Polymorphism Investigation of Key Regulating Regions of Calpastatin Gene In Common Carp (*Cyprinus Carpeio*)

M I Mir[1], Aashiq Hussain Bhat[2], Salma Khan[3]

ABSTRACT

The biochemical regulated mechanism of softening of muscle fibers is due to proteolytic enzymes especially calpain. In the present study 1445 bps of calpastatin gene an endocytolic inhibitor of calpain has been amplified using five set of primers. The bioinformatic tools were used for demarcation of exon intron margins and phylogenetic analysis. The published sequence accession numbers NP_001124063.2(ZF), NP_001118010.1(RT), NP_001167162.1(SS), gb/AA162761.1 (ZF), gb/AAY18570.1(RT) and gb/AFV08638.1(SS) were taken for marking out of exon intron boundaries and positioning of mutations which resulted change in amino acid within the conserved region for functional validations. The exon 11 and exon 12 showed mutation from serine (S=T**C**T) to tyrosine (Y=T**A**T) and valine (V=**GTT**) to alanine (A=G**C**T). The amplified fragment length of 316bp and 311bp of calpastatin gene in common carp contain two exons (14, 20) of calpain inhibitory activity. Sequence alignment with published reports showed that exon 14 (inhibitory domain 1) has glycine (G=GGT) positioned in common carp than that of glutamine (Q>394=CAA) in zebra fish, glutamic acid (E>491=GAG) in atlantic salmon and lysine (K>223=AAG) in rainbow trout. The inhibitory domain II (exon 20) retains glycine (G>548=GGT) in two study groups (A&B) of common carp while as the group C showed the substitution of aspartic acid (D>548=GAT) in place of glutamic acid at 548, 230 and 689 in reference reports of zebra fish, rainbow trout and atlantic salmon respectively. The exon 22 and exon 23 and intron in between 300bp functions to target proteins, substitution of proline(617th), lysine (622), serine(296) and aspartic acid(759) in exon 22 where as substitution of aspartic acid (650), theronine (323) and valine (809) in exon 23 functionally differentiate calpastatin gene in fishes. while as exon 26 and 27 of calpastatin renders protein for rapid destruction due to presence of PEST sequence and by selective proteolysis and mutation of arginine in place of serine requires functional validation. Apart from muscle proteiolysis, Calpastatin is immensely important in neuron degeneration, retarded growth, muscle callipage etc. further analysis at molecular level may generate a wider horizon of the study.

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1. Introduction

The meat quality is governed by the environmental as well as genetic factors. The meat quality like color, texture and tenderness are important for consumers. Although Meat quality is governed by number of physical and biological factors but calpastatin gene regulation in association with calcium ions are of great importance. The calpastatin is a cytosolic inhibitor of calpain also known as ical gene. Calpastatin gene has been studied in different species of vertebrates for improving meat quality through selective breeding programmes. The different structural and functional important exonic regions have shared different level of homology in the vertebrates. The association between single nucleotide polymorphism and meat quality has been widely studied in experimental and domesticated animals including ovines, bovines and caprines. A single nucleotide polymorphim C>T is significantly associated with meat quality in rabbits and is also associated with low intramuscular fat deposition (Wang Jie et al., 2017). Studies have reported association of SNPs with meat quality in terms of shear force of muscle fibers and have shown mutations targeting the gene in breeding specific manner (Ramayo-Caldas et al., 2016). The role of protein domains has been studied for protein proteolysis like KFERQ sequences, cyclin destruction boxes, PEST regions, ATP dependent proteasome and calcium dependent calpain are

Author Information:

1. Dr Iqbal: Senior Research Fellow, under National surveillance programme for aquatic Animal diseases,Faculty of Fisheries, Shere Kashmir Agricultural University Kashmir-India, 190006
2. Dr Aashiq: PhD, Department of Clinical Biochemistry, Kashmir University-India, 190006
3. Dr Salma: Professor, Department of Biological Sciences, Rani Durgavatie University-Jabalpur India

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responsible for non-lysosomal target proteolysis (Dice J.F.1990, Glotzer et al. 1991, Rogers et al. 1986, Mykles et al. 1998).

