Short Communication

Characterization of calpastatin gene in Iranian Afshari sheep

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Abstract

Calpastatin is an endogenous inhibitor of calpain (calcium-dependent cysteine protease). Calpastatin activity is highly related to the rate of protein turnover and rate of meat tenderization. In order to characterize the structure of calpastatin in Iranian Afshari breed of sheep, intron 6 and partial exon 7 of the L domain were amplified and sequenced. A fragment of approximately 1.5 kb was identified. In this study, an Afshari calpastatin gene fragment that encoded L Domain amino acids was detected. Hence by detection of such conserved mutations, it is possible to use these polymorphisms in Marker-Assisted Selection (MAS).

Keywords: Calpastatin; Sequence analysis; Iran; Afshari sheep

Rate of muscle protein degradation has a very important role in the rate of extent muscle mass. Differences in the rate of muscle growth in domestic animals are often due to differences in the rate of muscle protein degradation, but with little or no change in the rate of protein synthesis (Amanda *et al.*, 2004). It was originally proposed that the *calpain* system was responsible for initiating metabolic turnover of the myofibrillar proteins and that it affected muscle protein degradation (Goll *et al.*, 1998).

Presently, the calpain system is known to be constituted of three well-characterized proteins which

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include the two Ca²⁺-dependent proteolytic enzymes, namely µ-calpain and m-calpain. Calpastatin is the third member of the calpain family, a multi-headed inhibitor capable of inhibiting more than one calpain molecule (Reynaud et al., 2005). Calpastatin is the variable component of the calpain system and skeletal muscle. Calpastatin activity is highly related to the rate of muscle protein turnover and rate of postmortem tenderization (Goll et al., 2003; Amanda et al., 2004). Calpastatin has a peculiar molecular structure consisting of one N-terminal region (L-domain) and four repetitive inhibitory units each containing three highly conserved regions called A, B, and C (Melloni et al., 2006). In Iran, approximately 25 populations of sheep have been identified so far based on their morphological characters and meat production. Afshari sheep represent one of the largest populations within Iranian sheep.

The main objective of this study was to identify the L-domain of the calpastatin gene structure as a polymorphic region which could be used for correlating the genetic variation with meat production and tenderness. Sequencing of an amplified fragment of the calpastatin gene revealed a high similarity with the reported bovine sequences of this gene.

Five Afshari sheep breeds were selected from an education farm at the Department of Animal Sciences, University of Zanjan, Zanjan, Iran. Genomic DNA was extracted from 1 ml anticoagulated blood, collected from jugular vein by a slight modification of the salting out method (Boom *et al.*, 1990). Two primers designated Cast 1F (Forward 5' AGCAGCCACCATCAGAGAAA 3'); and Cast 1R (Reverse 5' TCAGCTGGTTCGGCAGAT 3') which have been designed based

on the bovine calpastatin gene (GenBank accession No: AY834770) were used to amplify a fragment of approximately 1500 bp. PCR mixture (25 μ l) was consisted of 50-100 ng sheep genomic DNA, 10 pmol of each primer, 200 μ M dNTPs (Roche, Germanty), 1.5 mM MgCl₂, PCR buffer 1X, and 1 unit *Taq* polymerase (Sinagene, Iran).

The temperature cycling (initial denaturation 10 min at 95°C, 35 cycles; detaturation 1 min at 94°C, annealing 1 min at 62°C and extension 1.5 min at 72°C and final extension 10 min at 72°C) was performed in a DNA thermal cycler (Bio Rad, PDS-He1000, U.S.A). PCR amplified products were electrophoresed in a 1% agarose gel and fragments were subsequently visualized by a UV transilluminator (Uvidoc, UK) (Fig. 1).

Selected PCR products from 17AZI sample were re-amplified in a total volume of 125 μ l followed by purification from the gel using a gel extraction kit (Core-one TM, Seoul, South Korea), according to the manufacturer's instructions. Sequencing was performed on both sides of fragment with forward and reverse primers using an ABI sequencing machine (Kowsar Biotech, Tehran, Iran).

