Discussion

Complement system is a powerful host defense that comprises of about 35 individuals. Activation of complement system results in the generation of activated protein fragment that plays a role in microbial killing, phagocytosis, inflammatory reaction and antibody production (Kirschfink, 1997; Boshra *et al.*, 2006). Fish appear to possess activation pathway similar to mammals. However, understanding the complement system in fish and the role of the individual proteins in the host defense is still limited in some species such as common carp (Yano and Nakao, 1994; Nakao and Yano, 1998) and rainbow trout (Sunyer *et al.*, 1996; Sunyer *et al.*, 1998; Bayne *et al.*, 2001; Zarkadis *et al.*, 2001). Results of the EST from *Clarias macrocephalus* in the present work revealed that two genes were found to be related to the complement system, the C3 and C8γ.

An approximately four-fifth (4140 bp) of C3 cDNA from walking catfish, *C. macrocephalus,* was cloned and sequenced. 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. 5). The sequence also showed similarity to C3 molecules of mammals. However, 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 of 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 is composed of an α-chain (C-terminus) and β-chain (N-terminus) after post translational modification 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), have 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

X). 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 suggest that RXXR is the consensus sequence for the the β-α chain processing site (Castillo *et al.*, 2009). Two critical cysteine residues, which are 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 are 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 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).

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 consequencely 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. X, indicating that C3 convertase from many 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 cleavage site was also found in trout C3-1 and C3-3 (Zarkadis *et al.*, 2001). Furthermore, it has been reported that Trout C3-1 can be cleaved by factor I at Arg-Thr bond in the presence of

adequate cofactor (Alsenz *et al.*, 1992). These sequence data may suggest that *C. macrocephalus* C3 functions similarly to C3 molecules of other animals.

Tissue distribution analysis in adult walking catfish revealed that C3 was mainly expressed in liver. Expression appeared to be low in other tissues, including brain, heart and muscle. This result indicates extrahepatic synthesis of C3 in *C. macrocephalus*. However, C3 mRNA has been found only in liver hepatocytes of 30 day old walking catfish larva by *in situ* hybridization but this may have been due to amount of mRNA and sensitivity of the technique. Although hepatocytes are known as the primary source of C3 synthesis in mammals, but C3 has also been found to be expressed in a variety of tissues such as brain, kidney, lung, skin intestine, muscle and fat tissues (Morgan and Gasque, 1997). In other fish, extrahepatic synthesis of C3 has been detected in a wide range of tissues at different stage of larval development of Atlantic halibut and Atlantic cod (Lange *et al.*, 2004a, 2004b; Lange *et al.*, 2006). In rainbow trout, C3 mRNAs of all subtypes are also widely expressed in various tissues although their degree of expression is low when compared to liver (Løvoll *et al.*, 2007a). Similarly, transcript of C3 is observed in other tissues beside liver in Alantic salmon (Løvoll *et al.*, 2007b) and Indian major carp (Mishra *et al.*, 2009). Whereas, in spotted wolfish, C3 has been found to have limited expression only in liver of larvae and adult fish (Ellingsen *et al.*, 2005). These data suggest 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. However, in the future, the expression patterns of multiple isoforms should be further examined in other teleosts (Boshra *et al.*, 2006)

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, rainbow trout 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.*, 2006; Løvoll *et al.*, 2007b). These were in accordance with the presence of C3 protein in early stages of larval development determined in Alantic cod (Lange *et al.*, 2004a), Atlantic halibut (Lange *et al*, 2004b) and Atlantic salmon (Løvoll *et al.*, 2007b). These indicate that C3 plays an important role in the early immune response of fish larvae.

Many studies have examined the use of immunostimulants such as β-glucan to prevent fish and shellfish infectious diseases. β-glucan has been found to be able to enhance innate immune response and disease resistance in fish (Ai *et al.*, 2007). It has been reported that β-glucan are capable of activating the complement system in fish (Engstad *et al.*, 1992; Bagni *et al.*, 2005; Misra *et al.*, 2006). However, little is known about how the expression of the complement genes is affected by the introduction of immunostimulants. In this study, the results show that C3 expression in liver of walking catfish was significantly induced by β-glucan feeding. Similar result was observed in gilthead seabream fed with 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). Lipopolysaccharide (LPS) was also found to induce the expression of C3 (Wang *et al.*, 2008). Moreover, the expression of C3was up-regulated in liver of grass carp and common carp infected with the ectoparasites, indicating its behavior as acute-phase protein (Chang *et al.*, 2005; Santiago *et al.*, 2007). Therefore, the expression levels of C3 could be used as a reference marker for assessment of fish health (Mishra *et al.*, 2009).

