Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part B 148 (2007) 225–230
CBP
www.elsevier.com/locate/cbpb

Expression and distribution of fugu TIMP-2s (fgTIMP-2a and fgTIMP-2b) mRNAs in tissues and embryos

Hiroshi Tsukamoto [a], Yoshihiro Yokoyama [a,*], Tohru Suzuki [b], Shoshi Mizuta [a], Reiji Yoshinaka [a]

[a] *Laboratory of Food Chemistry, Department of Marine Bioscience, Faculty of Biotechnology, Fukui Prefectural University, Obama, Fukui 917-0003, Japan*
[b] *Laboratory of Bioindustrial Informatics, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 981-8555, Japan*

Received 19 September 2006; received in revised form 8 February 2007; accepted 9 February 2007
Available online 16 August 2007

Abstract

In teleosts, two distinct types of TIMP-2s occur, TIMP-2a and TIMP-2b, but little is known about their locations and quantitative expressions. Here, we examined pufferfish (*Takifugu rubripes*) TIMP-2a (fgTIMP-2a) and TIMP-2b (fgTIMP-2b) quantities and locations in fugu adult tissues and embryos. To compare the quantitative expression of fgTIMP-2s, we performed a quantitative real-time PCR (qPCR). FgTIMP-2a mRNA was constitutively expressed and significant differences in expression were not observed among adult tissues. Whereas, fgTIMP-2b mRNA was significantly differently expressed in ordinary muscle and gill compared to the expression level in whole blood ($P<0.05$). Although significant difference was not observed between brain and other tissues, both fgTIMP-2s mRNAs were abundant in the brain. In addition, we examined embryos during development using qPCR. Both fgTIMP-2s mRNAs gradually increased during embryonic development from 48 hpf. However, fgTIMP-2b mRNA was obviously abundant compared to fgTIMP-2a mRNA in embryos. We also examined the specific mRNA distribution in embryos. The fgTIMP-2s mRNAs showed the same distribution during development. Both fgTIMP-2s are expressed in adult fugu tissues and embryos but their expression levels clearly differ, suggesting that there is a predominance of fgTIMP-2b over fgTIMP-2a *in vivo*.

Keywords: Embryo; Fugu; Quantitative real-time PCR; *Takifugu rubripes*; Teleost; Tissue inhibitor of metalloproteinase (TIMP)-2a; TIMP-2b; Whole mount *in situ* hybridization

1. Introduction

Tissue inhibitors of metalloproteinases (TIMPs) are the primary inhibitors of matrix metalloproteinases (MMPs) in tissues (Woessner, 1991; Gomez et al., 1997; Brew et al., 2000; Baker et al., 2002). TIMP is composed of two domains, the N-terminal and C-terminal domains that have different functions. The N-terminal domain inhibits MMP activity; whereas the C-terminal domain functions differently among TIMPs (Baker et al., 2002). TIMPs not only inhibit MMPs but also have multifunctional activities (Lambert et al., 2004) in cell proliferation (Hayakawa et al., 1992; Hayakawa et al., 1994; Corcoran and Stetler-Stevenson, 1995), apoptosis (Brand et al., 2000; Baker et al., 1988), and tumor angiogenesis (Mignatti et al., 1989; Takigawa et al., 1990).

Four TIMP family members, TIMP-1, -2, -3 and -4, are found that have been characterized and reviewed for mammals (Gomez et al., 1997; Brew et al., 2000; Lambert et al., 2004; Baker et al., 2002). However, in the teleost fish fugu *Takifugu rubripes*, the TIMP-1 gene was lost but there are still four genomic TIMPs where two differing distinct types of TIMP-2s are found (Yu et al., 2003). In teleosts TIMP-2, Japanese flounder TIMP-2s (Kubota et al., 2003), zebrafish TIMP-2 (Zhang et al., 2003a), fugu TIMP-2s (Yokoyama et al., 2005), medaka TIMP-2s (Ogiwara et al., 2005) and red seabream TIMP-2 (Touhata et al., 2006) have been cloned. Additionally, Lodemel et al. (2004) show a TIMP-2-like protein in Atlantic cod muscle using a two-dimensional real-time reverse zymography. Two TIMP-2s were reported in the Japanese flounder (Kubota et al., 2003) and the medaka (Ogiwara et al., 2005) as well as in a pufferfish. Although it is not known if the TIMP-1 gene is lost in the other teleost fish species, because of the loss of the TIMP-1 gene in the fugu genome (Yu et al., 2003), there

* Corresponding author. Tel.: +81 770 52 6300; fax: +81 770 52 6003.
E-mail address: yokoyama@fpu.ac.jp (Y. Yokoyama).

1096-4959/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2007.02.016

Table 1
Primers used for the quantitative real-time PCR

Gene		Sequence(5′→3′)
TIMP-2a	Forward	CCTGATGATTGAGAAGCTGGTGCAC
	Reverse	TCCTCGCCGTCCAAGAACTCCTTC
TIMP-2b	Forward	TGGACGGACTGGGTGATGGAGGAAAG
	Reverse	GGGATCTTCGATGTCCATGAAGTCC
EF-1α	Forward	GCATGGTTGTCACCTTTGCTCCCG
	Reverse	GTCAGCTCCCTTTGGTGGGTCGTTC

The expected size of products for fgTIMP-2a, fgTIMP-2b and fgFE-1α using qPCR are 129, 141 and 191 bases, respectively.

may be different specialized mechanisms to metabolize ECM proteins in teleost as compared to mammals.