A Calpastatin gene in Atlantic salmon encodes a protein of 937 amino acid and has two inhibitory domain. Rainbow trout calpastatin contains two isoforms, calpastatin long variant and calpastatin short of total open reading frame of 2600bp coding for 497aa and 2246bp coding for 296aa, respectively (Saleem et al., 2005). Zebrafish calpastatin gene is located on chromosome 21(BX511081.8) encoding 839 amino acid from 30 exons (Lepage et al., 2008). The investigation of polymorphism within the coding region of calpastatin gene in fishes has not been done before as per literature available, thus the present study was designed. We presume that the genetic polymorphism within calpastatin gene might have effect on the fillet quality in fishes as reported in higher chordates. The present piece of work will give a preliminary insight to the SNP based marker assisted selection of brood stock for profitable farming of freshwater culture fisheries.

2. Material

In the present study fishes of same age group was captured from the well established government farms. Tissue samples (muscle) are collected in normal saline, genomic DNA was extracted by phenol-choloroform method, stored at -20^{0}C (Sambrook and Russel.,2001). The quantitative and qualitative analysis was done on spectrophotometer and agarose gel electrophoresis, respectively.

2.1 Bioinformatics and Polymerase Chain Reaction

The exon intron boundaries demarcation was performed by aligning calpastatin genomic sequence of zebra fish (Danio rio) retrieved from Zfin genomic data base with calpastatin cDNA sequences of rainbow trout (Oncorhynus mykiss) and atlantic salmon (Salmo salar) and primers from conserved region were designed for amplification of calpastatin gene in common carp (cyprinus carpio) testified for thermodynamic properties using Primer3 software. PCR was carried out using 25ul reaction containing 2ul genomic DNA, 1ul (10pM) of each primers, 2.5ul of dNTPs, 1.5ul $MgCl_{2,}$ 2.5ul PCR buffer, 0.2ul of taq polymerase (fermentas) and water. The PCR cycling conditions are hot start at 94^{0}C for 10mints, followed by 35 cycles of Denaturation 94^{0}C for 30sec, annealing 55-60 ^{0}C for 30 sec, elongation 72 ^{0}C for 30 sec, and final elongation 72 ^{0}C for 10 mints. The PCR products were separated on 2 per cent of Agarose followed by sequencing.

2.2 Sequence Analysis

The sequences were used for BLASTn for nucleotide identity in the NCBI gene database. The nucleotide to protein BLAST was performed for identification and demarcation of exon intron boundaries of the calpastatin gene in common carp; moreover the homology with already available sequences was also evaluated for identification of functionally important domains of the calpastatin protein.

2.3 Association of SNPs with fillet quality

The muscle tissue was used for texture analysis under international standards. The fishes were selected on random basis for texture analysis and molecular (SNP) analysis. The experiment was done in triplicate for validation of results. The association between the SNPs and texture quality was also performed.

3. Results

The samples of same age group are selected on random basis for polymorphism detection and validation. In the present study amplification and sequencing of 1445 bps of calpastatin gene in common carp was done by five set of primer pairs (table1).

S.No.	Amplified region	Primer sequence (5>3)	Fragment length	Temperature
1	E11-intron-E12	F: ACTGAAACAATAGGAGCAG R:TCTGTCTTTGCTTTGGCTGGTTGTG	265bp	60^{0}C
2	E13-Intron-E14	F:TNGCTGGACGCTCT R:TTATAGGAGGTGGACG	316bp	58^{0}C
3	E19-Intron-E20	F:TTCAGTGCTTTGGG R:CTTTATAGGAGG	311bp	55^{0}C

Author Information:

1. Dr Iqbal: Senior Research Fellow, under National surveillance programme for aquatic Animal diseases,Faculty of Fisheries, Shere Kashmir Agricultural University Kashmir-India, 190006
2. Dr Aashiq: PhD, Department of Clinical Biochemistry, Kashmir University-India, 190006
3. Dr Salma: Professor, Department of Biological Sciences, Rani Durgavatie University-Jabalpur India

