Short Communication

Characterization of calpastatin gene in Iranian Afshari sheep

Navid Dinparast Djadid[1*], Mehdi Nikmard[2], Sedigheh Zakeri[1], Saber Gholizadeh[1]

[1]Malaria and Vector Research Group, Biotechnology Research Center, Pasteur Institute of Iran, P.O. Box 1316943551, Tehran, I.R. Iran [2]Animal Science Department, College of Agriculture, University of Zanjan, P.O. Box 313, Zanjan, I.R. Iran

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 include the two Ca^{2+}-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

*Correspondence to: **Navid Dinparast Djadid, Ph.D.**
Tel/Fax: +98 21 66480780
E-mail: ndinparastdjadid@yahoo.com and navidmvrg@gmail.com

on the bovine calpastatin gene (GenBank accession No: AY834770) were used to amplify a fragment of approximately 1500 bp. PCR mixture (25 µl) was consisted of 50-100 ng sheep genomic DNA, 10 pmol of each primer, 200 µM dNTPs (Roche, Germanty), 1.5 mM $MgCl_2$, PCR buffer 1X, and 1 unit *Taq* polymerase (Sinagene, Iran).

The temperature cycling (initial denaturation 10 min at 95ºC, 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 µl 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 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.

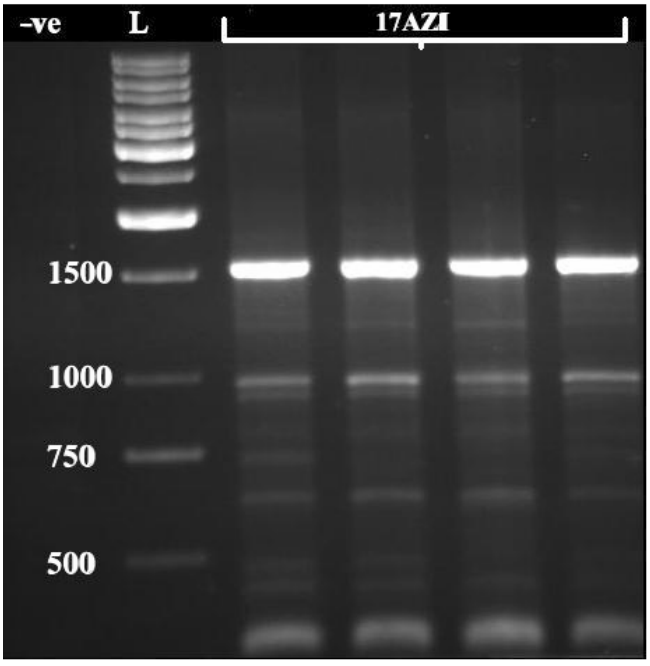

Figure 1. Electrophoresis analysis of PCR products amplified for Cast1 in sheep 17AZI. -ve and L are representing negative control and one kb (SMO311) marker.

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

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 Ca^{2+}, calpains proteolytic activity has inverse relation with calpastatin activity and Ca^{2+} concentration (Goll *et al*., 2003). It is generally accepted that the Ca^{2+} dependent interaction of calpain with calpastatin is the most relevant mechanism involved in the regulation of Ca^{2+} induced proteolysis. The calpastatin binding region is localized in the non-inhibiroty L-domain containing

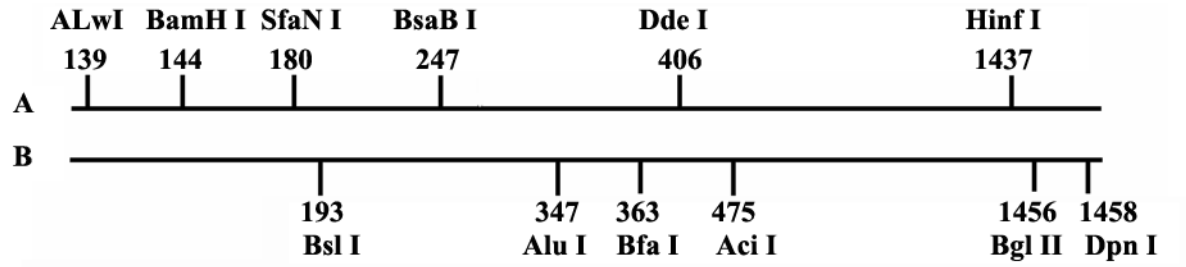

Figure 3. Comparison of the restriction maps of the calpastatin gene in A: sheep and B: bovine species. Selected restriction enzymes are shown on both fragments. The numbers show the sites cut by restriction enzymes.

