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GENETIC POLYMORPHISM OF BUFFALO μ-CALPAIN GENE USING PCR-RFLP

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ABSTRACT

Calpain is a ubiquitous cytoplasmic cysteine protease, its activity is dependent on calcium. Two genes of calpain- *CAPN1* (μ-calpain) and *CAPN2* (m-calpain) have been identified. The micromolar calcium-activated neutral protease (*CAPN1*) gene encodes a cysteine protease, μ-calpain that degrades myofibril proteins under postmortem conditions and appears to be the primary enzyme in the postmortem tenderization process. Regulation of μ-calpain activity has correlated with variation in meat tenderness. Genetic polymorphism analysis of *CAPN1* gene would likely aid in the development of selection criteria for improving meat tenderness. This study aimed to detect genetic polymorphism within intron 14 of buffalo *CAPN1* gene using PCR-RFLP technique.

Buffalo DNA was amplified using primers that were designed from the cattle *CAPN1* gene sequence. The amplified fragments obtained from all tested buffalo DNA (100 animals) at 670-bp were digested with *Fok*I endonuclease. All buffalo animals are genotyped as CC where all tested buffalo DNA amplified fragments were digested with *Fok*I endonuclease and gave two digested fragments at 530-bp and 140-bp due to the presence of C base at position 4685 in all tested animals and the absence of T base in this position within intron 14.

Keywords: Buffalo, μ-calpain gene, PCR, RFLP

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INTRODUCTION

Variation in meat tenderness has significant impact on consumer satisfaction with beef; however, classical selection techniques have not been effective in eliminating the small fraction of animals yielding undesirable palatability traits (Page et al., 2002). Establishing the genetic basis for variation in meat tenderness would likely aid in the development of selection criteria for improving meat tenderness in cattle (Page et al., 2004).

Calpain is a ubiquitous cytoplasmic cysteine protease, its activity is absolutely dependent on calcium (Sorimachi et al., 1997). Two genes of calpain- *CAPN1* (μ-calpain) and *CAPN2* (m-calpain) have been identified (Suzuki and Sorimachi, 1998). The micromolar calcium-activated neutral protease (*CAPN1*) gene encodes a cysteine protease, μ-calpain that degrades myofibril proteins under postmortem conditions and appears to be the primary enzyme in the postmortem tenderization process (Koohmaraie, 1992; 1994; 1996 and Beltran et al., 1997). Regulation of μ-calpain activity has been correlated with variation in meat tenderness (Geesink and Koohmaraie, 1999).

Bovine *CAPN1* has been mapped to the telomeric end of BTA29 (Smith et al., 2000) and recently, a QTL for meat tenderness was found to be segregating in this region of BTA29 (Casas et al., 2000 and Morris et al., 2001). The evaluation of *CAPN1* as a candidate gene for meat tenderness was supported by Meat Animal Research Center, MARC (AMSA, 1995).

In the *CAPN1* gene, more than 100 single nucleotide polymorphisms (SNPs) have been identified in cattle (Smith et al., 2000; Page et al., 2002 and Juszczuk-Kubiak et al., 2004). Among them, four polymorphisms, two non-synonymous SNPs (G316A and V530I), and two intronic SNPs (C4685T and C4751T) have been found to have significant effects on meat tenderness (Page et al., 2002; Page et al., 2004; White et al., 2005; Morris et al., 2006; Rincon and Medrano, 2006 and Van Eenennaam et al., 2007)

The relatedness between cattle and buffalo (*Bovinae* subfamily) is useful in studying the genetic polymorphisms and mapping of QTL candidate genes in buffalo. The chromosome banding similarity between cattle and

buffalo indicates gene mapping conservation and genetic polymorphism homology between these two species (Othman, 2005 and 2006). Our objective was to detect the genetic polymorphism within intron 14 of buffalo μ-calpain (*CAPN1*) gene, which has been associated with meat tenderness, using PCR and RFLP techniques.

