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Molecular cloning and expression of gelatinases (MMP-2 and MMP-9) in the pufferfish *Takifugu rubripes*

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Abstract

To determine the metabolism location of the extra-cellular matrix proteins in fugu (*Takifugu rubripes*), we cloned the cDNAs of the fugu gelatinases, matrix metalloproteinase-2 (MMP-2) and MMP-9, and examined their expressions in various adult tissues using a quantitative real-time PCR. The expression profiles of fugu gelatinases were different among tissues. FgMMP-9 mRNA was abundant in tissues that contain blood cells abundantly where fgMMP-2 mRNA was little expressed. We also examined the expression of these genes in fugu embryos during development using a whole mount *in situ* hybridization. Fugu MMP-2 mRNA was expressed in the pharyngeal area and mesenchyme in embryos at 80 hours post fertilization (hpf). While fugu MMP-9 mRNA was expressed in the vent at 140 hpf and the caudal end of the fin fold at 172 hpf. Although fugu MMP-2 mRNA was expressed in the pectoral fin bud at 120 hpf, fugu MMP-9 mRNA did not appear in this tissue until 10 days post fertilization (dpf). These data show expression profiles differ between the fugu gelatinases and suggest expressions of these genes are controlled at the matrix protein degradation site in fugu embryos during development.

Keywords: Embryo; Fugu; Gelatinases; Matrix metalloproteinase (MMP)-2; MMP-9; Quantitative real-time PCR; *Takifugu rubripes*; Teleost; Whole mount *in situ* hybridization

1. Introduction

The matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases that degrade extra-cellular matrix (ECM) components such as collagens and non-collagenous molecules (Massova et al., 1998; Nagase and Woessner, 1999; Visse and Nagase, 2003). Consequently, they are involved in various processes of ECM metabolism such as in embryonic development, morphogenesis, and tissue remodeling (Woessner, 1991; Matrisian, 1992; Nagase and Woessner, 1999). The MMPs are grouped by their ability to degrade molecules. Among the MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are thought to be especially important in collagen degradation through the digestion of denatured collagen (gelatin) generated by the collagenases (MMP-1, -8 and -13) that can cleave the triple helical regions of the fibrillar collagen molecule (Visse and Nagase, 2003). These two gelatinases also degrade other molecules, such as the type IV and V collagens (Shipley et al., 1996; Nguyen et al., 1993). Regulation of the MMP activities is essential to maintain homeostasis in a variety of extra-cellular environments. The expression of MMP activity is controlled both at the level of gene transcription and at the level of proenzyme activation after secretion into the ECM (Visse and Nagase, 2003). The down regulation of MMP activity in the ECM may occur through the interaction with specific inhibitors such as tissue inhibitors of metalloproteinase (TIMPs); and this balance suppresses undesirable proteolysis (Gomez et al., 1997; Brew et al., 2000; Baker et al., 2002).

In embryonic development, ECM remodeling is essential for both morphogenesis and tissue growth in order for cells to migrate to appropriate places (Blavier and Delaisse, 1995; Chin and Werb, 1997; Vu and Werb, 2000). These events are thought to be facilitated by the MMP activities as regulated by TIMPs. Moreover, the MMPs contribute to not only ECM remodeling but also to many other cellular functions (Vu and Werb, 2000).

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MMP activities are required in cell migration, cellular behavior and the release of active molecules that bind to the ECM suggesting that MMPs play important roles in the developmental process (Vu and Werb, 2000).

In utilization of fish muscle as a food, the firmness of fish muscle is correlated to the collagen content (Sato et al., 1986). The firmness of fish muscle immediately after death and the softening rate during storage differ among species (Hatae et al.,1985; Sato et al., 1986; Nakayama et al., 1990; Ando et al.,1993). Generally, the firmness of most fish muscle softens within 1–3 days during early storage at 5 °C (Ando et al., 1991). However, the fugu (*Takifugu rubripes*) muscle shows a tough texture with little tenderization even after 72 h at 5 °C (Ando et al., 1993). It has not been determined why fugu muscle shows this unique property. Reports showed that the degradation of ECM protein, especially type V collagen, is correlated with a decrease in the breaking strength of fish muscle (Sato et al., 1997; Shigemura et al., 2003) suggesting the gelatinases are the key proteinases that accelerate the softening of fish muscle.

