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Trans-generational enhancement of C-type lysozyme level in eggs of zebrafish by dietary β-glucan

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ABSTRACT

β-glucan has been shown to increase non-specific immunity and resistance against infections or pathogenic bacteria in several fish species, but information regarding its trans-generational immune-enhancing effects is still rather limited. Lysozyme is a maternal immune factor playing an important role in the developing embryos of zebrafish. Here we clearly showe that β-glucan enhanced the level of C-type lysozyme in eggs of zebrafish, and the embryos derived from β-glucan-treated zebrafish were more resistant to bacterial challenge than control embryos. Moreover, the transferred lysozyme was apparently linked with the antimicrobial defense of early embryos. In addition, we also showed that β-glucan induced a significant increase in the synthesis of C-type lysozyme in previtellogenetic oocytes. Therefore, we show for the first time that β-glucan can enhance the lysozyme level in offspring via both inducing the transfer of the molecule from mothers to eggs and stimulating its endogenous production in oocytes.

1. Introduction

Immunostimulants, or immunostimulators, are substances (drugs and nutrients) that stimulate the immune system by inducing activation or increasing activity of any of its components. One notable example is β-glucan which has been widely used in fish culture (Meena et al., 2013; Petit and Wiegertjes, 2016; Vetvicka and Vetvickova, 2016). β-glucan is a naturally occurring polysaccharide with β(1,3)-D-linkages in the backbone. Common sources of β-glucan include cell walls of bacteria, plants, algae, yeast, and mushrooms. Typically, studies investigating the effects of β-glucans have mainly focused on juvenile and adult fish (Ringø et al., 2012; Vetvicka et al., 2013). A large number of studies have shown that β-glucan is able to enhance non-specific immunity and resistance against infections or pathogenic bacteria in several fish species (Chen and Ainsworth, 1992; Nikl et al., 1992, Nikl, 1993; Raa et al., 1992; Robertsen et al., 1990, Robertson, 1994; Santarem et al., 1997; Sahoo and Mukherjee, 2001, 2002; Sealey et al., 2008; Welker et al., 2007). Moreover, β-glucan is also shown to be able to affect the expression of immune-related genes including cytokine, chemokine and antimicrobial peptide genes (Katzenback, 2015; Løvoll et al., 2007; Rodríguez et al., 2009) as well as production of antibody (Kamilya et al., 2006; Selvaraj et al., 2005). Very recently, Jiang et al. (2016) have proven that dietary β-glucan is able to enhance the contents of complement component 3 and factor B in eggs of zebrafish, providing the first evidences for the transfer of immune-relevant molecules from mother to egg. However, information regarding the trans-generational e ffect of β-glucan is still rather limited.

Eggs of most fishes are released externally, and their embryos are thus exposed to aquatic environment full of thousands of various microorganisms, including potential pathogens, before their immune system is fully developed. It has been proposed that fish embryos depend upon the maternal provision of immune-relevant molecules for protection against invading pathogens (Zhang et al., 2013). Actually, the lysozyme, IgM, zinc finger protein ZRANKB2, egg yolk protein phosvitin and fish-egg lectin have been proven to be maternally-transferred molecules stored in eggs, capable of protecting early embryos of zebrafish *Danio rerio* against

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bacterial attack (Wang et al., 2009; 2011, 2012, 2016a and 2016b). Previous studies also show that β-glucan can increase lysozyme activity in Atlantic salmon *Salmo salar* (Paulsen et al., 2003) and blue-fin porgy *Sparidentax hasta* (Al-Gharabally et al., 2013). We wonder if the administration of β-glucan on fish enhances the transfer of lysozyme from mother to eggs, thereby promoting the antibacterial activity of resulting embryos. The present study was thus performed to answer this question.

2. Materials and methods

2.1. Fish, glucan treatment and quantitative real-time PCR (qRT-PCR)

All the zebrafish *Danio rerio* used in the experiments were treated in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee of the Ocean University of China. Wild-type zebrafish at the age of about 3 months old were purchased from a local fish dealer and maintained in containers with well-aerated tap water at 27 ± 1 °C under a 14-h light/10-h dark photoperiod.

A total of 20 female *D. rerio* at age of about 3.5 months old were randomly divided into 2 groups (10 fish/group), and cultured in 2 identical tanks. Each group of the fish received one of the following diets: the control diet Miero Fish Food (RAINBOW, China) or the experimental diet Miero Fish Food supplemented with 1.25 g/kg β-1,3 glucan (purity>99%, Sigma) prepared as described by Jiang et al. (2016). The fish in each group were fed with respective diet during the trial at a rate of 2% body weight at day 1. After 1 week treatment, 3 specimens of fish were sampled from each group, and the liver, kidney, spleen and ovary were dissected out of the sampled fish. The preparation of total RNAs, cDNA synthesis and qRT-PCR were performed as described by Jiang et al. (2016). The gene *β-actin* was chosen as the reference for internal standardization. The primer sets P1 and P2, specific of C-type lysozyme gene, and P3 and P4, specific of β-actin gene, were designed using the primer premier 5.0 program (Table 1).