MATERIALS AND METHODS

Genomic DNA Extraction

The genetic polymorphism of *CAPN1* gene was analyzed using 100 blood samples from unrelated Egyptian buffaloes. Genomic DNA was extracted from the whole blood by phenol-chloroform method described by John et al. (1991) with minor modifications. Ten ml of blood taken on EDTA were mixed with 25 ml cold sucrose-triton and the volume was completed to 50 ml by autoclaved double distilled water. The solution was mixed well and the nuclear pellet was obtained by spinning and discarding the supernatant. The nuclear pellet was suspended in lysis buffer (10 mM Tris base, 400 mM NaCl and 2 mM sodium EDTA) pH 8.2, with 20% sodium dodecyl sulfate (SDS) and proteinase K (10 mg/ml), and incubated overnight in a shaking water-bath at 37°C.

Nucleic acids were extracted once with phenol, saturated with Tris-EDTA (TE) buffer (10 mM Tris, 10 mM NaCl and 1mM EDTA), followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1) until there was no protein at the interface. This was followed by extraction with chloroform-isoamyl alcohol (24:1). Equal volume of the solvent was added to each extraction, followed by thorough mixing and centrifugation for 10 min. at 2000 rpm. The top layer was carefully transferred to another Falcon tube for the next extraction.

To the final aqueous phase, 0.1 volume of 2.5 M Na acetate and 2.5 volume of cold 95% ethanol were added. The tubes were agitated gently to mix the liquids till a fluffy white ball of DNA was formed. The DNA was picked up with a heat-sealed Pasteur pipette and washed briefly in 70% ethanol.

The DNA was finally dissolved in an appropriate volume of 1X TE buffer. DNA concentrations were determined via spectrophotometer and diluted to the working

concentration of 50 ng/µl, which is suitable for polymerase chain reaction.

Polymerase Chain Reaction (PCR)

The primers used in this study were designed from the cattle *CAPN1* gene sequence (GenBank No. AF248054). The 670-bp DNA fragment (exon 14 to exon 18, the region coding for protein large subunit of domain IV and including introns) in the *CAPN1* gene was amplified (Juszczuk-Kubiak et al., 2004).

Primer Up: TTC AGG CCA ATC TCC CCG ACG (exon 14)

Primer Down: GAT GTT GAA CTC CAC CAG GCC CAG (exon 18)

A PCR cocktail consists of 1.0 µM upper and lower primers and 0.2 mM dNTPs, 10 mM Tris (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$ and 1.25 units of Taq polymerase. The cocktail was aliquot into tubes with 100 ng of buffalo DNA. The reaction mixture was overlaid with sterile mineral oil. The reaction was cycled for 1 min. at 94°C, 2 min. at 62°C and 2 min. at 72°C for 30 cycles. The PCR reaction products were electrophoresed on 3% agarose gel stained with ethidium bromide to test the amplification success.

RFLP and Ggarose Gel Electrophoresis

20 µl of PCR product were digested with 10 units of *Fok*I restriction enzyme -GGATG- in a final reaction volume 25 µl. The reaction mixture was incubated at 37°C in water bath for 5 hours. After restriction digestion, the restricted fragments were analyzed by electrophoresis on 2.5% agarose/1X TBE gel stained with ethidium bromide. The 100-bp ladder was used as molecular size marker. The bands were visualized under UV light and photographed with yellow filter on black and white film.

RESULTS AND DISCUSSION

Meat tenderness is a critical trait in determining consumer satisfaction, and there has been significant interest in genetic selection to decrease problems with meat tenderness variation. However, the problem of variability in meat tenderness has not diminished, in part because of an inability to accurately select for increased tenderness. Identification of genetic markers for meat tenderness variation would provide some selection criteria to facilitate genetic improvement in this trait (Page et al., 2004).

Calpain is a ubiquitous cytoplasmic cysteine protease, its activity is absolutely dependent on calcium (Sorimachi et al., 1997). Two isoforms of calpain have been identified; μ-calpain (*CAPN1*) that requires calcium at micromolar concentrations for activity and m-calpain (*CAPN2*), for which calcium at milimolar concentrations is needed (Suzuki and Sorimachi, 1998). Both calpains have been shown to degrade most of the myofibrillar protein excluding actin and myosin (Beltran et al., 1997).

