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Functional characterization of chitinase-3 reveals involvement of chitinases in early embryo immunity in zebrafish

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ABSTRACT

The function and mechanism of chitinases in early embryonic development remain largely unknown. We show here that recombinant chitinase-3 (rChi3) is able to hydrolyze the artificial chitin substrate, 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside, and to bind to and inhibit the growth of the fungus *Candida albicans,* implicating that Chi3 plays a dual function in innate immunity and chitin-bearing food digestion in zebrafish. This is further corroborated by the expression profile of *Chi3* in the liver and gut, which are both immune- and digestion-relevant organs. Compared with rChi3, rChi3-CD lacking CBD still retains partial capacity to bind to *C. albicans*, but its enzymatic and antifungal activities are significantly reduced. By contrast, rChi3-E140N with the putative catalytic residue E140 mutated shows little affinity to chitin, and its enzymatic and antifungal activities are nearly completely lost. These suggest that both enzymatic and antifungal activities of Chi3 are dependent on the presence of CBD and E140. We also clearly demonstrate that in zebrafish, both the embryo extract and the developing embryo display antifungal activity against *C. albicans*, and all the findings point to chitinase-3 (Chi3) being a newly-identified factor involved in the antifungal activity. Taken together, a dual function in both innate immunity and food digestion in embryo is proposed for zebrafish Chi3. It also provides a new angle to understand the immune role of chitinases in early embryonic development of animals.

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1. Introduction

Chitinases (EC 3.2.1.14) are hydrolytic enzymes that break down β-1,4-linked N-acetyl-D-glucosamine (GlcNAc) linkages in chitin, an important structural component of insect exoskeletons, shells of crustaceans, and fungal cell walls (Onaga et al., 2011). On the basis of amino acid sequence homology, chitinases are classified into two families 18 and 19 (Henrissat, 1991), and each family has a different three-dimensional structure and a catalytic mechanism (Monzingo et al., 1996; van Aalten et al., 2001), implicating that families 18 and 19 evolved independently during animal evolution (Bussink et al., 2007). Chitinases belonging to family 19 only include plant chitinases and *Streptomyces griseus* chitinase (Ohno et al., 1996), while those belonging to family 18 are found not only in chitin-containing organisms but also in widely diverse organisms that do not comprise endogenous chitin, including humans and mice (Mali et al., 2004). Members of family 18 chitinases typically contain a catalytic domain with highly conserved sequence FDGLDLDWEYP, and a linker region followed by a chitin-binding domain (CBD). The linker region is rich in serine and threonine, and may be extensively glycosylated (Zhu et al., 2008).

Family 18 chitinases take part in many biological processes. The first mammalian chitinase was identified in the plasma of patients suffering from Gaucher disease and named chitotriosidase because of its ability to hydrolyze 4-methylumbelliferyl β-chitotrioside (Boot et al., 1995; Hollak et al., 1994). Later, the chitotriosidase was shown to possess an antifungal property (van Eijk et al., 2005). A second mammalian chitinase, acidic chitinase (AMCase), characterized by its acidic pH optimum, was mostly expressed in gastrointestinal tract and lung (Boot et al., 2001), and has thus been proposed to be involved in digestion of chitin-containing food as well as in immunity (Boot et al., 2005; Suzuki et al., 2002; Zhu et al., 2004). Similarly, a dual function for chitinases in both food digestion and innate immunity has been proposed in invertebrates including insects, mollusks and shrimp (Badariotti et al., 2007; Mali et al., 2004; Dahiya and Pathak, 2006; Kramer and Muthukrishnan, 1997; Lundblad et al., 1976; Pan et al., 2005; Tan et al., 2000). In addition, chitinases also play a key role in the molting process during larval growth and development of arthropods (Watanabe et al., 1993; Arakane et al., 2003). Analogously, chitinase has been implicated in the normal development of trunk and tail in developing zebrafish larvae (Bakkers et al.,

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1997; Semino and Allende, 2000) and early embryonic development of the oyster *Crassostrea gigas* (Badariotti et al., 2007). These indicate involvement of chitinases in early development of both invertebrates and vertebrates, but information as such is rather scarce. Likewise, little is known about the functions and mechanisms of chitinases in early embryonic development.

The zebrafish (*Danio rerio*) has been an ideal organism for the study of developmental biology and biotechnology, but it is emerging as a model species for the study of immunology, human disease and cancer (Trede et al., 2004; van der Sar et al., 2004; Phelps and Neely, 2005; Meeker and Trede, 2008; Sullivan and Kim, 2008; Zhang and Cui, 2014). Eggs of zebrafish are released and fertilized externally, and the resulting embryos/larvae are therefore exposed to an aquatic environment full of potential pathogens capable of causing various types of diseases. For example, the fungi *Pseudoloma neurophilia* and *Pleistophora hyphessobryconis* can infect zebrafish including its eggs/embryos (Ramsay et al., 2009; Sanders and Kent, 2011), and *Candida albicans* can colonize and invade zebrafish at multiple anatomical sites and kill the fish in a dose-dependent manner (Chao et al., 2010). During the early stages of development, zebrafish embryos have little or only limited ability to synthesize immune relevant molecules endogenously, and their lymphoid organs are not yet fully formed (Lom and Nilsen, 2003). Moreover, early embryonic developmental stage is one of most vulnerable periods in life history (Wang et al., 2009). How zebrafish embryo/larvae survive the pathogenic attacks in such a hostile environment is one of the key issues for reproductive and developmental immunology. It has been supposed that embryos/larvae of fish including zebrafish depend upon the maternal provision of immune-relevant molecules for protection against invading pathogens before full maturation of immunological systems (Zhang et al., 2013). As chitinases are shown to be involved in immunity in both invertebrates and vertebrates, we wonder if they play a role in the immune defense of developing zebrafish embryos/larvae. Six genes encoding chitinase/chitinase-like proteins have been identified in *D. rerio*, and their expression patterns characterized at 10 different stages of embryonic development (Koch et al., 2014), laying a foundation for further elucidation of the roles of chitinases in zebrafish development. The aims of this study were therefore to functionally characterize chitinase-3 gene from *D. rerio*, designated as Chi3, and to explore its functions in developing embryo/larva. Such a study will shed light on the mechanisms of how early embryos/larvae of fish protect themselves from pathogenic attacks.

