Food Sci. Biotechnol. Vol. 14, No. 4, pp. 551 ~ 553 (2005)

RESEARCH NOTE

Food Science and Biotechnology

Presence of Carbonic Anhydrase III in Liver of Flounder, *Limanda yokohamae*

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Abstract Carbonic anhydrase III was found in liver of flounder, *Limanda yokohamae*. Protein was isolated from cytosolic extracts and identified using SDS-PAGE and isolectric focusing. Specific protein bands with molecular weight of 30 kDa and pIs of 7.0 and 6.5 were detected by Western blotting. This is the first report of identification of carbonic anhydrase III from *L. yokohamae*.

Keywords: carbonic anhydrase III, liver, flounder

Introduction

Carbonic anhydrase (CA; carbonate dehydrase, carbonate hydro-lyase, EC 4.2.1.1) is one of the zinc metallo-enzymes that catalyze the simple hydration/dehydration of CO_2 and H_2O to facilitate the blood transport and excretion of CO_2 (1). CA classes (CAs) are not only found in mamalian tissue but are also abundant in fish and archaea (1, 2). CAs are classified into α-CAs (eubacteria, green algae, and animals), β-CAs (eubacteria and plant chloroplasts), and r-CAs (archaeabacteria, eubacteria, and plants). In higher vertebrates, seven α-CAs isoenzymes have been described (3). The presence of multiple isozymes within a species underscores the importance of this enzyme.

CAs are involved in many different processes, such as ion exchange, acid-base balance, carboxylation, and decarboxylation reactions, and inorganic carbon diffusion between the cell and its environment as well as within the cell (1, 4). They are also involved in many aspects of reproduction and have a functional role in sperm maturation and capacitation by influencing bicarbonate secretion (5, 6). CA activity with acetazolamide inhibited acidification of epididymal fluid (3, 7), decreased fluid secretion in the testis, and impaired sperm function (1, 8).

CA III, one of the CA isozymes, has been identified in many tissues (9); however, the liver and red skeletal muscle (type I fibers) are the only tissues that have large amount of CA III (10). CA III is distinguished from the other isozymes by several characteristics, particularly by a lower specific activity (about 1% of isozyme II) and by its resistance to acetazolamide (4, 9). CA III has been found in the rat, the chicks and the archaea (3, 10), and it is present in large amounts in certain tissues, comprising about 5% of the soluble protein of male rat liver, about 8% of skeletal muscle, and up to 25% of adipocytes (4). The physiological function of CA III is still unknown, but these characteristics suggest that it may not act simply as CA.

In this study, we demonstrated the presence of CAIII in the liver of flounder. This is the first report of the identification of CA III from any teleosts.

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Received June 16, 2005; accepted June 29, 2005

Materials and Methods

Preparation of cytosolic protein The flounders, *Limanda yokohamae*, were caught in the Sunchon bay of Korea, and their livers were collected. Liver cytosolic protein was prepared as follows (3, 11). Liver was 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 extracts were collected.

Antibody production Purified rat liver CA III was prepared as described by Chai *et al.* (3). One hundred micrograms of purified CA III was injected subcutaneously at 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 at a 1:1 mix with the incomplete Freund's adjuvant (0.5 mL) was administered subcutaneously two weeks after the first injection. Antiserum was prepared via biweekly bleeding from the ear vein beginning one month after the initial injection of antigen. Antibody titer was determined by dot immunoassay (12), and a 1:500 titer was shown to be optimal for CA III detection.

SDS-PAGE and Western blot analysis Cytosolic protein was mixed with 5x sample buffer, boiled for 2 min, and subjected to SDS-PAGE using the method of Laemmli (13). For each sample, 4 μg protein was loaded. Samples were separated by SDS-PAGE on 12% gels. The separated proteins were transferred onto a PVDF membrane, and the membrane was blocked with 5% skimmed milk in 15 mM Tris-HCl (pH 7.6) containing 0.15 M NaCl (TBS). The membrane was immersed overnight in 5% skimmed milk containing rabbit anti-CA III antiserum at a 1:500 dilution. After washing with TBS containing Tween-20, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit antiserum (1: 10000 dilution) for 2 hr at room temperature. Bands were finally visualized with *p*-nitroblue tetrazolium chloide/5-bromo-4-

chloro-3-indolyl phosphate.

Isoelectric focusing (IEF) and Western blot analysis Cytosolic protein was reduced with 10 mM dithiothreitol for 30 min and focused on horizontal slab gels (4.5% acrylamide/1% ampholyte, pH 3.5-9.5) at 1500 V and 2.75 mA/cm, and the limiting power was 1.125 W/cm gel for 50 min. Protein bands were stained as described elsewhere (3, 14). Isoelectric focusing gels containing Netfix (Serva, Biochemicals Inc., City, NY, USA) were analyzed by Western blot methods as follows. Gels were transferred onto Immobilon-P (PVDF) (Millipore Inc., Bedford, MA, USA) membrane using a Semi-Dry Transfer Cell (Bio-Rad Lab., Hercules, CA, USA). The detection of CA III was performed using Western blot methods.

