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Molecular cloning, sequencing and tissue-level expression of complement C3 of *Labeo rohita* (Hamilton, 1822)

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ABSTRACT

Complement component C3 plays a central role in all known complement activation pathways. In the present study, we cloned, sequenced and analyzed the full-length cDNA sequence of *Labeo rohita* complement C3 (LRC3). The expression pattern of complement C3 mRNA in different tissues of healthy rohu and after challenge with *Aeromonas hydrophila* were evaluated using real-time PCR. The LRC3 cDNA sequence of rohu comprised of 5081 bp encoding a predicted protein of 1645 amino acids. The deduced amino acid sequence had the characteristic domain architecture. About eight domains specific to complement C3 are present in the sequence starting from signal peptide to netrin C345C (NTR) domain. The post-translational processing signal sequence (RKRR), the C3-convertase cleavage site sequence (LAR) and the canonical thiol-ester motif (GCGEQ) were found to be conserved in the LRC3. Real-time PCR analysis revealed the highest expression of C3 in liver and extra-hepatic expression of C3 was also observed in all the tissues studied. *A. hydrophila* challenge resulted in significant up-regulated expression of C3 transcripts in both liver and kidney at 6, 12, 24, 48 and 72 h post-infection.

1. Introduction

Complement system, one of the important effector systems of innate humoral immunity, is present in lower as well as higher animals and comprises of a group of about 35 soluble and membrane-bound proteins having multi-functional properties including pathogen recognition and elimination and clearance of apoptotic cells [1,2]. Teleosts are known to have a well-developed complement system with diversified complement proteins and increased complement activity, compensating them for the not so well-developed specific immunity. Bony and cartilaginous fish complement systems show a close functional similarity to those of mammals, with a few characteristic differences such as more heat labile, have lower optimal reaction temperature (10–27 °C) and high alternative complement pathway titer [3–5].

C3 is the most important and central complement component present in the plasma that participates in all three distinct but overlapping activation pathways. Mature C3 (190 kDa glycoprotein) molecule of most animals consists of two di-sulfide linked polypeptide chains (α and β), with a thioester bond between Cys and Gln within the highly conserved GCGEQ sequence motif in the α-chain. C3 is proteolytically activated to generate C3a (74 amino acids from the N-terminus of α-chain) and C3b (the residual 180 kDa portion), which undergoes a gross conformational change to expose and cleave the thioester bond, allowing its binding to a hydroxyl group on the target surface by transacylation [6]. Fish appear to have all the three complement activation pathways like mammals and the fish complement proteins identified thus far show many structural and functional homologies to their mammalian counterparts [7–10].

Mammalian C3 is encoded by single gene while most of the teleost fish were found to possess multiple isoforms of C3 [11–19]. In addition to being present in multiple isoforms, C3 has also been found to be polymorphic in carp [17]. The combination of diversity, high titer and activation at low temperature makes complement system one of the most effective immune parameters in fish [5].

Rohu (*Labeo rohita*), is an important candidate species in freshwater aquaculture in South East Asia. Intensive culture

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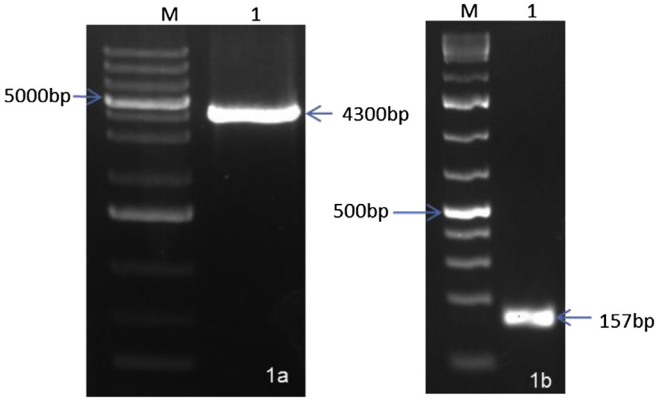

Fig. 1. Agarose gel electrophoresis of long PCR (1a) and 5′ RACE-PCR (1b) products. Lane M: DNA ladder; lane 1: PCR product.

practices have resulted in frequent disease outbreaks especially of bacterial origin. Hence efforts are on to characterize the immune genes of this species to understand their role in specific and non-specific immune defense mechanisms. Complement proteins play an important role in defense mechanisms of fish involving both specific and non-specific immunity. To date the full-length sequence of rohu complement C3 is not available except for a partial sequence of 154 bp [20]. Hence the present study was undertaken to clone and sequence the full-length cDNA of complement C3 of rohu and evaluate the structural features of C3 at deduced amino acid level. The relative expression of C3 mRNA was also studied to find the expression pattern of complement C3 in different tissues of healthy and *Aeromonas hydrophila*-infected fish.

2. Material and methods

2.1. Experimental animals

Seventy healthy rohu (*L. rohita*) weighing 30–40 g were obtained from a commercial fish farm near Mumbai, India. The fish were kept in 500 L FRP tanks (stocking density 6.1 kg m^{-3}) on continuous aeration with 20% water exchange daily. The water temperature was 28 ± 2 °C throughout the study and fish were fed twice daily with commercial pellet feed. The fish were acclimatized for one month before the experiments.

2.2. RNA extraction and first strand cDNA synthesis

Liver tissue collected from healthy rohu was homogenized in a tissue homogenizer (Tomy, Japan) and the total RNA was isolated using RNeasy Mini kit (Qiagen, USA) following manufacturer's protocol. The RNA was quantified using Nanodrop 2000 (Thermo scientific, USA) and used immediately for the cDNA synthesis. One microgram of total RNA was treated with DNaseI (Fermentas, USA) and subsequently reverse-transcribed into cDNA using random hexamer primers and Superscript III Reverse Transcriptase (Invitrogen, USA).