Table 1
Sequences of the primers used in this study.

Primers	Sequence(5′–3′)	Sequence information
P1 (sense)	ATGGGGAGACTTACACTTATA	Zebrafish chitinase 3
P2 (antisense)	CTAAGGTTTGGGCCAAACACA	Cloning primer
P3 (sense)	AAGGTGCCCTATGCCACAAAGAA	Real-time PCR primer
P4 (antisense)	TCATCCAGATCAAGTGCCCAAAC	(chitinase)
P5 (sense)	CCGTGACATCAAGGAGAAGC	Real-time PCR primer
P6 (antisense)	TACCGCAAGATTCCATACCC	(β-actin)
P7 (sense)	CGGAATTCATGGAAATGGCCTGCTAC	Primer for Chi3
P8 (antisense)	CCGCTCGAGAGGTTTGGGCCAAACACAG	Expression
P9 (sense)	CGGAATTCATGGAAATGGCCTGCTACA	Primer for Chi3-CD
P10 (antisense)	CCGCTCGAGCTGTCCAGCAAAGTCATCC	Expression
P11 (sense)	GACTGG**AAC**TATCCCGGAGCAAGAG	Mutation primer
P12 (antisense)	CAGATCCAGTCCATCAAATCCATG	(E140N)

Table 2
The accession numbers of chitinase genes used in this study.

Species	Name	Accession Nos.
Homo sapiens	Human AMCase	NP_970615.2
Homo sapiens	Human chitotriosidase	AAI05681.1
Mus musculus	Mouse AMCase	NP_075675.2
Mus musculus	Mouse chitotriosidase	NP_082255.1
Rattus norvegicus	Rat AMCase	NP_997469.1
Rattus norvegicus	Rat chitotriosidase	NP_001073157.1
Sus scrofa	Pig chitinase, acidic	NP_001245306.1
Bos taurus	Bovini acidic mammalian chitinase	NP_777124.1
Gallus gallus	Chicken AMCase like protein	NP_989760.1
Xenopus tropicalis	Frog chitotriosidase like protein	NP_001005792.1
Oncorhynchus mykiss	Rainbow trout chitinase	CAD59687.1
Thunnus orientalis	Pacific bluefin tuna chitinase 3	BAL14138.1
Danio rerio	Zebrafish chitinase, acidic.3	NP_998378.1
Branchiostoma japonicum	Amphioxus chitotriosidase-like protein	AEI59134.1
Lethenteron camtschaticum	Lamprey chitinase	ACF10400.1
Penaeus monodon	Black tiger shrimp chitinase 3	ADG22163.1

2. Materials and methods

2.1. Cloning, sequencing and sequence analysis of Chi3 cDNA

Total RNAs were extracted with RNAiso plus (TaKaRa) from *D. rerio*, and cDNAs were synthesized with reverse transcription kit (TaKaRa) with oligo (dT) primer, following the manufacturer's instructions. A pair of primers P1 and P2 specific for chitinase-3 (Table 1) were designed using primer premier program version 5.0 according to the chitinase sequence (NP_998378.1) obtained from NCBI (http://www.ncbi.nlm.nih.gov/). The PCR amplification reaction was carried out at 94 °C for 5 min, followed by 32 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 7 min. The amplification products were gel-purified using DNA gel extraction kit (AXYGEN), cloned into the pGEM-T vector (Invitrogen), and transformed into Trans 5α *Escherichia coli* (TransGen). The positive clones were selected and sequenced to verify for authenticity.

The cDNA sequence obtained was analyzed for coding probability with the DNATools program. Multiple protein sequences were aligned using the MegAlign program by CLUSTAL W method in DNASTAR software package. The SMART program (http://smart.embl-heidelberg.de/) was used to predict the functional sites and domains in the deduced protein sequence, the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) used to predict the signal peptide, and the ProtFun 2.2 Server (http://www.cbs.dtu.dk/service/) to predict posttranslational modification. The information of exon–intron organization was obtained from Ensembl database (http://asia.ensembl.org/index.html). The accession numbers of chitinases used were listed in Table 2.

2.2. Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted with RNAiso plus from the embryos/larvae at different developmental stages and the different tissues of adult *D. rerio*, including the eye, brain, heart, liver, spleen, gut, fin, gill, skin, testis and ovary. The cDNAs were synthesized with reverse transcription kit (TaKaRa), and were used as template for qRT-PCR, which was performed on ABI 7500 real-time PCR system (Applied Biosystems). The gene-specific primers P3 and P4 for Chi3 and P5 and P6 for β-actin were designed using Primer Premier 5.0 program (Table 1), used to amplify products of 150 bp and 200 bp, respectively. The β-actin gene was chosen as the reference for internal standardization. The analysis was performed using the software GraphPad Prism 5. The threshold cycle (Ct) value was calculated by $2^{-\Delta\Delta Ct}$ method.

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