Original Article

Sequence Information, Ontogeny and Expression Analysis of Complement Component C3 in Walking Catfish *Clarias macrocephalus*

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Abstract

Complement system is a powerful host defense mechanism that contributes to both innate and acquired immunities. C3 is the central component of all activation pathways and plays a crucial role in the early immune response. The aim of this present study was to look into ontogeny and expression of C3 in walking catfish *Clarias macrocephalus* by using molecular techniques. A 4140 bp part of the cDNA encoding the walking catfish *C. macrocephalus* complement component C3 was obtained by RACR-PCR amplification. The deduced amino acid sequence showed that this partial sequence of *C. macrocephalus* C3 exhibited 52-64% and 41-45% identity with fish and mammal orthologs, respectively. The *C. macrocephalus* C3 sequence contained many functionally important sites such as thioester site, cleavage sites for C3 convertase and factor I as well as properdin binding site, which are present in mammalian C3. Ontogeny study of *C. macrocephalus* showed that C3 transcripts were not present in unfertilized egg but present immediately after hatching and gradually increased with development. Tissue distribution analysis revealed that C3 transcript was mainly expressed in liver. In addition, the expression level of C3 mRNA was examined in catfish fingerlings fed on β-glucan for 1, 3, 7 and 14 days. Semi-quantitative PCR analysis showed that C3 expression in liver of walking catfish was significantly induced by β-glucan. The highest expression of C3 mRNA was observed in fish fed on β-glucan for 7 days. These results particularly represented that C3 played an essential role in innate immune responses and the expression profile of C3 could be used as a reference marker for assessment of fish health.

Keywords: *Clarias macrocephalus*, complement component C3, gene expression, ontogeny

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Thai J Vet Med. 2012. 42(4): 495-507.

บทคัดย่อ

ลำดับนิวคลิโอไทด์ การพัฒนาการ และการแสดงออกของคอมพลีเมนต์ C3 ในปลาดุกอุย *Clarias macrocephalus*

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ระบบคอมพลีเมนต์เป็นกลไกการป้องกันร่างกายของโฮสท์ที่มีประสิทธิภาพอย่างยิ่งอันหนึ่งซึ่งทำหน้าที่ส่งเสริมการทำงานของระบบภูมิคุ้มกันทั้งแบบไม่จำเพาะ และแบบจำเพาะเจาะจง โดยมีคอมพลีเมนต์ C3 ทำหน้าที่เป็นศูนย์กลางของทุกกลไกการกระตุ้นในระบบคอมพลีเมนต์ และยังมีความสำคัญมากต่อการตอบสนองทางภูมิคุ้มกันในระยะเริ่มแรก การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจสอบพัฒนาการ และการแสดงออกของคอมพลีเมนต์ C3 ในปลาดุกอุย *Clarias macrocephalus* ด้วยวิธีทางชีวโมเลกุล จากการศึกษาโดยวิธี RACR-PCR ได้ชิ้นส่วนของ *C. macrocephalus* C3 cDNA ความยาว 4140 คู่เบส และมีความเหมือนกันกับลำดับกรดอะมิโนในบริเวณที่สอดคล้องกันของ C3 ของปลาและสัตว์เลี้ยงลูกด้วยนมที่ระดับความเหมือนร้อยละ 52-64 และ 41-45 ตามลำดับ จากการเปรียบเทียบลำดับกรดอะมิโนของ *C. macrocephalus* C3 และ C3 จากสัตว์ชนิดอื่นพบว่า *C. macrocephalus* C3 ประกอบไปด้วยส่วนที่ทำหน้าที่สำคัญหลายส่วน เช่น thiolester site, cleavage sites สำหรับ C3 convertase และ factor I และ properdin binding site ซึ่งพบใน C3 จากสัตว์เลี้ยงลูกด้วยนมเช่นกัน จากการศึกษาพัฒนาการชี้ว่า ไม่พบการแสดงออกของยีน C3 ในไข่ที่ยังไม่ได้รับการปฏิสนธิ แต่พบการแสดงออกของยีน C3 ทันทีหลังจากที่ปลาดุกอุยฟักเป็นตัว และระดับการแสดงออกจะเพิ่มขึ้นเมื่อปลามีการพัฒนาหรือมีอายุมากขึ้น และพบว่าตับเป็นอวัยวะหลักที่มีการแสดงออกของยีน C3 นอกจากนี้การศึกษาการแสดงออกของยีน C3 ในตับปลาดุกอุยขนาดปลานิ้วหลังจากได้รับอาหารที่ผสม β-glucan เป็นระยะเวลา 1, 3, 7, 14 วัน ด้วยวิธี Semi-quantitative PCR ยังแสดงว่า β-glucan สามารถกระตุ้นการแสดงออกของยีน C3 ได้อย่างมีนัยสำคัญ โดยมีระดับการแสดงออกมากที่สุดเมื่อปลาได้รับอาหารที่ผสม β-glucan เป็นระยะเวลา 7 วัน ผลการศึกษาครั้งนี้ชี้ให้เห็นว่า C3 มีความสำคัญในการตอบสนองของระบบภูมิคุ้มกันแบบไม่จำเพาะ และ รูปแบบการแสดงออกของยีน C3 ยังสามารถนำมาใช้เป็นเครื่องหมายในการประเมินสุขภาพของปลาได้

คำสำคัญ: ปลาดุกอุย คอมพลีเมนต์ C3 การแสดงออกของยีน การพัฒนาการ

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Introduction

Complement system is a powerful host defense mechanism that contributes to both innate and acquired immunities (Song et al., 2000; Carroll, 2004). This system comprises about 30 serum proteins acting as a cascade reaction and is activated through one of the following three pathways: the classical-, the alternative-, and the lectin- pathways. Activation of any three pathways results in cleavage of the third complement component (C3), and consequently leads to formation of a large pore-forming complex inducing lysis of invading microorganisms. In addition, the activation of the complement system generates biological molecules that enhance phagocytosis, inflammatory reaction, and antibody production (Kirschfink, 1997; Boshra et al., 2006).