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		AGGAGT		
4	E22-Intron-E23	F: CTTCCGATTTT R:CCTTAACAACATC	300bp	60^0C
5	E26-Intron-E27	F:CAGCCATCAATGGA R:GGTTTCGGTTTCTG	278 bp	60^0C

The sequencing was performed by big dye termination method (Sanger). The intraspecies variability of cast gene was performed using sequence alignment softwere (CLUSTAL-W-Supplementary file). The amplified sequences were further subjected for identifying similar type of sequences in fishes of outgroup. The results showed maximum sequence homology with calpastatin gene of zebra fish, rainbow trout and atlantic salmon available in NCBI data base. Moreover, we have found that calpastatin gene is highly conserved in higher vertebrates also. The BLASTx was performed for identification and validation of protein transcript of the amplified fragments and important findings from the study are identification of two calpain inhibitor domains along with membrane targeting regions and target region of destruction in the calpastatin protein (Fig.1,2).

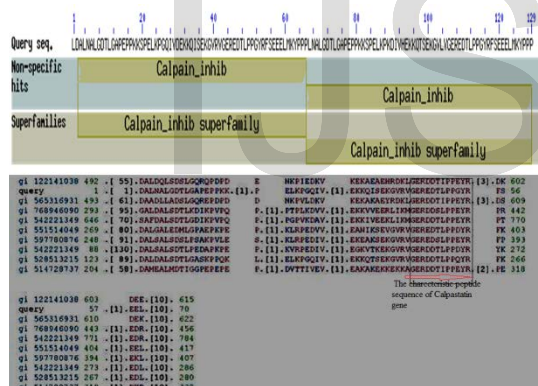


Fig. 1: The figure shows Insilco translational product of Calpastatin gene in common carp with two calpain inhibitory domains showing trade mark region of seven peptide sequence enclosed in box. The seven peptide sequence is highly conserved in higher vertebrates also.

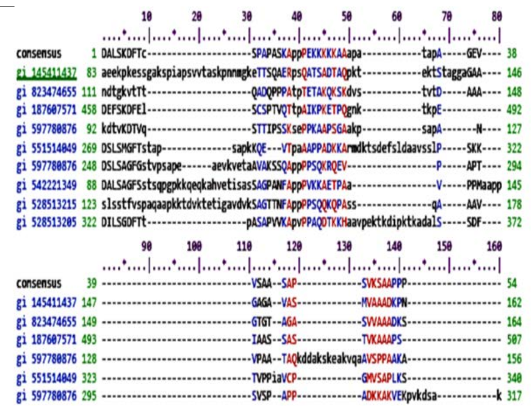
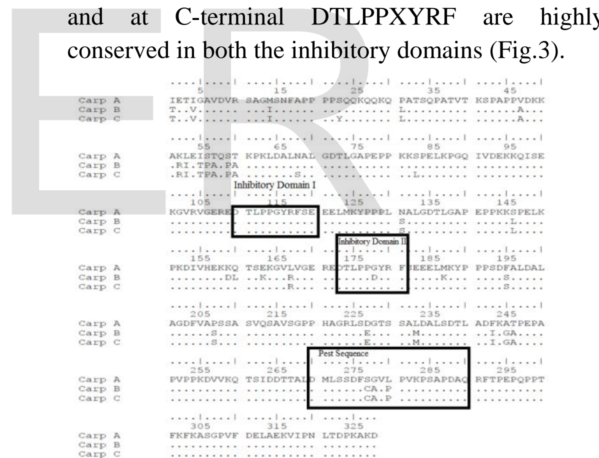


Fig. 2: Showed the membrane targeting regions and target region of destruction in the calpastatin protein amplified in common carp and its homology with sequences available in the NCBI data base.

The amplified fragment length of 316bp and 311bp of calpastatin gene in common carp attributed to inhibitory activity of exon 14 and exon 20 after aligned with zebrafish calpastatin gene.

