악 할 수 있었다.