C3 is one of the most important components of the complement system, which plays a central role in all three activation pathways. It is a major opsonin of the complement system that functions in coating microorganisms for phagocytosis (Matsuyama et al., 1992), and belongs to the acute phase proteins, which its synthesis increases in response to inflammation (Bayne and Gerwick, 2001). Similar to mammals, teleost fish C3 has been characterized as a glycoprotein, which is composed of the α and β disulfide linked chains, containing an intrachain thiolester bond in the α-chain (Abelseth et al., 2003; Lange et al., 2004c). Although C3 is mainly synthesized in liver hepatocytes, it can be produced by other cell types and tissues, such as skeleton muscle cells, osteocytes, epithelial cells of skin, brain, gills, gut and head kidney, which varies from species to species (Andrews et al., 1995; Morgan and Gasque,

1997; Magnadottir et al., 2005; Lϕvoll et al., 2007[a], 2007[c]). Furthermore, extrahepatic synthesis of C3 was found at different development stages of Atlantic halibut- and Atlantic cod- larval developments, indicating that C3 may play a role in the formation and generation of organs, in addition to its important functions in the early immune response (Lang et al., 2005, 2006).

Walking catfish (*Clarias macrocephalus*) is an air breathing freshwater catfish species which distributes primarily in South- and Southeast- Asia (Frosse and Pauly, 2012). This species is one of the important marketed freshwater fish in Thailand because of its high demand in local market. Also, *C. macrocephalus* is important as a female brood-fish for hybrid catfish production which accounted for 136.5 metric tons in 2008 and ranked the second of all cultured freshwater fish in the country. Culture of *C. macrocephalus* started in the early 1960s and rapidly expanded after the successful production of fish fry by artificial breeding. However, the production of *C. macrocephalus* has been limited by their low growth rate and disease outbreaks. As an alternative, the hybrid catfish, i.e. artificial cross-breeding between male *Clarias gariepinus* and female *C. macrocephalus*, is produced and presently become a dominant catfish species widely cultured throughout the country with over 90% of *Clarias* production in Thailand, making culture of *C. macrocephalus* important for providing female brood-stock. Due to its low disease resistance, *C. macrocephalus* is vulnerable to bacteria and parasite infections, particularly during larval rearing, which consequently results in shortage of female brood-fish. Therefore, it is important to establish strategies to promote an effective immune system in fish larvae to produce healthy fry and reduce use of antibiotics in the fish culture. However, the ontogenetic development of the immune system fluctuates among different fish species. Up to the present, no ontogenetic studies have been conducted in this species. Therefore, the present study aims to present the ontogeny and tissue expression of C3, an effective component of the immune system, in walking catfish *C. macrocephalus*, as well as the modulation of this component after β-glucan administration.

Materials and Methods

Cloning and sequencing of C. macrocephalus C3 cDNA: Total RNA was extracted from liver tissue of adult *C. macrocephalus* 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 primer, CmC3-RACE 1 (Table 1), was designed from the 3' end fragment of *C. macrocephalus* C3 cDNA obtained from the expressed sequence tags (EST) analysis (Panprommin et al., 2007). 5'-RACE-PCR reaction was carried out with the initial denaturation step at 94^{0}C for 120 sec, followed by 30 cycles of 94^{0}C for 30 sec, 68^{0}C for 30 sec and 72^{0}C for 4 min, and the final extension step at 72^{0}C for 5 min. Two more gene-specific primers, CmC3-RACE-2 and CmC3-RACE3, were designed based on the nucleotide sequence of *C. macrocephalus*. C3 cDNA was 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 service of First Base Laboratories Sdn Bhd, Malaysia.

To obtain a single fragment of the partial *C. macrocephalus* C3 cDNA, PCR amplification was performed by using two sets of gene-specific primers (CmC3-SF and CmC3-SR). The PCR condition was at 94^{0}C for 120 sec min; 30 cycles of 94^{0}C for 30 sec, 55^{0}C for 30 sec and 72^{0}C for 4 min, followed by 72^{0}C for 5 min. The amplification products were cloned and their plasmids were isolated and sub-cloned for sequencing as described above.

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 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.

Determination of C. macrocephalus C3 mRNA expression level during larval development: Catfish larvae were obtained by artificial breeding from Fishery farm unit, Ubon Ratchathani University, Thailand. The culture of larval stage was carried out under the routine larval rearing process. The larvae were continuously reared in a 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 phosphate buffer saline (PBS) and frozen in liquid nitrogen before homogenization with Trizol reagent. Total RNA was extracted and cDNA was synthesized and the expression level of *C. macrocephalus* C3 transcripts was determined by PCR as the procedure described above. Beta-actin was used as the internal control for cDNA levels in different ages.

Tissue expression analysis of C. macrocephalus C3: Total RNA from various tissues of healthy *C. macrocephalus* including brain, heart, kidney, liver, spleen, intestine, muscle and ovary were extracted following the protocol described above. Two

micrograms of total RNA were 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 *C. macrocephalus* C3 specific primers (CmC3-rtF and CmC3-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^{0}C for 120 sec, 25 cycles of 94^{0}C for 30 sec, 60^{0}C for 30 sec and 72^{0}C for 30 sec followed by 72^{0}C for 5 min. The products were analyzed on a 1.5% ethidium bromide-stained agarose gel.

In situ hybridization: *In situ* hybridization was conducted under RNase-free condition. All solutions were treated with 0.1% diethylpyrocarbonate (DEPC), which had been autoclaved before used.

Tissue preparation

The larval samples of fish at the age of 30 days post hatching were rapidly rinsed in PBS and immediately fixed in freshly-prepared 10% neutral buffered 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 4-6 µm, mounted on poly-*L*-lysine coated glass slides, air dried overnight and stored at 4^{0}C until used.

Riboprobe synthesis

Riboprobes were prepared from the plasmids containing *C. macrocephalus* C3 cDNA. 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. *C. macrocephalus* C3 cDNA containing vectors was 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).

Hybridization

Paraffin embedded sections were dewaxed in xylene and rehydrated in sequences of ethanol (96%, 70% and 30%, respectively). The sections were then treated with proteinase K (10 µg/ml in 10 mM Tris-HCl (pH 8), 1 mM EDTA) at 37^{0}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 hour at room temperature, the sections were prehybridized in a hybridization buffer (50% formamide, 5 x SSC and 10% dextran sulphate) at 55^{0}C for 2 hours. 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 hours at 55^{0}C in a humidified chamber. Then, the hybridized sections were washed with 2 x SSC at room temperature and treated with 20 µg/ml RNase A in 2 x SSC at 37^{0}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 hour at room temperature. The sections were then rinsed in Tris-buffer saline (TBS) to stop the reaction and mounted in 10% glycerol in TBS.

Determination of C. macrocephalus C3 mRNA expression levels after β-glucan feeding: Healthy *C. macrocephalus* fingerlings with an average weight of 10.5 g were acclimated under laboratory conditions for 1 week and fed on commercial diet at 5% of body weight per day. The fingerlings were subsequently fed daily on 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. Amplification of *C. macrocephalus* C3 was conducted with the same gene-specific primers as in RT-PCR (Table 1) under the condition as follows: 1 cycle of 94^{0}C for 120 sec, 25 cycles of 94^{0}C for 30 sec, 60^{0}C for 30 sec and 72^{0}C for 30 sec followed by 72^{0}C for 5 min. The products were subsequently electrophoresed on 1.5% agarose gel containing ethidium bromide.

