2. Materials and Methods

2.1. Cloning and sequencing of *C. macrocephalus* C3 and C8γ cDNA

Total RNA was extracted from liver tissue of adult catfish using Trizol Reagent (Invitrogen, USA), according to the manufacturer's protocol. Messenger RNA (mRNA) was purified from the total RNA using a QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, UK). One microgram of mRNA was reverse-transcribed to 5' RACE products using 5'-CDS primer and PowerScript reverse transcriptase with the SMART RACE cDNA Amplification kit (Clontech, USA), according to the manufacturer's protocol. The first gene-specific primers, CmC3-RACE 1 and CmC8γ-RACE 1 (Table 1), were designed from the 3' end fragment of *C. macrocephalus* C3 and C8γ cDNA obtained from the EST analysis (Panprommin *et al.*, 2007). 5'-RACE-PCR reaction was conducted with the initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 4 min (1 min in case of Cm C8γ-R1), and the final extension step at 72 °C for 5 min. More two gene-specific primers, CmC3-RACE-2 and CmC3-RACE3, were designed based on the nucleotide sequence of *C. macrocephalus* C3 cDNA isolated by the first and the second RACE PCR, respectively (Table 1). Amplified RACE-PCR products were gel-purified using a DNA purification Kit (Clontech, USA) and subsequently cloned into the pGEM-T easy vector (Promega, USA). The positive clones were selected for extraction of plasmid using MiniPrep Plasmid Purification Kit (Qiagen, USA) according to the manufacturer's protocol. The plasmid DNA was sequenced in both directions using M13 forward and reverse primers with an automated sequencer by a commercial service (First Base Laboratories Sdn Bhd, Malaysia)

To obtain a single fragment of the partial *C. macrocephalus* C3 and the full-length *C. macrocephalus* C8γ cDNA, PCR amplifications were performed by using two sets of gene-specific primers (CmC3-SF and CmC3-SR, and CmC8γ-SF and CmC8γ-SR). The PCR condition was 94 °C for 2 min; 30 cycles of 94 °C for 30 s., 55 °C for 30 s and 72 °C for 1 min (4 min incase of *C. macrocephalus* C3) followed by 72 °C for 5 min. The amplification products were cloned and plasmids were isolated and subcloned for sequencing as described above.

2.2. Sequence data analysis

The cDNA sequences were searched for homology using the NCBI BLAST search program (National Center for Biotechnology Information, available at http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1997). The nucleotide sequence and deduced amino acid sequence were analyzed with GENETYX version 7. The cleavage site of signal peptides was determined by the SignalP program (http://www.cbs.dtu.dk/services/SignalP) (Nielsen *et al.*, 1997). The NetNGlyc was used to predict the N-liked glycosylation site (http://www.cbs.dtu.dk/services/NetNGlyc) (Kornfeld and Kornfeld, 1985). Multiple sequence alignment was performed using the Clustal X program and subsequently used to generate phylogenetic tree using the Phylip program with the UPGMA method and 1000 bootstrap values.

2.3. Tissue expression analysis of *C. macrocephalus* C3 and C8γ

Total RNA from various tissues of healthy catfish including brain, heart, kidney, liver, spleen, intestine, muscle and ovary was extracted as described above. Two microgram of total RNA was used for first-strand cDNA synthesis using an AMV reverse first-strand cDNA synthesis kit (Fermentus, USA), according to the manufacturer's protocol. RT-PCR using 1 μl of cDNA was performed with two sets of *C. macrocephalus* C3 and C8γ-specific primers (CmC3-rtF and CmC3-rtR, and CmC8γ-rtF and CmC8γ-rtR). The β-actin primer set (Panprommin *et al.*, 2007) was used as an internal control for amount and quality of cDNA. The PCR program was done under the following condition: 94°C for 2 min, 25 or 30 cycles of 94°C for 30 s, 60 °C for 30 s and 72°C for 30 s followed by 72°C for 5 min. The products were analyzed on a 1.5 % ethidium bromide-stained agarose gel.

2.4. Determination of *C. macrocephalus* C3 and C8γ mRNA expression levels during larval development

Catfish larvae were obtained by artificial breeding at Fishery farm unit, Ubon Ratchathani University, Thailand. The culture of larval stage was carried out under routine larval rearing process. The larvae were continuously reared in concrete pond and were sampled at 1, 3, 5, 10, 20 and 30 days post hatching for RNA extraction. One hundred

micrograms of each age of catfish larvae were washed with PBS and frozen in liquid nitrogen before homogenization with Trizol reagent. Total RNA was extracted, cDNA was synthesized and the expression level of *C. macrocephalus* C3 and C8γ transcripts was determined by PCR as described above. Beta-actin was used as the internal control for cDNA levels in different ages.

2.5. In situ hybridization

In situ hybridization was performed under RNase-free condition. All solutions were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved before use.