Sequence data were checked by FASTA program (www.ebi.ac.uk/fasta33) and comparison and blast

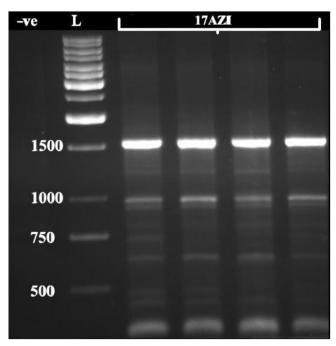


Figure 1. Electrophoresis analysis of PCR products amplified for Cast1 in sheep 17AZI. -ve and L are representing negative control and one kb (SMO311) marker.

analysis were performed with other sequence of Calpastatin gene at Genbank (www.ncbi.nlm.nih.gov/genbank). Gene runner version 3.05 (www.generunner.net), ClustalW (www.ebi.ac.uk/clustalw), ClustalX (www.clustal.org) programs were employed for determination of nucleic acids, deduced amino acid sequences and alignments.

A 1457 bp fragment was amplified in all the examined specimens of the Afshari breed. Now it is available in GenBank at National Center for Biotechnology Information (NCBI) with the accession number EF539858. Based on the purity of the DNA extracted from the 17AZI specimen, followed by successful PCR amplification, this sample was selected for further analysis. The respective fragment (Fig. 1) was excised from the gel was then purified, and sequenced with both primers. The resulting sequence corresponded to position 51 to 1478 of the Bos Taurus (Bos Taurus is scientific name of cattle), which was submitted to GenBank under accession number AF321530. An alignment of the identified calpastatin sequence from the Afshari sheep with the sequences deposited in GenBank revealed similarity with the bovine calpastatin genes (AY834770 and AF321530) (Fig. 2). The Percentage of identity between the two sequences and the Afshari sequence was 89% (Table 1) and Homology was 42% at the amino acid sequence.

In comparison to the bovine calpastatin gene, four insertions at positions 443-447, 573-586, 976-979, 1223-1236, and two deletions at positions 17-18 and 147-148 were detected in AF321530. The partial sequence of exon 7 was also determined at position 1420 to 1457, which comprises the QVT-GRDSGGGKS amino acid sequence (amino acid sequence that is coded by the exon 7). The restriction maps constructed based on the sequence of the calpastatin gene in both Afshari sheep and bovine indicate differences in the restriction sites of the two species (Fig. 3). These two restriction maps demonstrate the possible application of this fragment in designing a PCR-RFLP for species and sub-species differentiation of sheep or other related groups of animals.

Table 1. Total number of nucleotide substitutions, based on a 1457bp fragment of the calpastatin gene in Afshari sheep in comparision with similar sequences in GenBank.