The expression level of *C. macrocephalus* C3 gene was normalized relative to the β-actin gene expression levels using Genetools Analysis Software (Syngene). Statistical differences between groups were analyzed by one-way ANOVA. If there was significant difference Duncan's multiple range test was applied for group-comparisons. Statistical analysis was performed by using the Program R-statistics (R-Development Core Team, 2011).

Table 1 Primer sequences used in this study

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

Results

Sequence analysis of C. macrocephalus C3 cDNA: The partial cDNA sequence for *C. macrocephalus* C3 was obtained by RACE-PCR and consisted of 4140 bp, comprising an incomplete open reading frame of 3933 bp and a 3′ UTR of 233 bp containing the poly A tail (Fig 1a). The 3′ UTR contained a consensus polyadenylation signal (AATAAA) 18 bp upstream from the poly A tail. The incomplete open reading frame of *C. macrocephalus* C3 sequence encoded a part of protein consisting of 1311 amino acid residues. Three possible N-glycosylation sites were found at amino acid positions 507, 599 and 978 (Fig 1a).

The deduced 1311 amino acid sequence of the partial *C. macrocephalus* C3 was compared with the corresponding region of other known C3 sequences The partial *C. macrocephalus* C3 had greater similarity to the corresponding part of C3s from teleost fish (52-64% identity; 70-79% similarity) than to those of mammals (41-45% identity; 62-64% similarity), as shown in Table 2. Figure 2 shows amino acid sequence alignment of the partial *C. macrocephalus* C3 with the corresponding part of other known C3s using CLUSTAL X program, indicating the conserved amino acids and several functionally important sites. The alignment revealed that this part of *C. macrocephalus* C3 contained the partial β chain located at the NH_2-terminal end and the complete α chain located at the COOH-terminal. The potential β-α processing signal of *C. macrocephalus* C3 (RKRR motif) was found to align perfectly with the homologous position of other C3s. The cystein residues related with disulfide bonds between the β-α chains in human were conserved in *C. macrocephalus* C3 (Figs 1b, 2). Likewise, *C. macrocephalus* C3 possessed the factor I and C3 convertase cleavage sites (Arg-Ser) as found in human, with the exception of the second factor I cleavage site (Arg-Thr). Moreover, the thiolester site (GCGEQ) of *C. macrocephalus* was completely identical to those of other C3s. The key amino acids for other complement proteins, such as factor B, and factor H as well as properdin binding, were also conserved in *C. macrocephalus* C3 (Fig 2).

Table 2 Amino acid comparison between *C. macrocephalus* C3 and other C3s

Species	GenBank Accession no.	% identity/ similarity
Common carp, *Cyprinus carpio*	AB016210	64/79
Zebrafish, *Danio rerio*	XM002660575	61/78
Rainbow trout, *Oncorhynchus mykiss*	L24433	58/74
Japanese flounder, *Paralichthys olivaceus*	AB021653	54/71
Spotted wolffish, *Anarhichas minor*	AJ309570	53/69
Japanese medaka, *Oryzias latipes*	AB025576	52/70
Norway rat, *Rattus norvegicus*	NM016994	45/64
Cattle, *Bos taurus*	AM086793	45/63
Human, *Homo sapiens*	AY513239	41/62

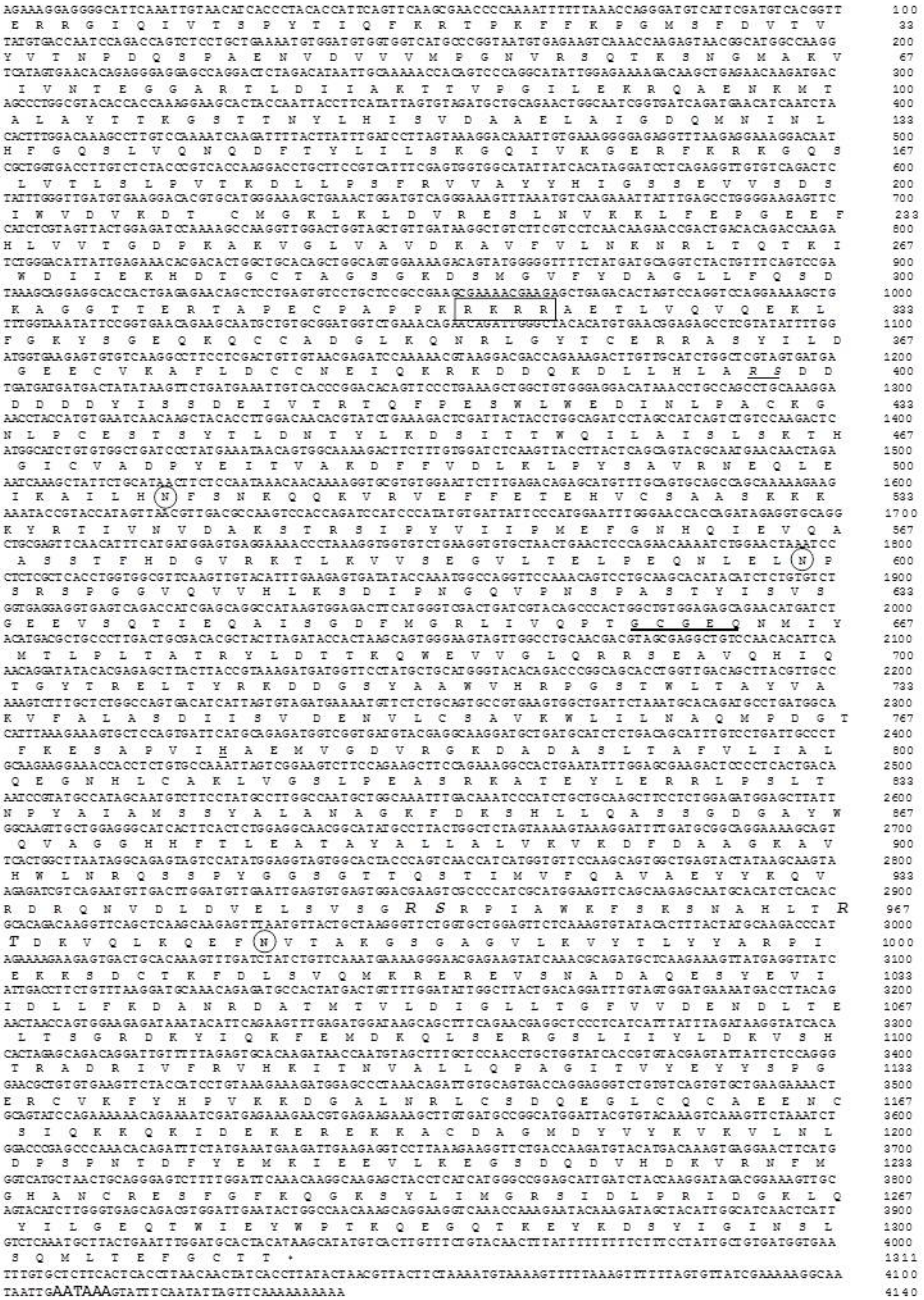
Phylogenetic analysis of C. macrocephalus C3: Phylogenetic analysis of *C. macrocephalus* C3 with C3 molecules from other fish species was performed using the Clustal software, based on the deduced amino acid sequence alignment of the C3 α-chains. The resulting tree showed that all the bony fish grouped a cluster (Fig 3). *C. macrocephalus* C3 was grouped with zebra fish and carp C3 indicating that the walking catfish (Order: Siluriformes) and other two fish species (Order: Cypriniformes) were phylogenetically close species. Moreover, among these three species, zebra fish and carp, which belong to the same order, are strongly genetically related.

