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Coagulation profile, gene expression and bioinformatics characterization of coagulation factor X of striped murrel *Channa striatus*

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ABSTRACT

A transcriptome wide analysis of the constructed cDNA library of snakehead murrel *Channa striatus* revealed a full length cDNA sequence of coagulation factor X. Sequence analysis of *C. striatus* coagulation factor X (*Cs*FX) showed that the cDNA contained 1232 base pairs (bp) comprising 1209 bp open reading frame (ORF). The ORF region encodes 424 amino acids with a molecular mass of 59 kDa. The polypeptide contains γ-carboxyglutamic acid (GLA) rich domain and two epidermal growth factor (EGF) like domains including EGF-CA domain and serine proteases trypsin signature profile. *Cs*FX exhibited the maximum similarity with fish species such as *Stegastes partitus* (78%), *Poecilia formosa* (76%) and *Cynoglossus semilaevis* (74%). Phylogenetically, *Cs*FX is clustered together with the fish group belonging to *Actinopterygii*. Secondary structure of factor X includes alpha helix 28.54%, extended strand 20.75%, beta turn 7.78% and random coil 42.92%. A predicted 3D model of *Cs*FX revealed a short α-helix and a Ca^{2+} (Gla domain) binding site in the coil. Four disulfide bridges were found in serine protease trypsin profile. Obviously, the highest gene expression ($P < 0.05$) was noticed in blood. Further, the changes in expression of *Cs*FX was observed after inducing with bacterial (*Aeromonas hydrophila*) and fungal (*Aphanomyces invadans*) infections and other synthetic immune stimulants. Variation in blood clotting time (CT), prothrombin time (PT) and activated prothromboplastin time (APTT) was analyzed and compared between healthy and bacterial infected fishes. During infection, PT and APTT showed a declined clotting time due to the raised level of thrombocytes.

1. Introduction

Fish depends on highly efficient homeostatic mechanisms to protect them from vascular damage and other mechanical injuries, since their gills are very delicate and the circulatory system is in close proximity to the aquatic environment. Generally, fish are highly susceptible to mechanical gill damage and most of the fish in nature and in culture farms exhibit damaged gills [1–3], making them more vulnerable to severe blood loss and infection [2,4]. Unless fish has an active mechanism to control blood loss through the damaged gills, it bleeds until death. Blood clotting mechanism plays a key role to prevent bleeding by a process called coagulation through the participation of thrombocytes.

Blood clotting mechanism is a fundamental process in both mammalian and non-mammalian vertebrates, which is similar to fish and higher mammals [5]. Generally, the initial event of coagulation involves the conversion of fibrinogen into an insoluble fibrin clot. This reaction is catalyzed by a serine protease called thrombin, which gets released after a cascade of proteolytic events. In fish, thrombin is generated by involving various coagulation factors which are mediated by vitamin K, thus forming fibrin clot.

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The homeostasis of coagulation mechanism is maintained by various methods such as inhibition of thrombin activation by various serine protease inhibitors and by fibrinolysis activity. Most of these inhibitor mechanisms are similar for both fish and mammals which are inhibited by the same inhibitors such as tissue plasminogen activator and urokinase [5,6].

The exact mechanism of blood coagulation to prevent the loss of fluid and cells following injury in vertebrates include three different processes: gelatinization of plasma, aggregation of blood cells, or combination of both gelatinization and aggregation [1,7]. In teleost, there is no significant difference in coagulation profile between marine and freshwater teleost suggesting the presence of similar haemostasis mechanisms among them [8].

Coagulation cascades are carried out in different processes such as intrinsic and extrinsic pathway; and recent studies have also proposed a cell-based model [9]. Initiation of coagulation by the intrinsic and extrinsic pathway is carried out through two distinct ways which involves various coagulation factors. However, both the cascades finally end up in inducing factor X which is further involved in the activation of thrombin. Coagulation factor X (Stuart factor) is a plasma glycoprotein (serine endopeptidase) that plays a chief role in blood coagulation pathway along with 30 different proteins involved. The activated form, factor Xa, catalyzes the conversion of prothrombin to thrombin in a reaction accelerated by factor V (proaccelerin), calcium ions and phospholipids [4,6]. Generally, coagulation starts with the extrinsic pathway resulting in the formation of a smaller amount of Xa and the extrinsic pathway gets turned off. The released Xa further activates the intrinsic pathway which involves many factors such as XII, XI and IX and finally leads to amplification of factor Xa generation. Since factor Xa is activated by both intrinsic and extrinsic pathways, they play a key role in the coagulation cascade [10].