The presence of multiple inhibitory domains in common carp plays important role in muscle turn over and calpain inhibition. The translated protein sequences showed that at N-terminal DTL**X**APEP and at C-terminal DTLPPXYRF are highly conserved in both the inhibitory domains (Fig.3).



whereas X is the hypervariable amino acid as in other fish calpastatin proteins and also in higher vertebrates. Sequence alignment with published reports showed that exon 14 (inhibitory domain 1) has glycine (G=GGT) positioned in common carp than that of glutamine (Q>394=CAA) in zebra fish, glutamic acid (E>491=GAG) in atlantic salmon and lysine (K>223=AAG) in rainbow trout (table 2). The inhibitory domain II (exon 20) retains glycine

Author Information:

1. Dr Iqbal: Senior Research Fellow, under National surveillance programme for aquatic Animal diseases,Faculty of Fisheries, Shere Kashmir Agricultural University Kashmir-India, 190006
2. Dr Aashiq: PhD, Department of Clinical Biochemistry, Kashmir University-India, 190006
3. Dr Salma: Professor, Department of Biological Sciences, Rani Durgavatie University-Jabalpur India

(G>548=GGT) in two study groups (A&B) of common carp while as the group C showed the substitution of aspartic acid (D>548=GAT) in place of glutamic acid at 548, 230 and 689 in reference reports of zebra fish, rainbow trout and atlantic salmon respectively (table 2).

Species		Shear force (g/g)	Amino acid	
Common carp A	Intraspecies	288.23±0.10 (Ph:6.06)	G=GGT* G=GGT**	
Common carp B		295.42±0.30 (Ph:6.18)	G=GGT* G=GGT**	
Common carp C		439.20±0.20 (Ph:6.18)	G=GGT* D=GAT**	
Rainbow trout	Interspecies		K=AAG* E=GAG**	Saleem et al. 2005
Atlantic salmon			E=GAG* E=GAA**	

*Amino acid inhibitory domain I, **Amino acid inhibitory domain II

The polymorphism in the trade mark region of calpastatin inhibitor regions showed that a single base mutation of A/G within the species and single base mutation of G/A and T/A between the families might have strong correlation with the fillet quality in terms of strength and postmortem proteolysis. There are several nucleotide mutations in the amplified fragments, most of them are silent mutations. The exon 11 and 12 in experimental fishes showed single nucleotide mutation S=T**C**T to Y=T**A**T, V=**GTT** to A=G**C**T resulted in change of amino acid in highly conserved region PPPPSQQKQ and VTKSPAPPVDKKA respectively.

The amplified fragment length of 300bp span exon 22, exon 23 and a short intervening intron and functional revelation membrane targeting of calpastatin gene in common carp substitution of aliphatic amino acid proline/Serine, basic amino acid lysine/serine (617th aa &622nd aa), hydroxylic amino acid seine/alanine (296th aa) and aspartic acid an acidic amino acid/glutamic acid (759th aa) in exon 22, whereas substitution of aspartic acid/glutamic acid (650th aa), theronine hydroxylic amino acid/seine (323rd) and valine an aliphatic amino acid/isoleucine (809th aa) in exon 23 might have functionally differentiated the calpastatin gene function in teleosts. The exon 26 and 27 region of calpastatin belongs to the target region of protein for rapid destruction, presence of PEST sequence playing critical role in selective proteolysis and presence of arginine in place of serine needs functional validation. Both regions belong to domin DI and DII including the inhibitory domain (Fig.3).

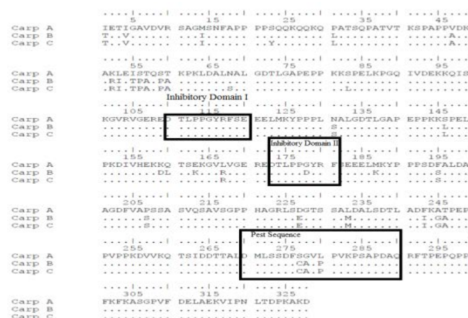


Fig. 3: The figure showed the complete translated sequence of amplified fragments and end joining of only exons. The sequences enclosed in rectangular boxes showed the presence of Inhibitory domain I & II and sequences at N-terminal DTLXAPEP and at C-terminal DTLPPXYRF are highly conserved in both the inhibitory domains. The stretch of amino acid within the rectangular box present at the terminal are PEST sequences required for targeting the protein.