Regulation of μ-calpain activity has been correlated with variation in meat tenderness (Geesink and Koohmaraie, 1999). Bovine *CAPN1* and *CAPN2* genes have been mapped to chromosome 29 (Smith et al., 2000). Only in few cases, the nucleotide sequence polymorphism has been detected in the bovine *CAPN1* gene.

In this study by using PCR and RFLP techniques, we aimed to detect the genetic polymorphism within intron 14 of buffalo *CAPN1* gene that has been associated with meat tenderness, which previously reported in bovine.

By means of PCR, the genomic buffalo DNA was amplified using oligonucleotide primers that were designed from the cattle *CAPN1* gene sequence (GenBank No. AF248054). The amplified fragments obtained from all tested buffalo DNA (100 animals) were at 670-bp (Fig. 1), from nt 4542 to nt 5212 according to cattle *CAPN1* gene sequence (GenBank No. AF248054, Juszczuk-Kubiak et al., 2004).

The PCR amplified fragments resulted from Buffalo DNA at 670-bp were digested with *Fok*I endonuclease to detect the genetic polymorphism within intron 14 of buffalo *CAPN1* gene. Depending on the alteration of presence C/T at position 4685 (within intron 14), we can easily differentiate between 3 different genotypes: TT with undigested one fragment at 670-bp, CC with two digested fragments at 530-bp and 140-bp and TC with three fragments at 670-bp, 530-bp and 140-bp.

All buffalo animals investigated in this study are genotyped as CC where all tested buffalo DNA amplified fragments were digested with *Fok*I endonuclease and gave two digested fragments at 530- and 140-bp. The restriction site is resulted due to the presence of C base at position 4685 in all animals and the absence of T base in this position within intron 14 (Fig. 2).

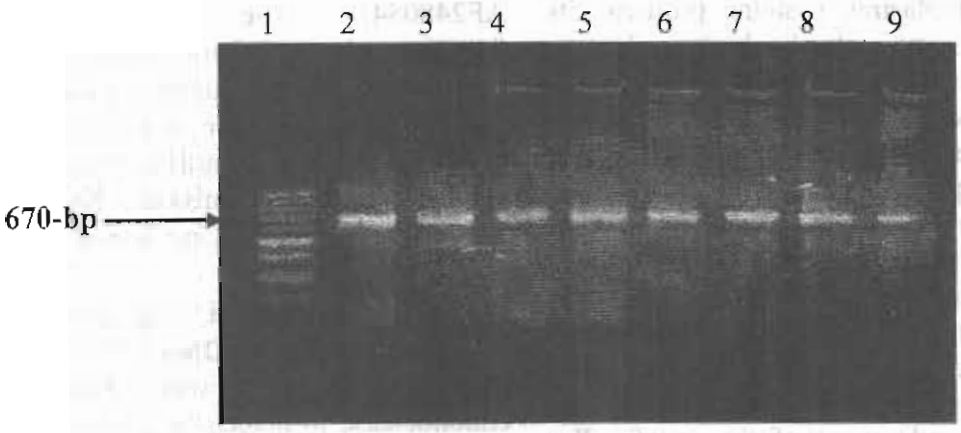

Fig. 1. Ethidium bromide-stained gel of amplified PCR products representing *CAPN1* primer