Parallel studies were also conducted for *C. macrocephalus* C8γ. The complete cDNA sequence of *C. macrocephalus* C8γ was 886 bp in size encoding 211 amino acid sequences. The deduced amino acid sequence showed the highest similarity to C8γs from rainbow trout and zebra fish. *C. macrocephalus* C8γ also showed high similarity to mammalian C8γs and slightly lower similarity to other lipocalin proteins α-1-microglobulin and prostaglandin D-synthase (Table 3). The sequence contained no potential *N*-glycosylation site as in human C8γ (Schreck *et al.*, 2000).

Alignment of amino acid sequences between *C. macrocephalus* C8γ and other known C8γs indicated that three conserved cystein residues involved in formation of disulphide bond in most C8γ proteins. The $Cys^{40}$of human C8γ, which involved in linkage to Cys^{164} of the C8α subunit and two additional Cys^{76} and Cys^{168} residue for intra disulphide bond, were conserved in *C. macrocephalus* C8γ. The high sequence similarity of *C. macrocephalus* C8γ with human C8γ implies their highly conserved conformation of the tree dimensional structure. *C. macrocephalus* C8γ seems to contain lipocalin domain as found in human C8γ.

Phylogenetic tree showed that *C. macrocephalus* C8γs was more closely related to C8γ of fishes and mammals than other lipocalins (Fig. 6).

Tissue distribution analysis revealed constitutive expression of C8γ in walking catfish's liver. Meanwhile, the weak expression was detected in kidney, spleen, intestine and muscle. C8γ mRNA was expressed in liver, kidney, spleen and heart of rainbow trout (Papanastasiou and Zarkadis, 2006). In human, liver and kidney were found to be the main source of C8γ expression (Trojer *et al.*, 1999). However, strong amplification observed in liver of trout and walking catfish points that liver is the major site of C8γ synthesis in teleosts. In addition, liver is also considered to be the main source of C8α, β, γ mRNA in trouts (Papanastasiou and Zarkadis, 2006a, 2006b).

Developmental expression study of C8γ in different stages of *C. macrocephalus* larvae showed that C8γ transcripts were immediately detected in *C. macrocephalus* after hatching and slightly increased over larval development. In addition, *C. macrocephalus* C8γ mRNA was not detected prior to fertilization. The results indicated that C8γ plays an important role in the early immune response of fish larvae. However, *C. macrocephalus* C8γ mRNA was not found in any tissues of 30 days fish larvae by *in situ* hybridization, which was likely caused by the small amount of mRNA and sensitivity of the technique. To confirm the results from *in situ* hydridization, the expression of C8γ mRNA was subsequently examined by dot blot hybridization with the same probes. The positive signal was observed at 5 microgram of total RNA prepared from liver of 30 days larvae. The results indicated that expression of *C. macrocephalus* C8γ mRNA appears to be very low in larval section but the level of C8γ mRNA seems to be greatly increased when fish grew up. In addition, semi-quantitative RT-PCR was performed to analyze the expression of C8γ gene in liver of catfish fingerling fed with β-glucan. The results revealed that C8γ transcripts were not regulated by β-glucan. The reason for this finding is still unclear and further studies are necessary.

In conclusion, two complement component *C. macrocephalus* C3 and C8γ were cloned and characterized. Expression analyses in different tissues of both genes indicated that liver is the major organ for complement protein synthesis. Their expression levels were immediately detected in *C. macrocephalus* after hatching and gradually increased over larval

development, suggesting that C3 and C8γ play an important role in the early immune response of fish larvae. Oral β-glucan administration enhanced the expression of C3, indicating an important role of C3 in immune system. However, the exact function of C8γ in immune system remains to be clarified.