Our laboratory studies the regulatory mechanism of proteolytic metabolism in the ECM in fish using the teleost fish, fugu (*T. rubripes*). Fugu is one of the most highly valued commercial fish species in Japan. Recently, a draft genomic database for fugu was established because the genomic size is much smaller than the human genome and consequently fugu is regarded as a model organism for the study of comparative genomics. Therefore, fugu is both a biologically and a commercially important species (Ando et al., 1991; Ando et al., 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).

Because there is a lack of information concerning MMPs and TIMPs in fish, we previously performed cDNA cloning of two fugu TIMP-2s (TIMP-2a and-2b) and examined their mRNA expressions using RT-PCR (Yokoyama et al., 2005). We also cloned fugu TIMP-3 cDNA and examined the gene expression of fugu TIMP-3 and-4 in fugu adult tissues and embryos (Tsukamoto et al., 2006). However, reports show the TIMP-1 gene is lost in the fugu genome (Yu et al., 2003) and two distinct types of TIMP-2 occur in some fish species (Kubota et al., 2003; Yu et al., 2003; Ogiwara et al., 2005) suggesting there is a unique mechanism of metabolism of ECM proteins in the teleosts including the fugu.

To further understand ECM metabolism in fugu, we cloned cDNAs of two fugu gelatinase genes (MMP-2 and MMP-9) and examined their expressions using a quantitative real-time PCR. This method enabled us to detect with higher sensitivity the expression levels of these gelatinases in the tissues. For embryos, we conducted a whole mount *in situ* hybridization (WISH) and examined the spatial and temporal gene gelatinase expressions in fugu embryos at different developmental stages.

Table 2
Summary of digoxygenin-labeled sense and antisense ribo-probes for WISH

Gene	Probe	Position of the sequense (base)	Riboprobe product (length, base)
MMP-2	Sense	548-1143	566
	Antisense	1143-548	
MMP-9	Sense	162-1750	1589
	Antisense	1750-162	

2. Materials and methods

2.1. Materials

The Japanese tiger pufferfish, *T. rubripes* (fugu) and fugu embryos were reared at the Research Center for Marine Bioresources of Fukui Prefectural University, Obama, Japan. Adult fugu about 32 cm long and 2 years-old were reared in an 8000-liter tank for several months before use. Fertilized fugu eggs were a kind gift from Dr. O. Tominaga, Fukui Prefectural University. Fugu embryos were kept in a 500-liter plastic tank with running seawater at 18 °C. A developmental series of fugu embryos at various hours post

Table 1
PCR primers used in cDNA cloning, RACE and quantitative real time PCR

Gene			Sequence (5'→3')
MMP-2	RT-PCR	Forward	TGGATGCCCACAAGACAGATGCAAC
		Reverse	CTGATCTCTCCCAACTTGACGATC
	5'-RACE	GSP	CTGATCTCTCCCAACTTGACGATC
		NGSP	CCGGGTTTTCATAGGCAGCGTCGA
	3'-RACE	GSP	GCAGATGCGTTGGAGAGAGGCTATC
		NGSP	GATGTCGACTCTGCCTTCAGTCTC
	Real-time PCR	Forward	GATGTCGACTCTGCCTTCAGTCTC
		Reverse	CTGATCTCTCCCAACTTGACGATC
MMP-9	RT-PCR	Forward	CATTGACATACAGCGCCGCAGTGGC
		Reverse	CTGAATGCTGGGAGGAAGGCCAAGC
	5'-RACE	GSP	CATCAGGAACACCACAGCGAGGTTG
		NGSP	TGTCCAGCTCTCCCGTCTCCTTCAG
	3'-RACE	GSP	CATTGACATACAGCGCCGCAGTGGC
		NGSP	TGTGCCATTTCCCCTTCCGCTTCC
	Real-time PCR	Forward	CATTGACATACAGCGCCGCAGTGGC
		Reverse	CATCAGGAACACCACAGCGAGGTTG
EF-1α	Real-time PCR	Forward	GCATGGTTGTCACCTTTGCTCCCG
		Reverse	GTCAGCTCCCTTTGGTGGGTCGTT C

The expected product size for MMP-2, MMP-9, and FE-1α, for real time PCR is 115, 152, and 191 bp, respectively.