	AY834770	Afshari
AF321530	24	165
AY834770	0	162

146 www.SID.ir

AF321530	AGAT GAAAGAAAGAAAGA AGGTGAAGTC TETCAATC GTATC CAACTCTTTGAGAC CECAT GGTT CCCCE AGGA ACCTA CEAG GTTCC TETGT CCAT	146
	AGAT BAAGAAGAAGAAGA AAGAAAGT BAAGT CTTT CAATT GTATC CAACT STTTGAGAC CCCAT GGTT CCCCC AGGAA CCTA CCAGG TTCC TTTGT CCAT	
	AGATGAAAGAAAGTGAAGTCTCTCAATCGTGTCCAACTCTTTGAGACCCCATGGTTCCCCAGGAACCTGCCAAGTTCCTCTGTCCAC	
Afshari	ADDA PARA PARA PARA PARA PARA PARA PARA	94
A F221520	GGASTTITC CAGGCAGGAGTGCAGGATTGGATTGCCATTTCCTTCTCCAGGGGATCTTCCCGACCCAGGGGAGTGAACCCAGGTCTCCTGCATTGCAGGCA	246
		6891
	GGAATITIC CAGGO AGGA GTGCAGGATTGGATTGC CATTIC CTICTOCAG GGGAT CTIC COGAC CCAG GGAGT GAACC CAGGTTOC TGCATTGCAGGCA	
Afshari	GGAATTIIC CAGGGAAGAGTGGAGTGGGTTGCCATTICCTTCTCCAGGGGATCCCGACCCAGGGGATTGAACCCAGGTGTCCTGGATTGTAGGCA	189
4 F221 520	***************************************	246
	GATGTTTIS COAGA CGGA TAGATGGGAAAACG CTAATGTCA GCTAG GGAATAAGA CACA GATCCTCTA CCTAC ACTGATGTGTGTAG GTGA CTCTT CGGA	346
	GATGTTTA CCAGACGGATAGAT GGGAAAACG CTAATGTCA GCTAG GGAATAAGA CACA GATCC TCTA CCTAC ACTGATGTGTGTA GGTGA CTCTT CGGA	6991
Afshari	GATGUTTAC CAGGUGAGATAGATG GGGAAACAU TAAT GUCAG CUAGG GGATAAGAT ACAG ATUUT UU CUATA CUGAT GUGT GCAGGUGAU TUTTU GGA	289
	1811 81111181 11881181181 1811 11811118811811	
AF321530	SCTATGCCAAATGTATCTAGGTAGGCAAGATTTGGTGCTGTCACTTCCCTGGTGTTGCAAATAGGCTTGAGGGGACCTGGGACTATGTAAGGCCTGAGG	446
AY834770	SCTATGCCAAATGTATCTAGTATAGTCAAGATTTGGTSCTGTCACTTCCCTGGTGTTGCAAATAGGCTTGAGGGGACCTGGGACTATGTAAGGCCTGAGG	7091
Afshari	COTTTCCCAAATGTATCT GGTATAGTC AAGATTTGGTGTTATCACTTTCCTGGTGTTGCAAATA GCCTTGAGG GGACTTGGGACTAT GTAA GGCCTGAGG	389
	*** ******** **** ******* ******* * ****	
AF321530	AGGGFATTA GACGGGTTT GGGGGATGTTCGCCCAGGCTCTTGTCACTGCCCAGCAGGAGTACTCAGTTGTGACCCAGGGAGCAGGCTTCAGTGTCTTA	539
AY834770	AGGGCATCA GACGGGTTT GGGGGATGTTCCGC CACGCCCTG TCACTGCCC AGCAGTACTC AGTTGTGACCTTGAGGGA GCAGGCTTC GGTGTCTTA	7187
Afshari	A GGGCATCAGATGGGTCTGAGGGATGTT CCATC ACAC CCTGCCAGCAGCCAGCAGCAGCCAGTGTGACCTTGAGGGAGCAGGCTTTGGTGTCTTA	489
2 1131141 1	18211382122	.07
AF321530	eaga gaggaagets sgaettseg sets eets eets eele ettetettse geastttasttsee aasteststega esett ge	626
	CAGA CAGGA AGETA GGAG TECAG GETG CETTE TECA CECT ETTETTETT GEAGTTTAGTTGCC AAGT GGTGT TGAC GETT GETGT	7274
Afshari	PAGA BAGBA AGETA GGAG TERAG GETG CETTETECA CECTT ETCATEGTGAT GTGGTTTAG TEGE CAAGITGTGTTGTGT GTAT GCAT GCAT GCAT GCAT GCAT	589
Aishaii	And the control of the control of the control of the state of the stat	309
AF321530	AGCC CACCA GACTA CTCT GTCCATGGGATTCT CCAG GCCAG GCCAGTGGA GTGGGTCAC CATTT CCTT CTCTA GGGGATCTT CCCAA CCCA GAGATTGAA	726
	AGCC CACCA GACTO CTCT GTCCATGGGATTCT CCAG GCCAG GCCACTGGAGTGGGTCAC CATTT CCTT CTCTA GGGGATCTT CCCAA CCCA GGGATTGAA	7374
Afshari		686
Alshari	AGCCCACCAGGGTTCCTTCTCGGGATTCTCCAGGCCAGAATACTGGAGTTGGTTCACCATTCCTTTAGGGGATCTTCCCGACCCAGGGATCGAA	UOU
A E221520	TCCGGGT CTCCTACATTTCAGGTAGATGATTTAC CGACT GAGCTATGAGGGAA GCCCTCTCC TAAT GGGCT GTGGGAATT -CAGATGCC CTGCCTGAT	022
		823
	${\tt TCCGGOTCTCCTACATTTCAGGTAGATGATTTACCCAACTGAGCTATGAGGGAAGCCCTCTCCTTATGGGCTGTGGGAATT-CAGATGCCCTGCGTGATAGATGCCCTGCTGATAGATGCCCTGCGTGATAGATGCCCTGCGTGATAGATGCCCTGCGTGATAGATGCCCTGCTGTGATAGATGCCCTGCTGTGATAGATGCCCTGCTGTGATAGATGCCCTGCTGTGATAGATGCCCTGCTGTGATAGATGCCCTGCTGTGATAGATGCCTGTGTGATGATGATGATGATGATGATGATGATGATGATGA$	7471
Afshari	ACCTEGGGT CTCCC ACATTGCGGGCAGATGATTTAC CGACTGAGCTATGA GGGAA GCCCTCTCCTTAT GGACTGTGGGAATTTCACATGCCCTGCGTGAT	786
4 E221 520	** ******* **** * ** ** ******** ******	000
	CTGCTCCTTACCTGGAGGTCTTAGAGTTTTATGGCTCTTACTGGAGACATCAACTTACAGAGCCAAAAAAATCAGAAATTATGTTGTGTATTTCCAGAACT	923
	etgetettachtgaggtettagatetttatggetettactgaagacateaaettacagagecaaaaaaateaaaatea-gtatatagtgtatttecagaact	7569
Afshari	CTGCT CCTTA CCTGGAGGTCTTAGAATTTTGTG GCTCTTACTGGAGA CATCAACTTACAGAGCCAARAR AATTA GAAAR ATATTCTGTATTT CCAGAAGT	886