Ontogenic appearance of C. macrocephalus C3 mRNA: Semi-quantitative RT-PCR was used to examine the expression level of *C. macrocephalus* C3 mRNA during catfish larval development. *C. macrocephalus* C3 was found to be expressed at a low level at 1, 3 and 5 days post hatching. Strong expressions were observed at 20 and 30 days after hatching. Overall, transcripts of *C. macrocephalus* C3 gradually increased as development progressed (Fig 4).

Tissue expression of C. macrocephalus C3: Expression profiles of *C. macrocephalus* C3 in various tissues were studied by RT-PCR. *C. macrocephalus* C3 mRNA expression was detected mainly in liver (lane 4: Fig 5). Expression was also observed at low level in brain (lane 1), heart (lane 2) and muscle (lane 7) for *C. macrocephalus* C3. Expression of β-actin used as internal control was observed in all tested tissues. Localization of *C. macrocephalus* C3 mRNA expression in fish larvae was also determined by *in situ* hybridization. Fish larval sections were prepared and hybridized with the sense and anti-sense probes for *C. macrocephalus* C3 mRNA. Hybridization performed with the *C. macrocephalus* C3 anti-sense probe showed staining of hepatocyte cells (dark purple hepatocytes) (Fig 6). There was no staining of the control sections hybridized with the *C. macrocephalus* C3 sense probe.

Effect of β-glucan feeding on C. macrocephalus C3 expression: The relative expression level of *C. macrocephalus* C3 was examined in catfish fingerlings fed on β-glucan for 1, 3, 7 and 14 days. Semi-quantitative PCR analysis showed significant up-regulation of *C. macrocephalus* C3 mRNA in all β-glucan feeding groups (Fig 7), compared to the 0 day unstimulated group ($p< 0.05$). The highest level of C3 gene expression was observed in catfish fingerlings fed on β-glucan for 7 days.

A



B

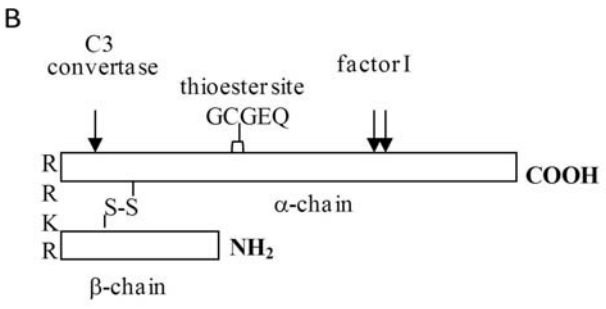
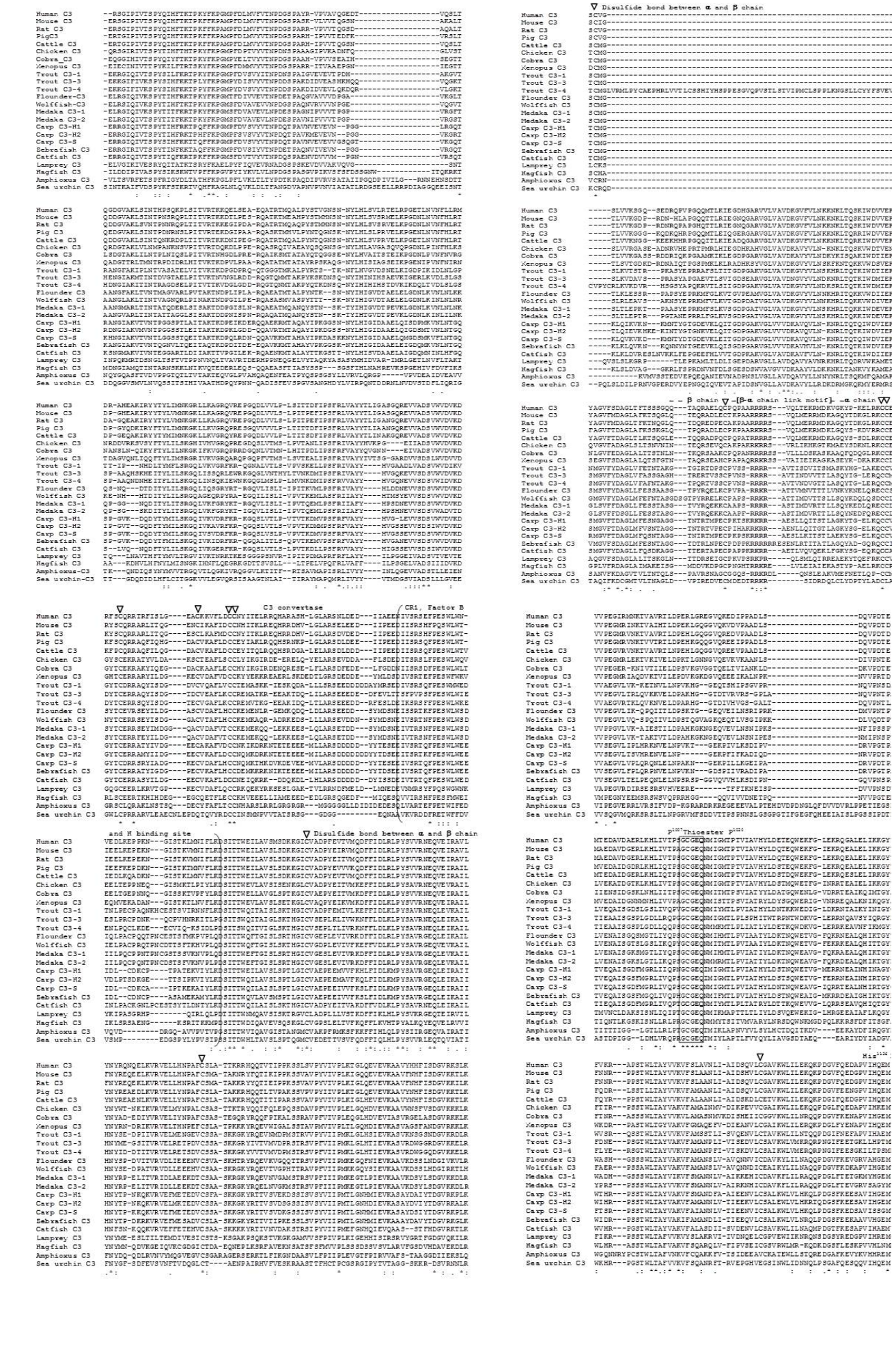
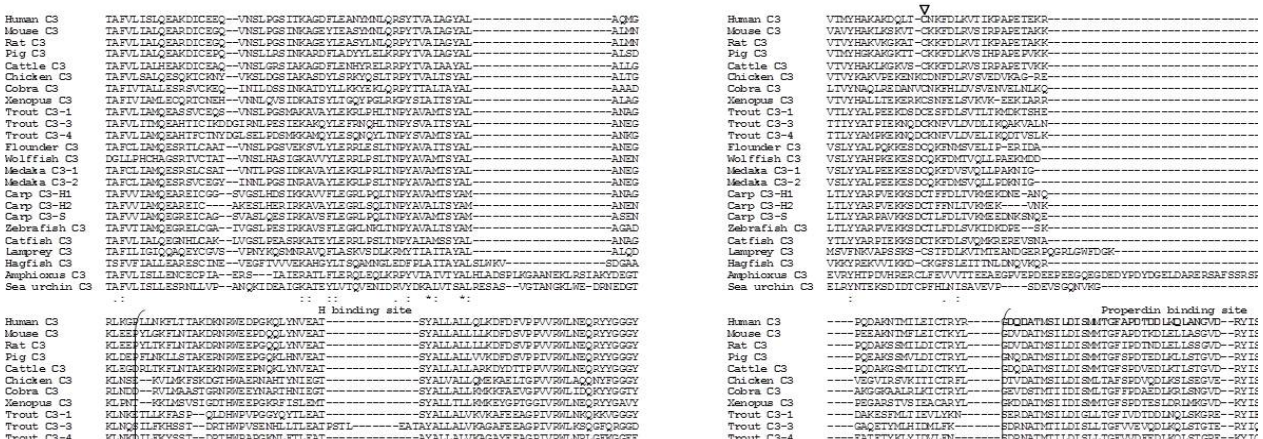