2.2. Mating and preparation of egg cytosol and serum

The remaining *D. rerio* were continuously fed with the control diet or the experimental diet at a rate of 2% body weight at day 1 for another week, and then mated with healthy males. In brief, sexually mature *D. rerio* from both experimental and control groups were placed in the late evening at a female to male ratio of 1:1, maintained at 27 ± 1 °C, and the naturally fertilized eggs were collected in the next morning. Each female *D. rerio* was mated with male fish three times at an interval of 2 days, and each time all the fertilized eggs were collected. After removal of the unhealthy eggs, the healthy fertilized eggs were pooled, and rinsed three times with double-distilled H_2O and then once with ice-cold double-distilled H_2O. After the excess H_2O was withdrawn, the eggs were immediately homogenized on ice for 30 s, and centrifuged at 15,000 g at 4 °C for 30 min. The supernatant, i.e. egg cytosol, was pooled and added with protease inhibitor cocktail (Roche, Indianapolis, IN), aliquoted and stored at −70 °C till used. After the third spawning, female *D. rerio* from both experimental and control groups were scarified to collect the blood by the method of Babaei et al. (2013). The blood was allowed to clot for 2 h at 4 °C, and then centrifuged at 3000 g for 10 min. The serum was pooled, aliquoted and stored at −20 °C until use. The protein concentrations of egg cytosol and serum were determined by BCA protein assay kit (CWBIO, China), using bovine serum albumin as a standard.

2.3. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to assay the concentrations of lysozyme in the egg cytosol and serum using the double-antibody sandwich ELISA kit (Product codes LYS: GYF9056; Guyan, Shanghai, China) according to the manufacturer's instructions.

2.4. Cloning and sequencing of C-type lysozyme gene

Total RNAs were extracted with TRIzol (TaKaRa, Dalian, China) from a whole *D. rerio*, and digested with RNasefree DNase (TaKaRa) to eliminate the genomic contamination. The first-strand cDNA was synthesized with reverse transcription system (TaKaRa) using oligo d(T) primer. To amplify the complete cDNA, a pair of specific primers P5 and P6 (Table 1) was designed according to the sequence of C-type lysozyme gene of *D. rerio* (GenBank accession number: BC162644.1; http://www.ncbi.nlm.nih.gov/) using Primer Premier 5.0 program. The PCR amplification reaction was carried out at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The amplification product was gel-purified using DNA gel extraction kit (AXYGEN), cloned into the pGEM-T vector (Invitrogen), and transformed into Trans5α *Escherichia coli* (TransGen). The positive clones were selected and sequenced to verify for authenticity.

2.5. Expression and purification of recombinant lysozyme

The cDNA region encoding mature protein of C-type lysozyme was amplified by PCR from *D. rerio* as above using the primer pairs P7 and P8 (Table 1). The PCR product was digested with *Eco*RI and *Xho*I and sub-cloned into the plasmid expression vector pET28a (Novagen, Darmstadt, Germany) previously cut with the same restriction enzymes. The recombinant plasmid was verified by sequencing, and named *pET28a/lys*. The cells of *E. coli* Transetta (DE3) were transformed with the plasmid *pET28a/lys* and cultured overnight in LB broth containing kanamycin (50 μg/ml). The expression, purification and refolding of recombinant C-type lysozyme (rCLys) was according to the methods of Lei et al. (2015).

The purified protein was analyzed by 12% SDS polyacrylamide gel electrophoresis (PAGE), and immunostained with the mouse

Table 1
Sequences of the primers used in this study.

Primer	Sequence (5′ to 3′)	Sequence information
P1 (S)	AGTGGTGTGATGACGGGACC	For qRT-PCR
P2 (AS)	AGCGGGACATCTTACGACCG	For qRT-PCR
P3 (S)	CCGTGACATCAAGGAGAAGC	For qRT-PCR
P4 (AS)	TACCGCAAGATTCCATACCC	For qRT-PCR
P5 (S)	AAGACACTGGGACGCTGTGATG	For C-type lysozyme gene cloning
P6 (AS)	TCAGGCTCGGAGGCTTTGTT	For C-type lysozyme gene cloning
P7 (S)	TACTCAGAATTCAAGACACTGGGACGCTGTGATG	For recombinant expression
P8 (AS)	TACTCACTCGAGTCAGGCTCGGAGGCTTTGTT	For recombinant expression