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Identification and expression of a novel carbonic anhydrase isozyme in the pufferfish *Takifugu vermicularis*

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ABSTRACT

Carbonic anhydrase (CA) is a key element for maintaining acid base balance in fish. In our present experiment, novel CA isozymes were identified from the pear puffer (*Takifugu vermicularis*). Based on the high homology of two predicted CA sequences of the tiger puffer (*Takifugu rubripes*), a 1715 bp novel cDNA was obtained from *T. vermicularis*. The open reading frame showed a complete coding sequence of 552 bp with a deduced peptide sequence of 183 amino acids that exhibited highest (97%) identity with pufferfish putative CA III and CA IV-like sequences. In addition, this translated protein sequence showed 36–37% identity with zebrafish CA IV-like, CA XVa, CA XVb, and CA XVc proteins. Phylogenetic analysis revealed that the pufferfish novel protein (pCA_n) was a membrane-bound CA protein. Alignment of multiple CA sequences illustrated that most of the putative active site residues of the pCA_n isozyme were situated at highly conserved regions of the CA sequences. Examination of motif distribution suggested that the pCA_n isozyme was very similar to the puffer predicted CA IV-like isozyme. Reverse transcription-polymerase chain reaction (PCR) analysis showed highly differential expression in the brain, gills, kidney, and muscle, whereas CA mRNA expression was almost absent in heart, liver, and intestine. Quantitative PCR expression of CA mRNA abundance suggested several-fold higher expression of pCA_n isozymes in the gills compared to other tissues tested. Our results suggest that the pCA_n isozyme might be related to CA IV-like isozymes. Further functional studies are needed to investigate the function of the pCA_n isozyme in *T. vermicularis*.

1. Introduction

Carbonic anhydrases (CAs), which are zinc-bound homeostatic metalloenzymes, regulate acid-base equilibrium by catalyzing the reversible reaction of carbon dioxide (CO_2) and water (H_2O) to produce bicarbonate (HCO_3^-) and a proton (H^+) (Lindskog and Silverman, 2000). There are five distinct classes of carbonic anhydrases, α-, β-, γ-, δ-, and ζ-CAs (Květoň et al., 2001; Krishnamurthy et al., 2008; Aggarwal et al., 2013), of which the α-CAs are the most studied, likely because this class of CA is found in vertebrates including humans (Pinard et al., 2015). Out of 16 different CA isoforms that are expressed in mammals, 13 are catalytically active whereas the remaining three are non-catalytic (Amores et al., 1998; Esbaugh and Tufts, 2007). The functions of these isozymes vary according to their molecular sequence, kinetic properties, tissue distribution, and sub-cellular localization (Hewett-Emmett, 2000; Lehtonen et al., 2004). Among the catalytically-active CA isozymes, CA I, II, III, VII, VIII, X, XI, and XIII are cytosolic; CA VA and VB are mitochondrial; CA VI is secreted; CA IX, XII, and XIV are transmembrane proteins, and CA IV and CA XV are glycophosphatidylinositol (GPI)-anchored (Zhu and Sly, 1990; Waheed et al., 1992; Hilvo et al., 2005; Frost, 2014). The non-catalytic CA isozymes, CA VIII, X, and XI, are known as CA-related proteins owing to the absence of one or more of the histidine residues that are essential for catalytic activity (Aspatwar et al., 2010).

The α-CAs were first reported in mammals and few CA proteins have been identified in non-mammalian vertebrates such as fish. CA activity has been found in many fish tissues (Dimberg et al., 1981; Sanyal, 1984; Conley and Malalatt, 1988; Henry et al., 1988, 1993; Lund et al., 2002; Esbaugh et al., 2005; Esbaugh and Tufts, 2006; Gilmour et al., 2007; Sattin et al., 2010; Ceyhun et al., 2010, 2011; Demirdag et al., 2015) but few CA isoforms have been characterized owing to a lack of information regarding the molecular structure of CA isozymes in fish (Esbaugh et al., 2005; Ceyhun et al., 2010, 2011; Demirdag et al., 2015). The CA isozymes of fish differ somewhat from those of mammals, at least where comparisons have been made, as in the case of CA IV and cytosolic isoforms. In mammals, single isoforms have been described for CA IV and CA XV whereas multiple isoforms (termed a, b, and c) are present for CA