Figure 1 (A) Partial nucleotide and deduced amino acid sequences of *C. macrocephalus* C3. The predicted glycosylation sites are indicated by circles. The potential β-α chain processing signal is enclosed in square. The putative cleavage sites for C3 convertase and factor I are shown in underlined italic letters and bold italic letters, respectively. The thioester site is underlined. The polyadenylation signal (AATAAA) is shown in bold letters at the end of 3′ UTR. (B) Schematic diagram of polypeptide structure of *C. macrocephalus* C3, showing the major functional sites.




H binding site
Properdin binding site
Factor I cleavage sites

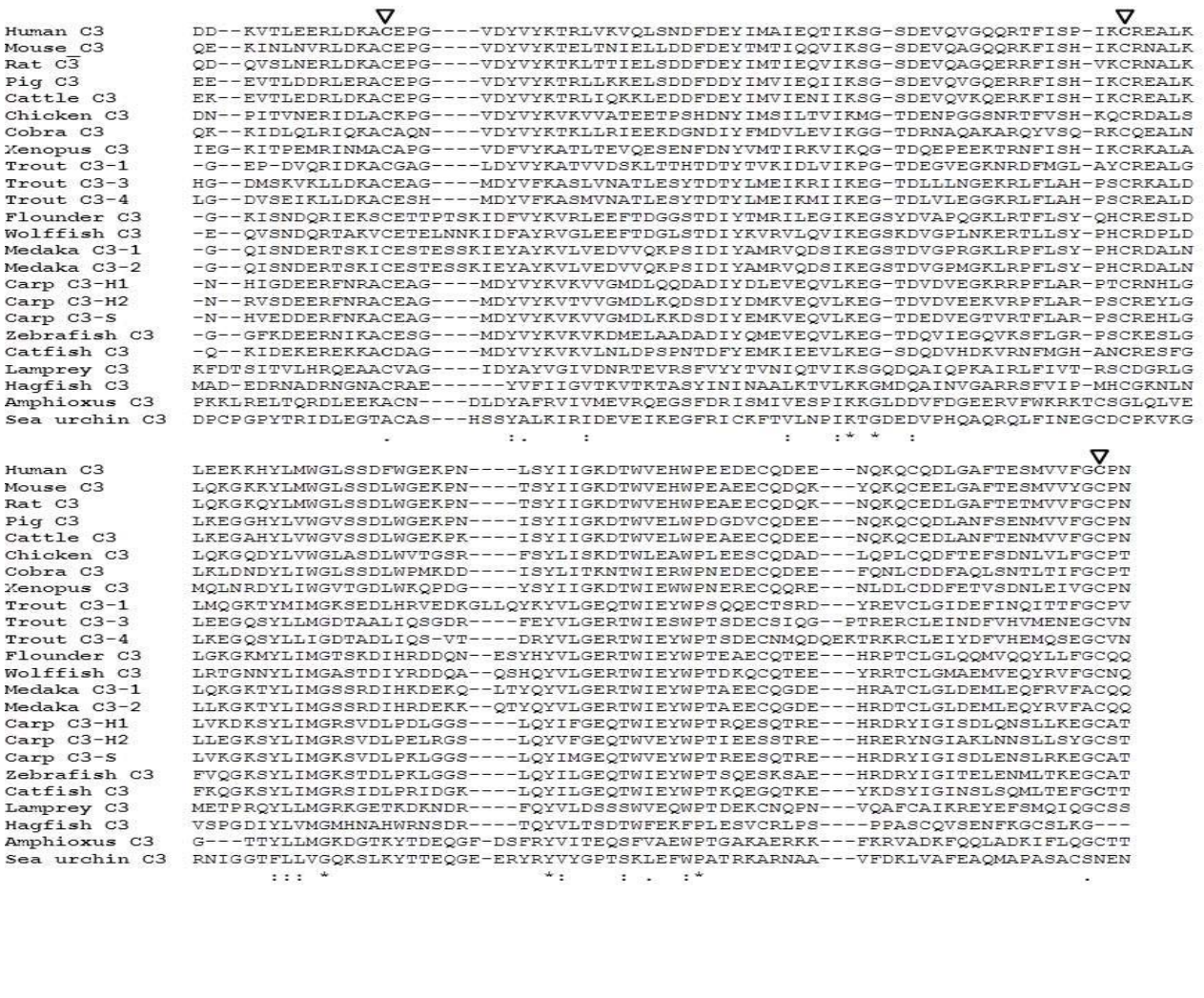

Human C3
Mouse C3
Rat C3
Pig C3
Cattle C3
Chicken C3
Cobra C3
Xenopus C3
Trout C3-1
Trout C3-3
Trout C3-4
Flounder C3
Wolffish C3
Medaka C3-1
Medaka C3-2
Carp C3-H1
Carp C3-H2
Carp C3-S
Zebrafish C3
Catfish C3
Lamprey C3
Hagfish C3
Amphioxus C3
Sea urchin C3

Figure 2 Alignment of C3 amino acid sequences between *C. macrocephalus* and other known sequences. The 1311 amino acid sequence of *C. macrocephalus* C3 (Catfish C3, Genbank accession no. AB636135) was aligned with the corresponding part of *Homo sapiens* C3 (Human C3, Genbank accession no. K02765), *Mus musculus* C3 (Mouse C3, Genbank accession no. NM009778), *Rattus norvegicus* C3 (Rat C3, Genbank accession no. NM016994), *Bos taurus* C3 (Cattle C3, Genbank accession no. NM001040469), *Gallus gallus* (Chicken C3, Genbank accession no. NM205405), *Naja naja* (Cobra C3, Genbank accession no. Q0331), *Xenopus tropicalis* (Xenopus C3, Genbank accession no. BC168633), *Oncorhynchus mykiss* (Trout C3-1, Genbank accession no. P98093), *O. mykiss* (Trout C3-3, Genbank accession no. U 61753), *O. mykiss* (Trout C3-4, Genbank accession no. AF271080), *Paralichthys olivaceus* C3 (Flounder C3, Genbank accession no. AB021653), *Anarhichas minor* C3 (Wolffish C3, Genbank accession no. AJ309570), *Oryzias latipes* C3-1 (Medaka C3-1, Genbank accession no. NM_001105082), *O. latipes* C3-2 (Medaka C3-2, Genbank accession no. NM_001105083), *Cyprinus carpio* C3-H1 (Carp C3-H1, Genbank accession no. AB016210), *C. carpio* C3-H2 (Carp C3-H2, Genbank accession no. AB016212), *C. carpio* C3-S (Carp C3-S, Genbank accession no. AB016213), *Danio rerio* C3 (Zebrafish C3, Genbank accession no. XM002660575), *Lethenteron japonic*um C3 (Lamprey C3, Genbank accession no. AY359861), *Eutatretus burger* (Hagfish C3, Genbank accession no. P98094), *Branchiostoma belcheri* (Amphioxus C3, Genbank accession no. AB050668), and *Strongylocentrotus purpuratus* (Sea urchin C3, Genbank accession no. NM214521) using the CLUSTAL X program. Conservation of amino acid identity is shown with an asterisk '*' whereas ':' and '.' indicate high and low levels of amino acid similarity, respectively. Conserved cystein residues forming disulfide bonds in *C. macrocephalus* C3 are indicated by reverse triangles (∇).

Discussion