3.1 Functional study validation of calpastatin gene

A thorough trimeric analysis of calpastatin activity was performed using PISA softwere in order to know calpastatin interaction with calpain and calcium for structural and functional understanding at cellular level. The calpain inhibitor is a multiprotein family and calcium is required as cofactor for the enzymatic kinetics. The number of inhibitory domain in the calpastatin protein signifies the calpain inhibition, more the number of inhibitory domain more will be the calpain inhibition (Fig.4).

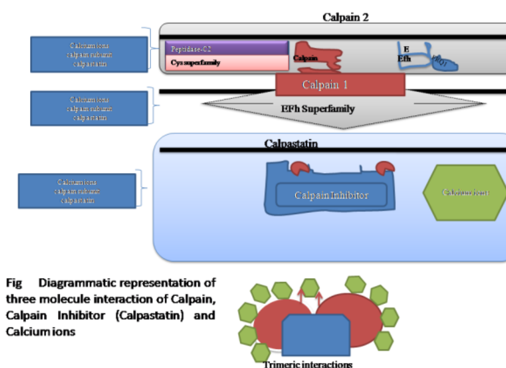


Fig Diagrammatic representation of three molecule interaction of Calpain, Calpain Inhibitor (Calpastatin) and Calcium ions

Author Information:

1. Dr Iqbal: Senior Research Fellow, under National surveillance programme for aquatic Animal diseases,Faculty of Fisheries, Shere Kashmir Agricultural University Kashmir-India, 190006
2. Dr Aashiq: PhD, Department of Clinical Biochemistry, Kashmir University-India, 190006
3. Dr Salma: Professor, Department of Biological Sciences, Rani Durgavatie University-Jabalpur India

Fig.4: Figure showed the complete diagrammatic understanding of Calpastatin interaction with Calpain protein and calcium ions. The number of inhibitory domain in the calpastatin protein signifies the calpain inhibition, more the number of inhibitory domain more will be the calpain inhibition

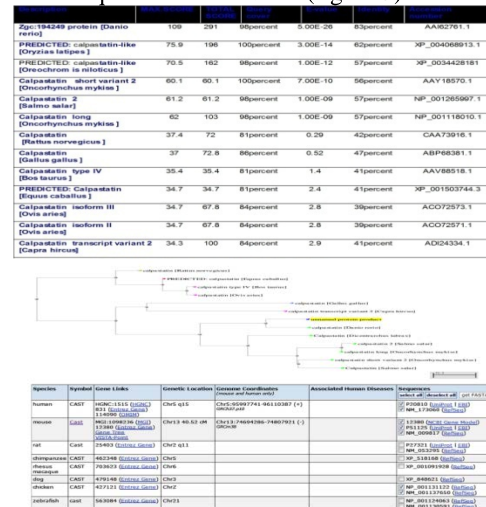
3.2 Association of SNPs with flesh quality

The meat quality in terms of share force (g/g) of common carp used for polymorphism validation ranges from 310.31g/g to 439.20g/g. The force valves are from lower to upper limit. The fishes were divided in to three groups according to fillet analysis (N=3) low firmness (group A: 288.23±0.10, group B: 295.42±0.30) and hard (group C: 439.20±0.20g/g) as shown in table 2.

3.3 Phylogenetic Analysis

The phylogenetic analysis was performed for the Calpastatin gene in vertebrates, tetrapods and teleosts by retrieving sequences available in NCBI database using sequences amplified in the present study. Among the retrieved sequences few of them where used for construction of tree by neighbor joining and maximum likelihood. The salmonidia and Cypernidae family form different branch. The zebra fish (*Danio rio*) and Common carp (*Cyprinus carpio)* belong to Cypernidae family. While as Rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) belongs to the Salmonidae family. The tree also showed that Danio *rio* (Cyprinids) is phylogenetically more close to mammalian counterpart than *Oncorhynchus mykiss* and *Salmo salar* (Salmonidae family) in terms of calpastatin gene.