	arca forficatatara gargatara-atotatgafar gasttitto fitat gascitotic gargacottitotataca acatgo otto	1019
	AACA GAGT CATATATAA GAAGAATAAA -ATC TATGAGAAG GAGTT CTTC GITAT GAGCT CCTG GAAGAC CGT GTATC TTGTATACAACA TGC CTTG	7664
Afshari	arca stast catatataa gargaataa scate est gagargarettitte attat gagetettg gargae catatat ete staskaer atrae etts	986
	***** ********** *** *** *** *** **** *** *** *** ****	
AF321530	${\tt TATCAGGSTTGCCGTGTGGCCCACTGACTGCAGCCACTTGTGTATGGTATTTTGGCTTACTGGAATTAAAATGAATTT-AATTAGCTGCTAACAATTACACACTGGAATTAAAATGAATTT-AATTAGCTGCTAACAATTACACACTGCAATTAAAATGAATTAAGATTAGCTGCTAACAATTACACACTGCAATTAAAATGAATTAAGATTAGCTGCTAACAATTAAAATGAATTAAGATTATAAGATTATAAGATTATAAGATTAAGATTATAGATTATATATATATATAGATTATATATATATATATATATATATATATATATATATAATAT$	1118
AY834770	TATE AGGSTTGCCGTGTGGCCCACTGACTGCAGCCACTTGTGTATGTTATTT-GGCTTACTGAATTAAAATGAATTTAAGTTGGTGCTAACAATTACACTGCTACACACTGACTG	7764
Afshari	${\tt TATEAGGATTGCTGTGGCCCACCAGCTACAGCCACTGTGTGTTTCTATTT-GGCTTACCATAATGAAATGA$	1085
	181118111118 11118811811	
AF321530	STCA GAATA GTTCA TATA AAATG CCCATTTCTAGCTTCTTGAAAAACTGGAAT CGCT GGCAG CAGT GGGCC CCCAT GGCAACACCTGACTGGCGTCCT	1218
AY834770	STCA GRATA STTCA TATA ARATGE CARTTET AGCTTETETT GRARA RET GGART COUT GGCAG CAGT GGGCE CCCAT GGCAACAC CT GAC TGGCGTCCT	7864
Afshari	STER BRATA STEER CATS RATA RARTGE CARTGET AGENT CTUTT GRAG RACT GGERT CGC AGE GGGC CC CAT GGERR CRUTT GREET GGERT CCC	1185

AF321530	TACA DOCTO GTOTO AGOO COCCO GOOD CACOT CACOT GALLOT	1305
	TACA SCITS STOTE ASSISTED SCIENCES CARCET CARCET SACETSCET SGCIT STOTA GGCIT STORE STICK ASSISTED STORE CARCET SACE SACET SACE SACE SACET SACE SACE SACE SACE SACE SACE SACE SACE	7951
Afshari	TGCA GCCTG GTCTGAGCC TCCCC GGCC CACTT CACC TGTGT GTTTTACCC ACGTG GCCT CTGTA AGCT CTGGA GTCCA GCTCTGTGT CCCC CACATTGCA	1285
- 21011111	448.4 8 464.4 644.4	
AF321530	TOAT STORAGEAGET AGTGAC CATTTOC CTACAAGATG CTCACTAGG GGAAA CGGG CTCC C TGGT AACATTTGAAATGTTTGAT CTTG AAAAT GGTC	1405
	TCATGTCCA GCAGAAGCTAGTGACCATTTCCCTACAAGATGCTCACTAGGGGAAACGGGCTCCCTGGTAACATTTGAAATGTTTGATCTTGAAAATGGTC	
Afshari	TOAT GTOCA GCAGAAGUT AGTGAC CATTTCTTACAAGATG TOCAC GAGG GGAAA CGGG CTCCC TGGT AACATTTGAAATGAT CTTG AAAAT GGGC	
AISHAIT	len billa dicha diamandi indi intini intini intini ana manaman budili di dinamandi intini intini intini manama	1303
AF321530	ATT CARACTTRACTTCGTCCGATCTTTTAGACCARGTCRCAGGGC ARGATCTCC GGTGGTGGAAAGAGC 1478	
	ATT CARACTTAR TTAGTTCTGTCGATCTTTTAGATCAAGTCACAGGACAAGATCTCCGGTGGTAGAAGAGGC 8124	
	4.400	
Afshari	ATTICARAGITTAGITTITGTICGATCITTICAGAICAAGGICACAGGAIGAGAT-TICGGITGGAAAGAGC 1457	