2.5.1. Tissue preparation

The larval samples of fish at age 30 days post hatching were rapidly rinsed in phosphate buffer saline (PBS) and immediately fixed in newly-prepared 10% formalin in PBS at room temperature overnight before being transferred to 70% ethanol. The samples were then dehydrated with a graded ethanol (70, 85, 95 and 100%), cleared in xylene, infiltrated with paraffin and embedded in paraffin wax. The paraffin sections were cut at 5 μm, mounted on poly-L-lysine coated glass slides, air dried overnight and stored at 4 °C until used.

2.5.2. Riboprobe synthesis

Riboprobes were prepared from the plasmids containing *C. macrocephalus* C3 and C8γ cDNAs. Briefly, PCR reactions were conducted by using the gene-specific primer sets (Table 1) used in RT-PCR. The amplified products were cloned into pGEM-T easy plasmid vector. Both *C. macrocephalus* C3 and C8γ cDNA containing vectors were enzymatically linearized with *Sac* I or *Sac* II, followed by phenol extraction and precipitation under RNAse free condition. Digoxigenine (DIG) labeled anti-sense and sense riboprobes were synthesized by *in vitro* transcription using RNA-labeling Kits (T7 and SP6 RNA polymerase) according to the manufacture's protocol (Roch, Germany).

2.5.3. Hybridization

Paraffin embedded sections were dewaxed in xylene and rehydrated in sequences of ethanol (96, 70 and 30%). The sections were then treated with proteinase K (10 μg/ml in 10 mM Tris-HCl (pH 8), 1 mM EDTA) at 37 °C for 30 min, incubated in 0.1 M glycine in PBS for 10 min and post-fixed with freshly prepared 4% paraformaldehyde in PBS for 15 min before being washing in PBS. After acetylation in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8), dehydration and air drying at least 1 h at room temperature, the sections were prehybridized in a hybridization buffer (50% formamide, 5 X SSC and 10% dextran sulphate) at 55 °C for 2 h. Fleshy prepared hybridization reaction containing Dig-labeled riboprobes, hybridization buffer, salmon sperm DNA, 10 mg/ml tRNA and 100 mM dithiothreitol dig-labeled riboprobes were added into the sections and allowed to hybridize for 16 h at 55 °C in a humidified chamber. Then, the hybridized sections were washed with 2 × SSC at room temperature and treated with 20 μg/ml RNase A in 2 × SSC at 37 °C for 30 min. For detection of hybridization, the sections were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase overnight and the hybridized signals were visualized by nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) containing 1mM lavamizole for 1 h at room temperature. The sections were then rinsed in TBS buffer to stop the reaction and mounted in 10% glycerol in TBS.

2.6. Determination of *C. macrocephalus* C3 and C8γ mRNA expression levels after β-glucan feeding

Healthy catfish fingerlings with an average weight of 10.5 g were acclimated under laboratory conditions for 1 week and fed with commercial diet at 5% of body weight per day. The fingerlings were subsequently fed daily with diet containing 5 g of β-glucan kg^{-1} dry feed for 14 days. Three fingerlings were randomly sampled on day 1, 3, 7 and 14 after β-glucan feeding. Samples taken on day 0 were used as a non-stimulated control. Their liver tissues were individually taken and collected in Trizol reagent (Invitrogen, USA). Total RNA isolation and first strand cDNA synthesis were performed for each fingerling liver sample of each group as described previously. The first strand cDNAs were used as templates for semi-quantitative RT-PCR. Amplifications of *C. macrocephalus* C3 and C8γ were conducted with the same gene-specific primers as in RT-PCR (Table 1) under the condition as follows: 1 cycle of 94 °C for 2 min, 25 cycles of 94°C for 30 s, 60 °C for 30 s and 72°C for 30 s

followed by 72°C for 5 min. The products were subsequently electrophoresed on 1.5 % agarose gel containing ethidium bromide. The expression levels of *C. macrocephalus* C3 and C8γ gene were normalized relative to the β-actin gene expression levels using Genetools Analysis Software (Syngene). Statistical differences between groups were analyzed by one-way ANOVA (SYSTAT 8.0 software, SPSS), followed by Duncan's multiple rang test for comparisons of means by group. Values were considered to be significant at $P < 0.05$.

Table 1 Primer sequences used in this study

Primer	Sequence (5'-3')
CmC3-RACE 1	TTGACCTTCCTGCTTTGTTGGCCAG
CmC3-RACE 2	CTAAGTAGCGTGTCGCAGTCAAGG
CmC3-RACE 3	GTTCTCTCAGTGGTGCCTCCTGC
CmC8γ-RACE 1	GGAAAACAACATCCAGGCCCAGATC
CmC3-SF	AGAAAGGAGGGGCATTCA
CmC3-SR	CCTATTGCTGTGATGGTG
CmC8γ-SF	GTGTGGAGGTATGTTGCGTG
CmC8γ-SR	CTCTTCAGGCCATTACGAGC
CmC3-rtF	GGTCTGTGTCAGTGTGCTGA
CmC3-rtR	GCACACTGACACAGACCCTC
CmC8γ-rtF	TTAGTCTCTGGGAGCCTGTG
CmC8γ-rtR	GTAGAAAGCGGCCCAGATTC
β-actin F	CTGCTGGAAGGTGGACAG
β-actin R	AACCTCTCATTGCCAATGGTG