The phylogenetic study between tetrapods and teleosts was done by BLAST searching tool of NCBI using sequences identified as inhibitory domain of calpastatin. The sequence showed highest similarity hits with Danio rio (83 percent) followed by Oryzias latipes (62 percent) and least with Salmo salar (57 percent) followed by Oncorhynchus mykiss short variant (56 percent) among teleosts; Gallus gallus (47 percent) followed by Rattus norvegicus (42 percent) and Bos Taurus (41 percent) followed by Ovis aries (39 percent) respectively. Gallus gallus ((Avain) is presumed to be the closest among the tetrapods followed by Rattus norvegicus to Cyprinus carpio. The phylogenetic tree was constructed on the basis of these sequences as shown in (figure 5).



Description	MAX SCORE	TOTAL SCORE	Query cover	E-value	Identity	Accession number
Zgc:194249 protein [Danio rerio]	109	291	98percent	5.00E-26	83percent	AAI62761.1
PREDICTED: calpastatin-like [Oryzias latipes]	75.9	196	100percent	3.00E-14	62percent	XP_004068913.1
PREDICTED: calpastatin-like [Oreochrom is niloticus]	70.5	162	98percent	1.00E-12	57percent	XP_0034428181
Calpastatin short variant 2 [Oncorhynchus mykiss]	60.1	60.1	100percent	7.00E-10	56percent	AAY18570.1
Calpastatin 2 [Salmo salar]	61.2	61.2	98percent	1.00E-09	57percent	NP_001265997.1
Calpastatin long [Oncorhynchus mykiss]	62	103	98percent	1.00E-09	57percent	NP_001118010.1
Calpastatin [Rattus norvegicus]	37.4	72	81percent	0.29	42percent	CAA73916.1
Calpastatin [Gallus gallus]	37	72.8	86percent	0.52	47percent	ABP68381.1
Calpastatin type IV [Bos taurus]	35.4	35.4	81percent	1.4	41percent	AAV88518.1
PREDICTED: Calpastatin [Equus caballus]	34.7	34.7	81percent	2.4	41percent	XP_001503744.3
Calpastatin isoform III [Ovis aries]	34.7	67.8	84percent	2.8	39percent	ACO72573.1
Calpastatin isoform II [Ovis aries]	34.7	67.8	84percent	2.8	39percent	ACO72571.1
Calpastatin transcript variant 2 [Capra hircus]	34.3	100	84percent	2.9	41percent	ADI24334.1

Species	Symbol	Gene Links	Genetic Location	Genome Coordinates (mouse and human only)	Associated Human Diseases	Sequences select all deselect all get FASTA Go
human	CAST	HGNC:1515 (HGNC) 831 (Entrez Gene) 114090 (OMIM)	Chr5 q15	Chr5:95997741-96110387 (+) GRCh37.p10		P20810 (UniProt | EBI) NM_173060 (RefSeq)
mouse	Cast	MGI:1098236 (MGI) 12380 (Entrez Gene) Gene Tree VISTA-Point	Chr13 40.52 cM	Chr13:74694286-74807921 (-) GRCm38		12380 (NCBI Gene Model) P51125 (UniProt | EBI) NM_009817 (RefSeq)
rat	Cast	25403 (Entrez Gene)	Chr2 q11			P27321 (UniProt | EBI) NM_053295 (RefSeq)
chimpanzee	CAST	462348 (Entrez Gene)	Chr5			XP_518168 (RefSeq)
rhesus macaque	CAST	703623 (Entrez Gene)	Chr6			XP_001091928 (RefSeq)
dog	CAST	479148 (Entrez Gene)	Chr3			XP_848621 (RefSeq)
chicken	CAST	427121 (Entrez Gene)	ChrZ			NP_001131122 (RefSeq) NM_001137650 (RefSeq)
zebrafish	cast	563084 (Entrez Gene)	Chr21			NP_001124063 (RefSeq) NM_001130591 (RefSeq)

Fig. 5: phylogenetic tree construction of different vertebrates classes for understanding the dynamic behavior of calpastatin gene.