Lane 1: 100-bp ladder marker

Lanes 2-9: 670-bp PCR products amplified from Egyptian buffalo DNA

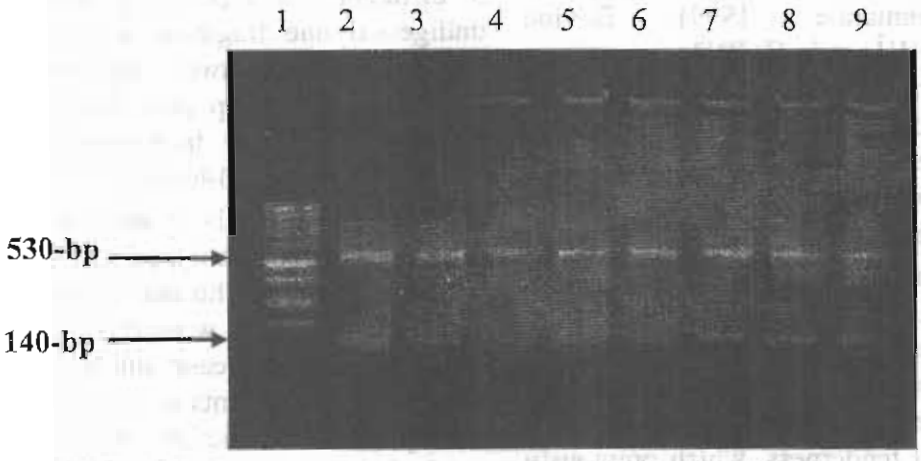

Fig. 2. The electrophoretic pattern obtained after digestion of PCR amplified buffalo *CAPN1* with *Fok*I

Lane 1: 100-bp ladder marker

Lanes 2-9: Homozygous CC genotypes showed two restricted fragments at 530- and 140-bp.

Thirty-eight SNPs (Single Nucleotide Polymorphism) in bovine *CAPN1* were identified by sequencing all the 22 exons and 19 of 21 introns in two sires: Piemontese × Angus and Jersey × Limousin (Page et al., 2002). The majority of the 38 SNPs was found in introns or was synonymous substitutions in the coding regions. They identified six SNPs in introns being two substitutions T/C (intron 7), one substitution C/T in intron 8 and three mutations: T/C, C/T and G/A (intron 18). Two SNPs in exon 6 were synonymous substitutions: G/A and T/C.

The two SNPs were reported by Page *et al.* (2002 and 2004) in bovine *CAPN1* gene representing exon 9 (A/G) and exon 14 (G/C) predicted amino acid changes: substitution in exon 9 (Gly^{316}/Ala^{316}) and exon 14 (Val^{530}/Ile^{530}). Analysis of genotypes and shear force values in the two sires: Piemontese × Angus and Jersey × Limousin (Page *et al.*, 2002) revealed a difference between *CAPN1* alleles in which the allele encoding isoleucine at position 530 and glycine at position 316 associated with decreased meat tenderness (increased shear force values) relative to the allele encoding valine at position 530 and alanine at posiyion 316 ($p<0.05$).

The genetic analysis of these both SNPs at 316 and 530 positions was done in two bovine populations by Page et al. (2004) and indicated an association of genotype and phenotype for both markers. Animals homozygous for the C allele at marker 316 had lower shear force ($p=0.02$) than animals of CG or GG genotype, and animals with homozygous GG genotype at marker 530 had lower shear force ($p=0.04$) than animals of AG or AA genotype.

Juszczuk-Kubiak *et al.* (2004), with the same primers used in our study, they reported the appearance of three SSCP (single-strand conformation polymorphism) patterns- within intron 14 of bovine *CAPN1*- in 141 tested animals belonging to seven cattle breeds. These three SSCP patterns were confirmed by RFLP and gave three different genotypes, CC (35%), CT (35%) and TT (30%) due to the transition of C→T at position 4685 in tested animals which showed T allele. The allele frequency was 0.62 and 0.38 for C and T allele, respectively. The authors reported

that the TT genotype was significantly associated with a higher lean share in valuable cuts; the advantage over the CC genotype was 4.5% ($p \leq 0.01$).

Casas et al. (2005) assessed the association of four SNPs in the bovine *CAPN1* gene with meat trait in Brahman population. Two markers, CAPN316 (C/G) in exon 9 and CAPN530 (A/G) in exon 14, were previously reported (Page et al., 2002 and 2004) and two new markers: CAPN4753 (A/C) in intron 21 and CAPN5331 (A/T) in intron 1. The CAPN530 marker was uninformative in this population, the G allele seems to be fixed or at high frequency. The marker CAPN316 was associated ($p<0.05$) with sensory panel tenderness score. The three markers CAPN316, CAPN4753 and CAPN5331 were associated with hump height ($p<0.02$). Another new SNP marker in this population was reported by White et al. (2005). This marker, CAPN4751, was associated with shear force on postmortem d 7 ($P<0.01$), 14 ($P= 0.015$) and 21 ($P<0.001$) in this population, demonstrating that genetic variation important for tenderness segregates in cattle at or near *CAPN1*.