Abbreviations: aa, amino acid; CA, carbonic anhydrase; cDNA, complementary DNA; GPI, glycophosphatidylinositol; pCA_n, puffer novel carbonic anhydrase; q-PCR, quantitative polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

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IV and CA XV in some teleost fish (Lin et al., 2008). Furthermore, there are at least nine CA IV-like isoforms that have been found to be expressed in some teleost fish (Lin et al., 2008; Gilmour and Perry, 2009). Additionally, several novel isozymes have also been recently identified in fish (unpublished results).

In fish, as in other vertebrates, the cytosolic CA isoform of the red blood cell actively participates in CO_2 excretion (Gilmour et al., 2002). A role for CA in CO_2 excretion has also been described for CA IV, found in the gill of elasmobranch fish. The gill is also the primary site for maintaining acid-base balance in fish (Marshall and Grosell, 2005). CA, which is found in high quantities in gill epithelial cells, catalyzes the hydration of CO_2 to supply the H^+ and HCO_3^- needed for regulating acid-base balance (Perry and Laurent, 1990). This CA also participates in ionic and osmotic regulation at least in freshwater fish, because the excretion of H^+ and HCO_3^- is linked to the active uptake of Na^+ and Cl^-, respectively (Evans et al., 2005; Gilmour and Perry, 2009). The presence of CA enzymes in other tissues has also been reported and CA has been shown to be engaged in a number of different physiological processes including bone formation, calcification, ion transport, acid-base balance, and carbon dioxide transport (Gilmour and Perry, 2009).

Many studies related to CA isozymes have been performed in different fish species but to date, no reports on the characterization of CA isozymes in the pufferfish species *T. vermicularis* have been published. Pufferfish, belonging to the genus *Takifugu*, are regarded as economically important organism in northeast Asia. Beginning in the 1960s, the artificial culture of this genus has been performed (Watabe and Ikeda, 2006). The pufferfish (*T. vermicularis*) is an excellent model for new gene discovery and the study of vertebrate evolution owing to its small and compact genome (Brenner et al., 1993; Venkatesh et al., 2000). In particular, the predicted CA isozymes of a different pufferfish species, *Takifugu rubripes*, obtained from the National Center of Biotechnology Information (NCBI) database, provide us with a good platform to identify and molecularly characterize the CA isozymes in the pufferfish *T. vermicularis*.

2. Materials and methods

2.1. Experimental fish

Pufferfish (*T. vermicularis*) were collected from Yeosu Bay and transported to the Department of Fisheries Science, Chonnam National University. To obtain tissue samples, the pufferfish were euthanized by immersion in an anesthetic solution (ethyl 3-aminobenzoate methanesulfonate, MS-222: 1 g/l; Sigma-Aldrich, St. Louis, MO, USA). The cardiac cavity was then opened and blood samples were withdrawn by using a syringe and immediately placed in liquid N_2; these were stored at −80 °C until use. Next, the body was immediately perfused with 50 ml heparinized saline to flush out erythrocytes from the various tissues. For perfusion, pufferfish were injected by cardiac puncture with 250 IU heparinized saline (NaCl 280 mM; KCl 6 mM; $CaCl_2$ 5 mM; $MgCl_2$ 3 mM; $NaSO_4$ 0.5 mM; $NaHPO_4$ 1 mM; $NaHCO_3$ 8 mM; urea 350 mM; glucose 5 mM; and trimethylamineoxide 70 mM) (Forster et al., 1972) to harvest brain, gill, heart, liver, intestine, kidney and white muscle tissues. After collection, tissues were immediately frozen in liquid N_2, and stored at −80 °C for subsequent use.