4 Discussion

The culture fishery is practicized globally to increase the fish production for the sustainability of aquaculture industry. Fishes in wild have good fillet quality than those under captivity as they have to toil for food, chased by predators and flow of water. The fillet loss and fillet strength after capture are key issues to be addressed in culture fisheries. The genetic polymorphism play important role in governing the quality of meat in mammals. Calvo et al, 2014 reported 37 polymorphism out of which three SNPs in intron 5, exon 7 and intron 12 of calpastatin gene were significantly associated with meat tenderness. The allele frequencies of A to G base shift in different genotypes of bovines showed considerable association for meat related traits such as shear force, myofibrillar fragmentation index, rib eye area (Enriquez-Valencia, 2017). Lindholm-Perry et al, (2009) reported in pigs three genetic markers within calpastatin gene on chromosome 2, associated with tenderness and advocate calpastatin as candidate gene. The effect of SNPS in calpin1, calpastatin and diacyleglycerol O-acyltransferase 1 selected for growth and beef related traits were confirmed by tait et al, 2014.

Researchers have studied expression profile of calpastatin gene in only a few commercially important fishes. The whole genomic study of zebra fish is available in Zfin genomic data base. The identification of SNPs in the functionally

Author Information:

1. Dr Iqbal: Senior Research Fellow, under National surveillance programme for aquatic Animal diseases,Faculty of Fisheries, Shere Kashmir Agricultural University Kashmir-India, 190006
2. Dr Aashiq: PhD, Department of Clinical Biochemistry, Kashmir University-India, 190006
3. Dr Salma: Professor, Department of Biological Sciences, Rani Durgavatie University-Jabalpur India

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important regions of calpastatin gene in fishes in general and common carp in particular has not been studied before as per literature survey. In the present study, the selected genomic sites of calpastatin gene were amplified and transcribed in to corresponding protein sequences for identification and characterization. We have amplified and identified two inhibitory domains, a membrane targeting and target destruction region in common carp. The variability of sequence in the expressed part (exons) within highly conserved region is of interest as they might have direct effect on the catalytic activity of the protein. In the present study we did not find any mutation in the seven peptide sequence (TLPPXYR) which is a family protein characteristic in exon homologous to inhibitory domain1 as glycine (G=GGT/X) is conserved in the study group. Where as in rainbow trout (K=AAG) is present as reported by saleem et al.,2004, 2005, and glutamic acid (E=GAG) in atlantic salmon(NCBI).

In exon-20 corresponds to inhibitory domain II, we have observed that glycine is replaced by aspartic acid in few fishes of the study group (G/A). We have observed excellent fillet quality in fishes having substitution of aspartic acid in place of glycine in the highly conserved inhibitory domain II of calpastatin gene (table2). Lepage et al.(2008) reported that expression of the capns1a, capns1b and cast splice variant of exon11-20 is highly regulated after the onset of zygotic transcription, which provide the clue towards the inhibitory role of calpastatin gene since from early development and growth. Saleem et al (2005) also reported multiple inhibitory domains in the calpastatin gene of rainbow trout. Emori et al (1987) and Takano et al (2000) reported that calpastatin N-terminal domain L and four repeating homologous sequences at C-terminal are functionally unique as each repeating unit is responsible for inhibiting the activity of one calpain molecule. The number of inhibitory domains in calpastatin gene signifies the potential of inhibition and the carcase quality. The studies in mammalian counterpart have shown a strong correlation between any variability in inhibitory region and meat/carcase quality. At low temperature fishes undergoes a period of starvation and for energy demands needs a source of energy. The calpain in association with hepatic proteins causes proteolysis of muscle proteins and hence the weight loss. Huang and Forsberg (1998) describe the antemortem role of calpain in degradation of myofibrillar protein and similar observation of postmortem proteolysis by these proteases impact on muscle texture by Goll et al. (1992). Thus we can say that any mutation in the inhibitory region have pronounced effect on the inhibitory activity of the gene.