Figure 2. ClustalW alignment of the Cast1 sequence in Afshari sheep and the two highly similar sequences of the bovine calpastatin gene (AF321530 and AY834770) that are available in GeneBank.

Comparison of sequences showed multiple nucleotide substitutions. The number of nucleotide substitutions in calpastatin gene of the three sequences is shown in Table 2. It has previously been revealed that the binding of the calpains to calpastatin required Ca²⁺, calpains proteolytic activity has inverse relation with cal-

pastatin activity and Ca²⁺ concentration (Goll *et al.*, 2003). It is generally accepted that the Ca²⁺ dependent interaction of calpain with calpastatin is the most relevant mechanism involved in the regulation of Ca²⁺ induced proteolysis. The calpastatin binding region is localized in the non-inhibiroty L-domain containing

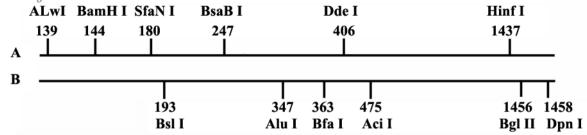


Figure 3. Comparison of the restriction maps of the calpastatin gene in A: sheep and B: bovine species. Selected restriction enzymes are shown on both fragments. The numbers show the sites cut by restriction enzymes.

Table 2. Pairwise percentage of similarity between sequences of Cast1 (Afshari) and the two sequence of the bovine calpastatin gene (AF321530 and AY834770).

Seq A	Name	Len (nt)	Seq B	Name	Len (nt)	Score
1	AF321530	1522	2	AY834770	13252	98
1	AF321530	1522	3	Afshari	1457	89
2	AY834770	13252	3	Afshari	1457	89

the amino acid sequences encoded by exons 2-8 (Melloni *et al.*, 2006). The L-domain function is unknown (Reynaud *et al.*, 2005) but it has been suggested that calpastatin L-domain has central role to regulate Ca^{2+} channel (Hao *et al.*, 2000).

In the present study, the partial sequences of intron 6 and exon 7 were determined. These sites encoded L-domain amino acids; hence by detection of the conserved mutations in these regions, especially in exon, it is possible to use such polymorphisms in marker-assisted selection (MAS).

Marker-assisted selection uses polymorphism-related information in livestock selection programs (Davis and DeNise, 1998). However, some studies have reported no association between the calpastatin alleles and meat tenderness in cattle (Barendse 2002; Chung *et al.*, 2002; 1999) and pigs (Kocwin-Podsiadla *et al.*, 2003).

Recently it has been reported that calpastatin can be a candidate gene for meat quality and rate of muscle extension in livestock (Schenkel *et al.*, 2006). Recent studies have reported the association between calpastatin polymorphism and meat quality in beef (Casas *et al.*, 2006; Ciobanu *et al.*, 2004). An experiment which has been carried out by Chung and Davis (2001) has shown that the different alleles of calpastatin have affected the growth traits in Angus bulls.

Calpastatin plays a major role in postmortem meat ten-

derization. Attention to this function and detecting polymorphisms in the calpastatin gene, may help to find the sheep alleles effective in meet production, especially in areas such as Iran that the genetic composition of their sheep breeds have not been studied properly. On the other hand, the 89% identity in the nucleotide sequence of the calpastatin gene L-domain as observed between the bovine species and sheep indicates that they have homology thus providing a comparative genome-based data for further related studies. Besides, this also proves that in the absence of a published sequence data in databases, regarding the target animal (in this study, sheep), using the counterpart sequences in related taxa (bovine, porcine, etc.), is applicable for detection of those genes, whereby the conserved nature of the desired sequence can play a major role in the successful amplification of the target genome.

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148 www.SID.ir

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