2.2. Sequence retrieval

Predicted pufferfish (*T. rubripes*) CA isozyme sequences were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/gene), but no published reports were available related to the identified isozymes. The CA sequences (XM_011621962.1, XM_011619795.1, M_011607386.1, XM_011618951.1, XM_011610113.1, XM_011610116.1, XM_003964268.1, M_003965527.2, XM_003978524.2, XM_003975510.2, XM_003971040.1, XM_003978720.2, M_011620084.1, XM_011608113.1, XM_011620083.1, XM_011620082.1, XM_011604933.1, M_011620639.1, XM_011621456.1, and XM_011620041.1) so obtained were then aligned using CLUSTALW (Thompson et al., 1997) and two sequences, predicted CA III (XP_011618386.1) and predicted CA IV-like (XP_011619758.1), were finally selected for use in homology-based cloning strategies based on their high similarity to each other (~98%).

2.3. RNA extraction

Total RNA was extracted from the eight tissues (brain, gill, heart, liver, intestine, kidney, muscle, and blood) of pufferfish using an RNeasy mini kit (Qiagen, Hilden, Germany). RNA was treated with RNase-free DNase (Promega, Madison, WI, USA) to eliminate any genomic DNA contamination. cDNA synthesis was conducted using the Superscript® III First-Strand synthesis kit (Invitrogen, Carlsbad, CA, USA). All steps were carried out according to the manufacturers' instructions.

2.4. Molecular cloning of pufferfish CA isozymes

For molecular cloning, reverse transcription (RT) primers (forward: 5′-TCTCAAGTCCTTCACCTTCAC-3′ and reverse: 5′-GAGTAGTCTTTCTAAATTGTC-3′) were designed based on the conserved portion of the two predicted CA sequences retrieved from the NCBI database. RT-polymerase chain reactions (PCR) were performed using an EmeraldAmp® GT PCR Master Mix (2× Premix) (Takara, Shiga, Japan). PCR was carried out using 1 μl cDNA template obtained from gill tissue in reaction mixtures containing 1 μl (20 pmol) of each forward and reverse primer, 8 μl Master Mix, and sterile distilled water (dH_2O) in a total volume of 20 μl. The PCR conditions for reaction mixtures involved initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 1 min, with a final extension for 5 min at 72 °C. The PCR products were purified using a gel extraction kit (Promega) and then ligated into the pTOP Blunt V2 vector (Enzynomics, Daejeon, Korea) and transformed into competent *Escherichia coli* DH5α cells (Enzynomics). The plasmid DNA was extracted using a plasmid mini kit (Qiagen) and sequenced by Macrogen Online Sequencing System (Macrogen, Seoul, Korea). To obtain the full-length sequence, 5′ rapid amplification of cDNA ends (RACE) PCR was performed based on the cloned sequence. The cDNA for 5′ RACE PCR was synthesized by RT from gill total RNA using the 5′ end-phosphorylated RT primer 5′-ATCCATAATCACTCG-3′ and the 5′-Full Race Core Set (Takara); PCR was carried out according to the instructions provided by the manufacturer. Two-step PCR was performed using two pair of primers (forward: 5′-ATGTTCCCAGCTCAGGCAGG-3′ and reverse: 5′-ACGTGTTTCAGACCTCCACC-3′; forward: 5′-CGTCCGGTGCCAAAACTTCC-3′ and reverse: 5′-ATGATGTATTCGATGGCGTG-3′) for the first and second steps, respectively, using Takara LA Taq at the annealing temperature of 65 °C. After the second PCR, the PCR products were ligated into the pTOP TA V2 vector (Enzynomics) and sequenced as described previously. The sequenced RACE products were then combined by overlapping with the initial cloned cDNA fragment.

2.5. Sequence analysis

A protein homology study was conducted using the Basic Local Alignment Search Tool (BLASTP) in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). The web tool “SMART” from European Molecular Biology Laboratories (EMBL) (smart.embl-heidelberg.de) was used to identify the CA domain in the predicted pCA_n protein. The primary structures of the genes were analyzed using ProtParam (http://expasy.org/tools/protparam.html) and the protein location within the cell determined by using Protcomp (http://linux1.softberry.com/berry.phtml). A multiple protein sequence comparison study of pCA_n was constructed with different CA proteins from humans and fish using GeneDoc (www.psc.edu/biomed/genedoc) (Nicholas and Nicholas,