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 tenderization. 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.

Acknowledgements

We wish to thank colleagues at the Malaria and Vector Research Group (MRG) for their technical assistance, especially Miss F. Farhoomand and Miss Z. F. Ganji.

References

Amanda W, Thompson VF, Goll DE (2004). Interaction of calpastatin with calpain: a review. *Biol chem*. 385: 465-472.

Barendse WG (2002). DNA markers for meat tenderness. *International patent application* No. PCT/AU02/00122.

World Intellectual Property Org. Int. Publication No. WO 02/064820 A1.

Boom R, Sol CJ, Salmans MM, Jansoen CL, Wertheim-van Dillen PM, Noorda JVD (1990). Rapid and simple method for purification of nucleic acids. *J Clin Microbiol*. 28: 495-503.

Casas E, White SN, Wheeler TL, Shackelford SD, Koohmaraie M, Riley DG, Chase JrCC, Johnson DD, Smith TPL (2006). Effects of calpastatin and μ-calpain markers in beef cattle on tenderness traits. *J Anim Sci*. 84: 520-525.

Chung HY, Kim CD, Cho CY, Yeon SH, Jin HJ, Jeon KJ, Kim HC, Lee HJ, Hines HJ, Davis ME (2002). Effects of calpatatin gene polymorphisms on growth and carcass traits of Korean native cattle. Proc. Of the 7th World cong. Genet. Appl. Livest. Prod., Montpellier, France. CD-ROM Communication No.11-33.

Chung HY, Davis EM (2001). Effect of calpain and calpastatin genotypes on growth of angus bulls. Ohio State University-Research and Reviews: Beef and sheep. Available: http://ohioline.osu.edu/sc181/sc181_5.html

Chung HY, Davis ME, Hines HC, Wulf DM (1999). Relationship of a PCR-SSCP at the bovine calpastatin locus with calpastatin activity and meat tenderness. Ohio State University-Research and Reviews: Beef and sheep. Available: http://ohioline.osu.edu/sc170/sc170_3.html

Ciobanu DC, Bastiaansen JWM, Lonergan SM, Thomsen H, Dekkers JCM, Plastow GS, Rothschild MF (2004). New alleles in calpastatin gene are associated with meat quality traits in pigs. *J Anim Sci*. 82: 2829-2539.

Davis GP, DeNise SK (1998). The impact of genetic marker on selection. *J Anim Sci*. 76: 2331-2339.

Goll DE, Thompson VF, Taylor RG, Ouali A (1998). The calpain system and skeletal muscle growth. *Can J Anim Sci*. 78: 503-512.

Goll EG, Thompson VF, Li H, Wei W, Cong J (2003). The calpain system. *Phys Rev*. 83: 731-801.

Hao LY, Kameyama A, Kuroki S, Takano J, Takano E, Maki M, Kameyama M (2000). Calpastatin Domain L is involved in the regulation of L-type Ca channels in guinea pig cardiac myocytes. *Biochem Biophys Res Comm*. 279: 756-761.

Kocwin-Podsiadla M, Kuryl J, Krzecio E, Zybert A, Przybylsky W (2003). The interaction between calpastatin and RYR1 genes for some pork quality traits. *Meat Sci*. 65: 731-735.

Melloni E, Averna M, Stifanese R, Tullio RD, Defranchi E, Salamino F, Pontremoli S (2006). Association of calpastatin with inactive calpain. *J Biol Chem*. 281: 24945-24954.

Raynaud P, Jayat-vignoles C, Laforet ML, Leveziel H, Amarger V, (2005). Four promoters direct expression of the calpastatin gene. *Arch Biochem Biophys*. 437: 69-77.

Schenkel FS, Miller SP, Jiang Z, Mandell IB, Ye X, Li H, Wilton JW (2006) Association of a single nucleotide polymorphism in the calpastatin gene with carcass and meat quality of beef cattle. *J Anim Sci*. 84: 291-299.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acids Res*. 22: 4673-4680.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res*. 24: 4876-4882.