C3 is a member of the α_2-macroglobulin family, which includes several related proteinase inhibitors, C4 and C5 (Armstrong and Quigley, 1999). It is one of the most important components of the complement system, which plays a central role in all activation pathways leading to inflammatory reaction, opsonisation and lysis of pathogens (Boshra et al., 2006). The molecule has been isolated and characterized from several teleost fish such as rainbow trout (Sunyer et al., 1996), common carp (Nakao et al., 2000), seabream (Sunyer et al., 1997), spotted wolfish (Abelseth et al., 2003), Atlantic cod and Atlantic halibut (Lang et al., 2004[c]). Ontogeny and source of expression of C3 in these fish are different from species to species (Magnadottir et al., 2005). Here, the cloning and expression of C3 from walking catfish, *C. macrocephalus* are described. Approximately four-fifth (4,140 bp) of C3 cDNA from walking catfish, *C. macrocephalus,* was cloned and sequenced. There was the 5′ end of about 1026 bp, which encoded for 342 amino acid residues of *C. macrocephalus* C3 cDNA remained in this study. The fragment constitutes a signal peptide sequence that is responsible for the membrane transportation of C3 protein. Although the study could not get over the full-length sequence of *C. macrocephalus* C3 cDNA, the obtained sequence is adequate to generate basic information of *C. macrocephalus* C3. The deduced amino acid sequence of the obtained C3 showed high sequence similarity to known teleost C3 proteins by the BLAST program (Table 2) and was clearly grouped as a cluster with teleost C3 upon phylogenetic analysis (Fig 3). The sequence also had high similarity to C3 molecules of mammals. Nevertheless, the sequence had a little similarity to other complement protein C4 which is a component derived from a common ancestor gene.

Alignment of amino acid sequences between the obtained partial *C. macrocephalus* C3 and the corresponding part of other known C3 indicated that the obtained *C. macrocephalus* C3 contained several functionally important sites. Conservation of the potential β-α processing site among *C. macrocephalus* C3 amino acid sequence indicated that the walking catfish C3 was composed of an α-chain (C-terminus) and β-chain (N-terminus) after post translational modification (Fig 1b) as found in mammals (de Bruijin and Fey, 1985), other bony fish (Zarkadis et al., 2001; Abelseth et al., 2003; Lange et al., 2006) and cartilage fish (Dodds et al., 1998). Meanwhile, C3 proteins of the lower evolutionary animals such as hagfish (Fujii et al., 1995), lamprey (Nonaka, 1994), amphioxus (Suzuki et al., 2002), carpet-shell clam (Prado-Alvarez et al., 2009) and horseshoe crab (Zhu et al., 2005) had been reported to have two processing sites on amino acid sequence that lead to a three-chain protein. However, the sequence for these processing sites varies among C3 molecules (Fig 2). In human C3, tetra-arginine, RRRR, acts as a cleavage site for the β-α chain, whereas the putative processing site, RKRR, occurs in the C3 molecules of walking catfish, pig and chicken; RRKR occurs in the C3 molecules of xenopus, Japanese flounder, hagfish, amphioxus and sea urchin; and RKPR occurs in the Japanese lamprey. These findings suggest that RXXR is the consensus sequence for the the β-α chain processing site (Castillo et al., 2009). Two critical cysteine residues, responsible for making disulfide bond between β and α chain in all C3 molecules, are also conserved in *C. macrocephalus* C3.