Results and Discussion

CAs have many different physiological functions, and seven distinct isozymes have been characterized in mammals (2, 10). CA III is a cytoplasmic enzyme that exhibits a low carbonate dehydrase activity (9). However, the biochemical function of this enzyme is not yet known. To obtain basic data for biochemical properties of CA III, we investigated this protein from the flounder liver using SDS-PAGE and IEF. The sensitivity and specificity of a heterologous antiserum for CA III was also compared on blots of SDS-PAGE and IEF gels. When cytosolic protein was analyzed by SDS-PAGE and Western blotting, a significant protein band with molecular weight of 30 kDa was detected from the liver of flounder (Fig. 1). The protein detected with CA III antiserum was virtually the same size as those of spiny dog fish, carp, and *Cyprinus carpio* (30 kDa) (12, 15, 16).

The cytosolic protein was resolved by IEF and Western blotting. Specific bands of CA III with pI 7.0 and 6.5 were detected on the blot of IEF gel (Fig. 2). Because IEF gel was run under non-denatured conditions and no evidence

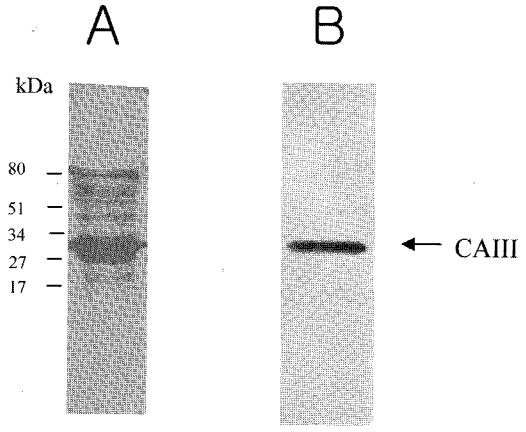

Fig. 1. SDS-PAGE and Western blot analysis of cytosolic protein from the liver of flounder, *Limanda yokohamae*. (A) Approximately 4 μg of proteins were analyzed on 12% polyacrylamide gel. (B) Immunodetection of CA III on a blot of PVDF membrane.

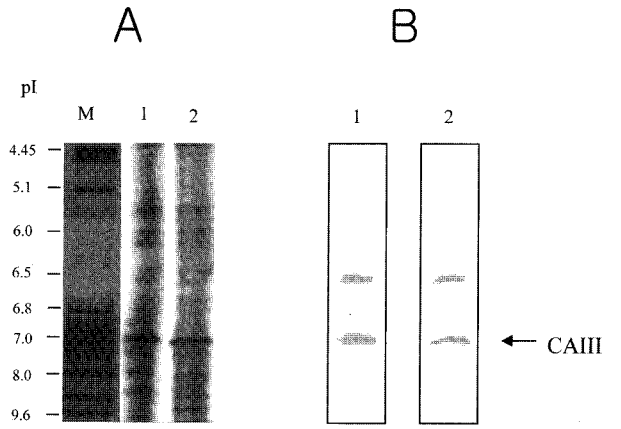

Fig. 2. Isoelectric focusing and Western blot analysis of cytosolic protein from the liver of flounder, *Limanda yokohamae*. Isoelectric focusing and blotting were described under Materials and Methods. (A) Approximately 10 μg (lane 2) and 20 μg (lane 1) of proteins was focused on isoelectric focusing gel and detected by silver-staining except pI marker. (B) Immunodetection of CAIII on a blot of PVDF membrane.

was found for the cleavage by IEF and Western blots, the potential for conformational isomers were considered.

Potentially, S-glutathiolation is an important modification of CA proteins resulting from oxidative stress (10, 17). A simple test for S-glutathiolation of CAs can be accomplished by thiol exchange reaction with glutathione. Although this reaction may be a minor mechanism for S-glutathiolation in intact cells, it is easily controlled *in vitro* and does not produce undesirable reactions. Previous detection of the glutathione level in the cytosolic extracts revealed no glutathione (data not shown). In addition, when protein concentration was increased by Amicon centrifuge, the visual concentration of the major band (pI 7.0) generally increased in proportion to the protein concentration (Fig. 2, lanes 1 and 2). Similar finding was observed in liver and muscle of male rat where reduced form of CA III had the same pI value as 7.0 (18). Furthermore, protein sulfhydryls can be oxidized to several states that can be demonstrated in intact proteins with various pI values (9, 10). Based on the findings that this immunoreactive protein had the same molecular weight as that of the rat liver CA III (30 kDa) (3, 9) and this protein can be modified to several states with different pI values (pI 7.0 and 6.5) (10), it is suggested that the specific protein bands of pI 7.0 and 6.5 found in the liver of flounder are the reduced and possibly the modified forms of CA III, respectively.

This is the first report of the presence of CA III in the liver of flounder, *L. yokohamae*. Further studies on its biochemical characteristics are needed to elucidate the physiological functions of CA III in the flounder liver.

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation (KOSEF, 2005) and partly by research grant from Sunchon National University (2002-0079).

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