The occurrence of a second TIMP-2 was first identified in the Japanese flounder and expression patterns differed in tissues between the jfTIMP-2s using RT-PCR (Kubota et al., 2003). We also report cDNA cloning of fugu TIMP-2s (fgTIMP-2a and fgTIMP-2b) and examined their expression levels using RT-PCR (Yokoyama et al., 2005). The reports suggest there are different expression patterns of TIMP-2s in tissues, but quantification has not been performed. Consequently, it is unknown if the expression profiles of TIMP-2s are different in tissues. This requires a more accurate and sensitive method than RT-PCR. The expression of zebrafish TIMP-2 mRNA in embryos was studied by Zhang et al. (2003a); however it is unknown whether both types of TIMP-2s mRNAs are expressed in embryos during development.

Our laboratory investigates the regulatory mechanism of ECM metabolism in fugu muscle. Fugu is a highly prized commercial fish species in Japan being harvested in the wild and cultured. Recently, a fugu draft genomic database was established because fugu genomic size is much smaller than human. Thus, the fugu is regarded as both a biologically and commercially important fish species (Ando et al., 1991; 1993; Sato et al., 1997; Yu et al., 2003; Yokoyama et al., 2005; Kurokawa et al., 2005; Tsukamoto et al., 2006; Christoffels et al., 2006; Noguchi et al., 2006; Tsutsui et al., 2006; Watabe et al., 2006).

Previously, we reported cDNA cloning of fgTIMP-3 and gene expressions of fgTIMP-3 and -4 in adult tissues and embryos (Tsukamoto et al., 2006) as well as the occurrence of MMP-2 and MMP-9 in pufferfish (Tsukamoto et al., 2007). Here we determined the expressions of fgTIMP-2s in adult fugu tissues and during the course of embryonic development using a quantitative real-time PCR (qPCR). This is a superior analytical method to determine the expression quantitatively. In addition, to clarify the distributions of fgTIMP-2s mRNAs, we also examined spatial and temporal gene expression of fgTIMP-2s in embryos using whole mount *in situ* hybridization (WISH).

2. Materials and methods

2.1. Materials

Japanese tiger puffer fish, *T. rubripes* (fugu), adults and embryos were reared at the Research Center for Marine Bioresources of Fukui Prefectural University, Obama, Japan. Adults (about 32 cm long and 2 years old) were reared in an 8000-liter tank with running seawater for several months before use. Fertilized eggs of fugu were a kind gift from Dr. Tominaga O., Fukui Prefectural University. Embryos were maintained in a 500-liter plastic tank with running seawater at 18 °C as previously described (Suzuki et al., 2002). They were intermittently collected as samples for RNA extraction and a whole mount *in situ* hybridization as previously described (Tsukamoto et al., 2006). Morphological features and stages of fugu embryos were determined as those of zebrafish described by Kimmel et al. (1995).

2.2. Quantitative analysis of TIMP-2s mRNAs in tissues and embryos

Adult tissue cDNAs from three individuals ($n=3$) were prepared as previously described (Tsukamoto et al., 2006). Briefly, primer sets for fgTIMP-2s (GenBank accession no. TIMP-2a, AB161713; TIMP-2b, AB154540) and fgEF-1α (AB193485) were constructed and are shown in Table 1. The specificity of the primer sets was confirmed using standard PCR. EF-1α was used as an internal control where the expression levels of the other genetic components were compared to the normal expression of EF-1α mRNA as a reference. qPCR analysis was performed using the iCycler (Bio-Rad) and Realtime PCR Master Mix (TOYOBO) as previously described (Tsukamoto et al., 2006). Measurements were performed three times and the relative expression of the mRNA was compared to EF-1α and calculated as described elsewhere (Zhang et al., 2003b).

A series of fugu embryos at 48, 56, 72, 92, 124, 140 and 168 hour post-fertilization (hpf) were collected. cDNAs from embryos at different stages were prepared and qPCR was performed. Measurements were performed three times and the relative expression of the mRNA was compared to EF-1α and calculated.

2.3. Whole mount in situ hybridization (WISH)

Embryos at different stages were fixed in 4% paraformaldehyde in phosphate-buffered saline (PFA) at 4 °C overnight. After washing with phosphate buffered saline (PBS) twice, the embryos were dehydrated and stored in pure methanol at −30 °C. Digoxygenin-labeled sense and anti-sense ribo-probes were synthesized from TA vectors (Qiagen) containing each

Table 2
Digoxygenin-labeled sense and anti-sense ribo-probes used for WISH

Gene	Probe	Restriction enzyme	Promoter	Position of the sequence (base)	Ribo-probe product (length base)
TIMP-2a	Sense	*Pst* I	SP6	53–634	582
	Anti-sense	*Sal* I	T7	634– 53	
TIMP-2b	Sense	*Kpn* I	SP6	10–675	666
	Anti-sense	*Sal* I	T7	675– 10	