Moreover, exon-11,12,22,23,26,27 and introns are important regions of calpastatin gene which are amplified in common carp and are selected based on the functional information available in the literature. The exon 22, 23 and intervening intron functionally act as membrane targeting region and exon 26,27 and intron in between act as region of rapid destruction of muscle protein. Hao et al (2000) reported, presence of ten amino acids in a stretch of domain L regulated the calcium channels for calpastatin activity. Any mutation within the highly conserved region have consequent effect on the enzymatic activity of the protein. The mutation pattern (F=TTT to S=TCT/TCA) might have direct effect on the protein. Similarly, with in the target protein for destruction region have pest sequence and instead of serine there is replacement of arginine in common carp. These pest sequences are functioning as regulatory role in half life of cytosolic protein. Saleem et al (2005) reported similar type of pest sequence in rainbow trout and suggests role in membrane targeting and destruction of the protein. Schenkel et al (2006) reported SNP at domain L in beef responsible for shear force, postmortem ageing and increased tenderness in meat. SNPs in coding region modifies expression of genes by linkage disequilibrium and if present in the promoter region by alternative splicing mechanisms (Debeljak et al., 2000; Murani et al., 2009).

The process of proteolysis apart from muscle wasting during starvation and postmortem help in maintaining structural and metabolic integrity of cells in several ways. Few mutations if incorporated alter the conformation, denaturation pattern, premature chain termination and rapid degradation of proteins. The presence of premature terminating codons might have role in formation of multiple forms of calpastatin protein in fishes as well in higher vertebrates (Goll et al & Saleem et al). The selective proteolysis, half life of protein and rapid degradation of few enzymes are governed by the presence of pest sequence. The presence of pest sequence in calpastatin gene might have played role in postmortem proteolysis and muscle wasting particularly in study group. That is why; after fish kill the impact of calpain inhibition by calpastatin is declining in dramatic pattern. The components of intracellular degradative pathway recognize the protein for rapid destruction by pest sequences. Studies have shown sequences which targets the

Author Information:

1. Dr Iqbal: Senior Research Fellow, under National surveillance programme for aquatic Animal diseases,Faculty of Fisheries, Shere Kashmir Agricultural University Kashmir-India, 190006
2. Dr Aashiq: PhD, Department of Clinical Biochemistry, Kashmir University-India, 190006
3. Dr Salma: Professor, Department of Biological Sciences, Rani Durgavatie University-Jabalpur India

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protein for rapid destruction so that the protein undergoes proteolytic degradation. Hershiko et al demonstrated the presence of unblocked aminoacid terminus for ubiquitin dependent proteolysis.

In conclusion, the study was focused for identification of SNPs in inhibitory, membrane targeting and destruction of target protein regions of calpastatin gene. The identification of SNP in fishes for calpastatin functional important regions were not studied so for as per literature received from different authentic source. The SNP identified and testified will help in selection of brooders for breeding purpose for better fillet quality as fillet loss and juiciness have negative impact on profitable marketing of fishes and economical loss to the grower.

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Author Information:

1. Dr Iqbal: Senior Research Fellow, under National surveillance programme for aquatic Animal diseases,Faculty of Fisheries, Shere Kashmir Agricultural University Kashmir-India, 190006
2. Dr Aashiq: PhD, Department of Clinical Biochemistry, Kashmir University-India, 190006
3. Dr Salma: Professor, Department of Biological Sciences, Rani Durgavatie University-Jabalpur India

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Author Information:

1. Dr Iqbal: Senior Research Fellow, under National surveillance programme for aquatic Animal diseases,Faculty of Fisheries, Shere Kashmir Agricultural University Kashmir-India, 190006
2. Dr Aashiq: PhD, Department of Clinical Biochemistry, Kashmir University-India, 190006
3. Dr Salma: Professor, Department of Biological Sciences, Rani Durgavatie University-Jabalpur India