Similar to other C3s, *C. macrocephalus* C3 contains an active thiolester site (GCGEQ) in the α-chain, which reacts with amino or hydroxyl group present on foreign cell surfaces upon activation and a conformation change in C3 molecule (Holland and Lambris, 2002). The thiolester site is surrounded by hydrophobic amino acids, as found in other C3. Pro^{1007} and Pro^{1020} residues that have been suggested to be essential for stability of thiolester formation in human C3 conserved in *C. macrocephalus* C3 (Isaac and Isenman, 1992). Furthermore, *C. macrocephalus* C3 contains a catalytic His residue located on 113 amino acids downstream of the thioester site and a Glu residue located on two amino acids downstream from the catalytic His residue (His^{1126} and Glu^{1128} of human C3). *In vitro* mutagenesis of human C3 has shown that both the His and Glu residues are important in determining the thioester-binding specificity of C3 to its target surface (Gadjeva et al., 1998).

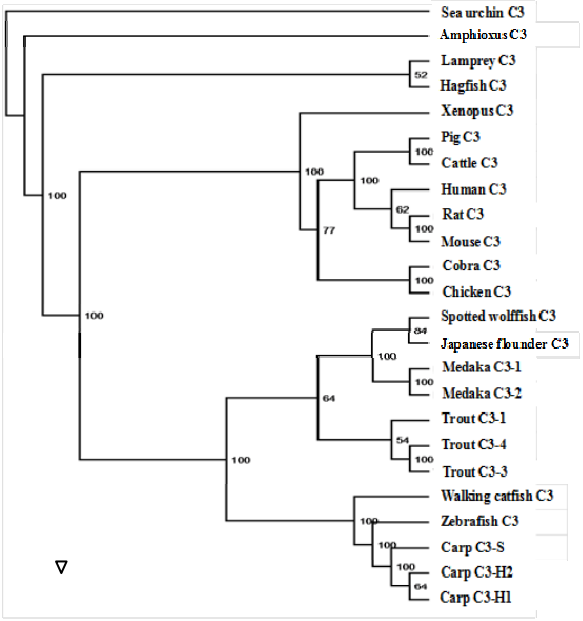


Figure 3 Phylogenetic relationships of C3 proteins from different species. The tree was conducted by the neighbor-joining method, based on the deduced amino acid sequence alignment of the α chains. C3 Catfish C3, (Genbank accession no. AB636135), Human C3 (Genbank accession no. K02765), Mouse C3, (Genbank accession no. NM009778), Rat C3 (Genbank accession no. NM016994), Cattle C3 (Genbank accession no. NM001040469), Chicken C3 (Genbank accession no. NM205405), Cobra C3 (Genbank accession no. Q0331), Xenopus C3 (Genbank accession no. BC168633), Trout C3-1 (Genbank accession no. P98093), Trout C3-3 (Genbank accession no. U 61753), Trout C3-4 (Genbank accession no. AF271080), Flounder C3 (Genbank accession no. AB021653), Wolffish C3 (Genbank accession no. AJ309570), Medaka C3-1 (Genbank accession no. NM_001105082), Medaka C3-2 (Genbank accession no. NM_001105083), Carp C3-H1 (Genbank accession no. AB016210), Carp C3-H2 (Genbank accession no. AB016212), Carp C3-S (Genbank accession no. AB016213), Zebrafish C3 (Genbank accession no. XM002660575), Lamprey C3 (Genbank accession no. AY359861), Hagfish C3 (Genbank accession no. P98094), Amphioxus C3 (Genbank accession no. AB050668), Sea urchin C3 (Genbank accession no. NM214521).

The comparison of the C3 sequences identifies the cleavage sites for C3 convertase and factor I, which play important regulatory roles in controlling the biological activity of C3 (de Bruijn and Fey, 1985). Cleavage of C3 convertase leads to the release of anaphylatoxin C3a and consequently creates the major C3b fragment. A putative C3 convertase cleavage site in *C. macrocephalus* C3 has the same specific sequence Arg-Ser as human C3 and most of C3s from other species aligned in Fig 2, indicating that C3 convertase from many fish species have similar binding specificities to that of human complement (Zarkadis et al., 2001). Two cleavage sites for serine proteinase factor I conserved in *C. macrocephalus* C3 were Arg-Ser in corresponding to Arg-Ser at position 1281 of human C3 and Arg-Thr instead of Arg-Ser at position 1298 of human C3. Replacement of Arg-Thr residue at the second factor I

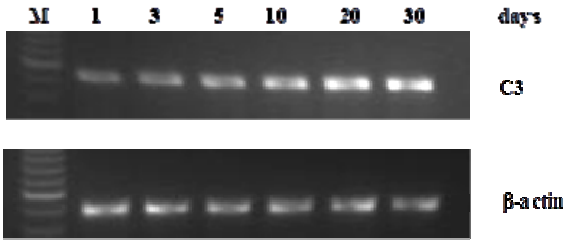


Figure 4 Semi-quantitative RT-PCR analysis of C3 in the different larval stages of *C. macrocephalus*. M indicates the molecular weight marker (100 bp ladder).

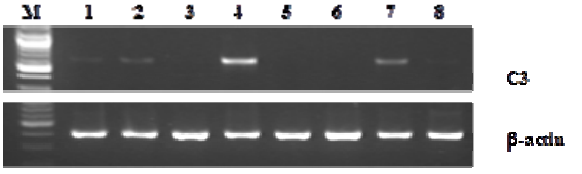


Figure 5 Expression of *C. macrocephalus* C3 gene in various tissues. Lane 1, brain; lane 2, heart; lane 3, kidney; lane 4, liver; lane 5, spleen; lane 6, intestine; lane 7, muscle; lane 8, ovary. M indicates the molecular weight marker (100 bp ladder).