To study the expression of complement C3 in different tissues, kidney, liver, spleen, gills, heart, skin, muscle and intestine were collected aseptically from nine healthy fish and the tissues from three fishes were pooled. Total RNA was isolated and reverse-transcribed into cDNA using oligo d(T)18 primer and first strand cDNA synthesis kit (Fermentas, USA) following the manufacturer's protocol.

To study the expression of complement C3 in response to bacterial infection, 54 fish were injected intraperitoneally with *A. hydrophila* isolated in this laboratory. The bacteria were grown on brain heart infusion broth, washed and resuspended in phosphate buffered saline (pH 7.4). The bacteria were injected at a dose rate of 10^8 CFU $fish^{-1}$ in 100 µl. Liver and kidney tissues were collected aseptically from nine fish each at 0, 6, 12, 24, 48, and 72 h post-challenge. Three samples were pooled to get three samples for each time-point. Total RNA was extracted and cDNA was synthesized using oligo d(T)18 primer.

2.3. Primer design and synthesis

Primers were designed from the conserved regions of complement C3 sequences of closely related species; *Cyprinus carpio*

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1     MDVKLLFLTVVLLSSPLLTLCDPLYIMSAPNLLRVGSSENVFVEAQDYSEVDLNVKIIIKNHPQKDREIL
71    SKSVSLTANNNFQILTDIKIPDDQNFFSEDPDEKQYVYLQAQFPSVTLEKVVMLSFQSGYIFVQTDKPIY
141   TPASTVQYRIFSLTPNLEPLKSQSGITVEIMNPQNITVLSEKIFPVKGMKSGKYAIPEVASSGIWKVVTM
211   FSNTPQKTFTANFEVKEYVLPTFEVKLKPHKSFFYVRDESLEVDIEAKYLFGQKVEGNAFVTFGVIKSEN
281   KKTSIPSSLQKVQIIQGEGTAELTNQMIIKTFPNINQLVGQSIYVSVSLLTESGSEMVEAERRGIQIVTS
351   PYTIHFKKTPQFFKPGMPFDISIYVTNPDQTPAENVEVEVNPGLVKGQTRANGIAKVTVNTLGGSNTLEI
421   TAKTKDPQLRDEQQAVKKMTAQAYKTKGGSHNYLHIGIDAAELEIGDSVKVNLNTGQSPGVKDQDYTYMI
491   LSKGQIVQVHRFKRRGQALVTLPVTVTKDMVPSFRFVAYYHVGSSEVVSDSVWVDVKDTCMGKLQLKVKD
561   KMNTYGTGDEVKLQITGDPGARVGLVVVDKAVHVLNKNRLTQTQIWDVIEKHDTGCTAGGGRESMGVFTD
631   AGLMFHSNTAGGTDTRTISNCPTPAKRKRRAESLQQITVTLAGKYSGDLKQCCVDGMKDNILGYTCERRA
701   TYIVDGPECAKAFLHCCNEMKTRKGMKAEESEMILARSDDDDDFYTDSEDIVSRTQFPESWLWEEVDLCD
771   KCQTPTTEKVLYLKDSITTWQILAVSLSPTLGICVAEPEELVVFKQLFIDLKIPYSAVRGEQLEIKAIIH
841   NYTPKKQKVRVEFMETEDVCSSASKKGKYRTIVNVDKGSSTSASYVIIPMTSGKHMIEVKASAYDAVYTD
911   GVRKPLKVVPEGVLTQLERGKVELNPVKNGEKPAIFRSEIPPDWVPDTPANTYISITGEEITQTVEQAIS
981   GDFMGRLIVQPSGCGEQNMIYMTIFGIATHYLDSTNQWEAVGMERRNEAITHINTGYQRQLGYRKTDGSY
1051  AAWKERPSSTWLTAYVSKVFAMANNLVVIDENVLCSALKWLVLHKQLPDGSFKEDAFVIHGEMVGDVRGK
1121  DAEVSLTAFVVIAMQEGSEICAASVSSLHESIRKAVAFLEGRFPKLTNPYAVAMTSYAMANVGKLNKDIL
1191  MKHSTKLEAGVSWTVPGQHHHSLEATAYAVLALVKAKDFDRAGEAVHWLGKQQSHYGGSGTTQATIMVFQ
1261  AVAEYRTQVNDRQNFNLEVELSVAGRSKPVKYTIKNDNKHLTRSDKMDINKDFNVTARGTGTATLSVLTL
1331  YYARPAEKKSDCKLFDLTVKMEKENEPKQQGAIETYKLTMEFYYKSDKYDATMTILDIGIPTGFTVDSRD
1401  LEELSTGKERYIQKFEMDKVLSERGSLILYLDMVLHTETERIIFRMHKVQNVGLLQPAAVTIYEYYSPDA
1471  RCTKFFHPEREDGALYRLCKGDLCQCAEENCSYQKKNHIEDDERFNKACEAGMDYVYKVTVVGMNLKKDS
1541  DIYEMKVEQVLKEGTDEDVEGNVRPFLGRPNCRIPLGLVKGKSYLIMGKSVDLPKLGGSLQYILGEQTWV
1610  EYWPTRQESQIREHRDRYIGISELQNSLLKEGCAT
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Fig. 2. Predicted amino acid sequence (1645) of *Labeo rohita* C3 (LRC3) cDNA. Thioester motif (GCGEQ) (boxed) and neighboring proline residues (circled) are known to be important for the thioester domain stability. The bold and underlined amino acid residues represents ANATO domain. The first 21 amino acids representing signal peptide is bold and italicized. The catalytic histidine residue His^{1126} and Glu^{1128}, the potential processing site (RKRR) for the cleavage of the alpha and beta subunits are shown in grey background.