Vitamin K-dependent gamma-carboxylation is one of the important processes that is essential for the activation of coagulation factors especially FX and prothrombin which makes them functional [11]. This process is involved in the coagulation cascade of both intrinsic and extrinsic pathways, thus making Vitamin K as a necessary component for their activity. Deficiency of vitamin K significantly reduces the blood coagulation time in salmonids, illustrating their predominant role in coagulation [12]. Exposure of warfarin, an anticoagulant causes spontaneous bleeding in *Danio rerio*, which demonstrate their role in fibrin formation [13].

Fish occupy an intermediate evolutionary position between vertebrates and invertebrates which clearly represent the development of vertebrate hematologic characteristics [14]. However, the hemostatic mechanism in teleost is not clear, since very few elements of blood coagulation were characterized, and based on those reports it could be suggested that fish blood coagulation mechanism was similar to that of mammals [6,15]. In teleost fish, the thrombocytes are the key elements involved in coagulation mechanisms. Thrombocytes are released by kidney and spleen, which survive for between 4 and 70 days that are later removed and replaced to maintain hemostasis [16]. Studies on lower non-mammalian vertebrates may provide clues for the relative appearance of some of the clotting factors during the evolution of vertebrate blood coagulation [5,15]. At an applied level, studies on blood coagulation are recognized as having potential for fish disease diagnosis [17] and further investigation into the hemostatic process is needed to develop practical preventive and therapeutic anticoagulation methods for fish culture [18]. In fish, there are evidences for fibrinogen [19,20] and prothrombin [21], however, the presence of a protease cascade and the exact mechanism has not been established.

Channa striatus is a widely cultured teleost in the Asia-Pacific region which holds high commodity value for its tasty meat and high nutritional and medicinal values such as anti-inflammatory, post-pregnancy immune booster and wound healing property [22,23]. Due to increased fish farming activities and contamination of water bodies, a diverse group of pathogens induce skin lesions in murrel. One such infection is epizootic ulcerative syndrome (EUS) which is primarily caused by an oomycete, *Aphanomyces invadans*, whereas the severity of ulcers are increased by bacteria such as *Aeromonas hydrophila*. The ulcers caused by EUS result in bleeding from wounds. Outbreaks of EUS induce mass mortality of *C. striatus* in culture industry and results in significant economic losses to fish farmers [24]. Bacterial infections are associated with coagulation disorders, resulting in disseminated intravascular coagulation and causing systemic infections. So, we hypothesized that increase in coagulation factor X expression acts as a laboratory marker for bacterial infections in blood. So far, there are only a few reports about the factors involved in coagulation in teleosts and notably there are no reports elucidating the role of coagulation factor X at the time of pathogenic infections, especially in *C. striatus*. This study provides a clear understanding on the molecular characterization of coagulation factor X and also explains the coagulation mechanism during bacterial infection in *C. striatus*.

The aim of the present study is to focus on the characterization and immunological role of coagulation factor X in the hemostatic system of *C. striatus*. In order to accomplish that, we have reported the structural and functional features of a full length murrel coagulation factor X (*Cs*FX), which is primarily, identified from the constructed cDNA library of *C. striatus*. In addition, tissue-specific expression of *Cs*FX and its accent upon EUS causing pathogenic agents were investigated. Furthermore, the immunological role of *Cs*FX has been determined by analyzing and comparing the whole blood clotting time (CT), prothrombin time (PT) and activated prothromboplastin time (APTT) between healthy and bacterial infected *C. striatus*.

2. Materials and methods

2.1. Bioinformatics analysis

A normalized *C. striatus* cDNA library was constructed from total RNA isolated from liver, spleen, muscle, kidney and gills of murrel. The detailed procedure on *C. striatus* cDNA library establishment was explained in our earlier studies [25,26]. A cDNA encoding *Cs*FX was identified from *C. striatus* cDNA library by BLASTx analysis as described in our earlier report [27]. The identified full length *Cs*FX cDNA sequence was analyzed for sequence characterization using DNAssist 2.2 [28]. Homologous sequences that are similar to *Cs*FX protein sequence were searched on NCBI Database using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast). Conserved domains and motifs present throughout the *Cs*FX sequence were analyzed on PROSITE Database (http://prosite.expasy.org/scanprosite/). Presence of signal peptide sequence was analyzed using the SignalP (http://www.cbs.dtu.dk). Conserved residues between the homologous sequences were identified by performing multiple sequence alignment using ClustalW on BioEdit (ver. 7.1.3.0). Evolutionary relationship of *Cs*FX with other homologous sequence was analyzed by constructing a phylogenetic tree using Neighbor-Joining Method in MEGA 6 [29]. Two dimensional structure of the *Cs*FX protein was constructed and further analyzed on Polyview server (http://polyview.cchmc.org). The three dimensional model of the amino acid sequence of *Cs*FX was predicted by I-Tasser program (http://zhanglab.ccmb.med.umich.edu/I-TASSER) and the structural analysis was performed using PyMol Molecular Graphics System (ver.1.5).