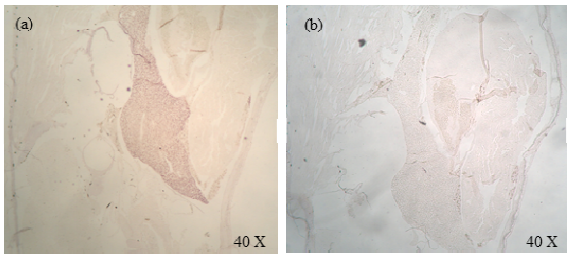


Figure 6 Expression of C3 mRNA in liver hepatocytes performed by *in situ* hybridization with anti-sense probe (a) and sense probe (b)

cleavage site was also found in trout C3-1 and C3-3 (Zarkadis et al., 2001). Furthermore, it was reported that Trout C3-1 could be cleaved by factor I at Arg-Thr bond in the presence of adequate cofactor (Alsenz et al., 1992). The results of the study suggest that *C. macrocephalus* C3 functions similarly to C3 molecules of other animals.

Developmental expression study of C3 in different stage of *C. macrocephalus* larvae showed that C3 transcripts were immediately detected in *C. macrocephalus* after hatching and gradually increased as development progressed. In addition, *C. macrocephalus* mRNA was not detected in eggs prior to fertilization. Similar studies on spotted wolfish and Atlantic salmon also revealed that C3 mRNA was steadily increased from embryo toward hatching and no C3 mRNA was observed in unfertilized eggs (Ellingsen et al., 2005; Løvoll et al., 2007[c]). These were in accordance with the presence of C3 protein in early stages of larval development determined in Alantic cod (Lange et al., 2004[a]), Atlantic halibut (Lange et al., 2004[b]) and Atlantic salmon (Løvoll et al., 2007[b]). These indicate that C3 plays a crucial role in the early immune response of fish larvae.

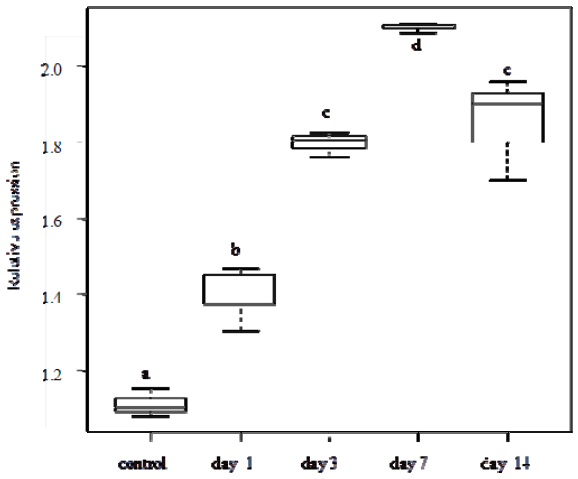


Figure 7 Relative expression of *C. macrocephalus* C3 in liver from *C. macrocephalus* administered with β-glucan. Fish were collected at 1, 3, 7 and 14 days post β-glucan feeding. *C. macrocephalus* mRNA levels were determined by semi-quantitative RT-PCR and standardized according to the respective β-actin mRNA levels. Statistically significant differences are indicated by different letters ($p< 0.05$).

Tissue distribution analysis in adult walking catfish revealed that C3 was mainly expressed in liver, but low in other tissues, including brain, heart and muscle. This result indicates extrahepatic synthesis of C3 in *C. macrocephalus* suggesting that C3 may play a crucial role in local inflammatory process. However, C3 mRNA has been found only in liver hepatocytes of 30 day-old walking catfish larva by *in situ* hybridization, but this may be due to amount of mRNA and sensitivity of the technique. Although hepatocytes are known as the primary source of C3 synthesis in mammals, C3 has also been found to be expressed in various tissues such as brain, kidney, lung, skin intestine, muscle and fat tissues (Morgan and Gasque, 1997). In other fish, extrahepatic synthesis of C3 was observed in a wide range of tissues at different stage of larval development of Atlantic halibut and Atlantic cod (Lange et al., 2005, 2006). In rainbow trout, C3 mRNAs of all subtypes were also widely expressed in various tissues although their degree of expression was low when compared to liver (Løvoll et al., 2007[a]). Similarly, transcript of C3 was observed in other tissues beside liver in Alantic salmon (Løvoll et al., 2007[b]) and Indian major carp (Mishra et al., 2009). Contrastly, in spotted wolfish, C3 was found to have limited expression only in liver of larvae and adult fish (Ellingsen et al., 2005). These data indicate that pattern of the expression of C3 in teleosts is species specific (Boshra et al., 2006) and liver is the major organ for C3 production.

Many studies had examined the use of immunostimulants such as β-glucan to prevent fish and shellfish infectious diseases. β-glucan was found to be able to enhance innate immune response and disease resistance in fish and shrimp (Ai et al., 2007; Zhao et al., 2012). It was reported that β-glucan are able to activate the complement system in fish (Engstad et al., 1992; Bagni et al., 2005; Misra et al., 2006). However, there is little information on how the expression of the complement genes is affected by the introduction of immunostimulants. The present study showed that C3 expression in liver of walking catfish was significantly induced by β-glucan feeding. Similar results were observed in gilthead seabream fed on live yeast as a source of β-glucan (Reyes-Becerril et al., 2008). In rainbow trout, C3-1 (the most promiment subtype) and C3-3 were induced after β-glucan stimulation although a moderate down regulation of C3-4 was also observed (Løvoll et al., 2007[b]). Lipopolysaccharide (LPS) was also found to induce the expression of C3 (Wang et al., 2008). Moreover, the expression of C3 was up-regulated in the liver of grass carp and common carp infected with the ectoparasites indicating its behavior as acute-phase protein (Chang et al., 2005; Gonzalez et al., 2007). These results particularly presented that C3 played a crucial role in innate immune responses and the expression profile of C3 could be used as a reference marker for assessment of fish health.

In conclusion, the partial *C. macrocephalus* C3 sequence isolated, from this study, had high similarity to the corresponding part of C3 of other teleost and contains several functional sites found in other species. Liver is the major site of C3 synthesis in *C. macrocephalus* and the expression of C3 mRNA transcripts can also be found in extra-hepatic tissues. The synthesis of this component was immediately detected in *C. macrocephalus* after hatching and gradually increased as development progressed, indicating the important role of C3 in the early immune response of walking catfish larvae. Moreover, the expression of C3 gene is up regulated in the liver of waking catfish fingerling fed on β-glucan. These results can be subsequently exploited for any studies to establish strategies for prophylaxis and control of disease in *C. macrocephalus* culture.

Acknowledgements

This work was supported by grants from the High Education Commission and the Thailand Research Fund (TRF) (MRG5080040).

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