By direct DNA sequencing of 24 unrelated Korean cattle, Cheong et al. (2008) identified 39 polymorphisms in Korean cattle *CAPN1* gene within exons and their flanking regions. Among them, 12 common polymorphic sites were selected for genotyping in the beef cattle (n=421). Associations of *CAPN1* polymorphisms with cold carcass weight (CW) and marbling score (MS) were analyzed. One polymorphism *(479C→T)* showed significant associations with MS. The T allele revealed an additive effect on MS, i.e., the lowest MS was found in T/T (MS = 0.94), intermediate in C/T (MS = 1.56) and the highest in C/C (MS = 2.34) ($p = 0.02$).

In conclusion, the genetic polymorphisms of the buffalo *CAPN1* gene provided the possibility for existence the association between restriction sites and amino acid substitutions with meat tenderness variations. The evaluation of *CAPN1* as a candidate gene for meat tenderness in buffalo would likely aid in the development of selection criteria for improving meat tenderness.

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الطرز الوراثية المتعددة لجين μ-calpain في الجاموس باستخدام PCR-RFLP

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يعتبر جين ال μ-Calpain من الجينات الهامة التى لها إرتباط وثيق بإنتاج اللحوم فى حيوانات المزارع وقد وجد أن الطرز الوراثية المتعددة لهذا الجين لها تأثير مباشر على نوعية اللحوم المنتجة ومن هنا تظهر الأهمية الإقتصادية الكبيرة لدراسة الطرز الوراثية المتعددة لهذا الجين فى الجاموس المصرى والذي يعتبر من المصادر الرئيسية لإنتاج اللحوم فى مصر. وقد إستهدفت هذه الدراسة تحديد الطرز الواثية المتعددة لجين μ-Calpain فى الجاموس المصرى بإستخدام تقنية PCR-RFLP.

وقد تم فى هذه الدراسة إستخلاص الحمض النووي من عينات الدم لعدد ١٠٠ من حيوانات الجاموس المصرى وذلك بطريقة الفينول كلوروفورم. وقد استخدم في هذه

الدراسة بادئ جزيئي لهذا الجين مصنع على حسب التسلسل النيوكليتيدي لهذا الجين في الأبقار ونتيجة الإرتباط والتشابه بين التسلسل النيوكليتيدى للجينات فى الأبقار والجاموس فإن هذا البادئ الجزيئي قد تم إستخدامه بنجاح فى الجاموس المصرى.

وقد تم إجراء تفاعل إنزيم البلمرة المتسلسل على عينات الحمض النووى للجاموس بإستخدام البادئ الجزيئ الخاص بهذا الجين. وقد أظهرت نواتج تفاعل إنزيم البلمرة المتسلسل لجميع عينات الحمض النووى المختبرة وجود **amplified fragments** عند مستوى **670-bp** وبعد ذلك قد تم معاملة هذه النواتج بإنزيم القطع ***FokI*** وتم بعد ذلك التفريد الكهربائى لنواتج القطع على الأجاروز جل ثم مشاهدة الحزم بالأشعة فوق البنفسجية وتصويرها للتعرف على الطرز الوراثية المتعددة لهذا الجين فى الجاموس المصرى . وقد أظهرت النتائج أن جميع حيوانات الجاموس المختبرة لها طرز وراثى متشابه وهو **CC** لجين **μ-Calpain**. حيث أن نتيجة معاملة نواتج تفاعل إنزيم البلمرة المتسلسل بإنزيم القطع قد أظهر وجود عدد إثنين من **digested fragments** عند مستوى **530-bp** و **140-bp** وهذا هو الشكل الجزيئي المميز للطرز الوراثى **CC** لجين **μ-Calpain**.