

A novel RFLP/*AluI* polymorphism of the bovine calpastatin (CAST) gene and its association with selected traits of beef*

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Calpastatin (CAST) is a specific inhibitor of the ubiquitous calcium-dependent proteases – μ -calpain and m-calpain, found in mammalian tissues. The level of components included into the calpain-calpastatin system determine the rate of *post mortem* tenderization of meat.

In the coding region of the bovine CAST gene (*CAST*) the new nucleotide sequence polymorphism was found being a substitution G→C at position 61 nt within the exon 1C (consensus sequence – GenBank AF117813). This sequence fragment of SNP position has already been deposited in the GenBank database under accession no. AY258325. Consensus of bovine *CAST* sequence with that of human (GenBank M86257 and M28230) revealed that G→C substitution was located at position 1460 nt of exon 12. Computer analysis of the mutation showed the Ser→Thr substitution at position 20 of amino acid sequence of CAST protein. The mutation creates a new *AluI* restriction site and, therefore, can be easily detected with PCR-RFLP.

The *CAST* RFLP-*AluI* polymorphism was studied in 138 bulls of seven breeds, including the native Polish Red (PR, preserved), and Polish Black-and-White (BW) breed. The frequency of alleles C and G varied between the breeds considered, the mean reaching 0.69 and 0.31, respectively. No homozygous genotype GG was found in Red Angus, Charolaise and Hereford bulls.

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Analysis of meat of 84 BW bulls revealed differences between animals of different genotypes. Meat of GC bulls showed higher cooking loss and was darker in colour than meat of both homozygous genotypes. Between GC and CC bulls significant difference appeared in the total content of hem pigment of meat. The message from these results is that CAST gene may probably be considered as a candidate marker for beef quality.

KEY WORDS: beef / calpastatin / SNP/*AluI* / tenderization / sensory evaluation

Calpastatin (CAST), which is an endogenous calpains' inhibitor, plays a central role in regulation of calpains activity in the cell [Ouali and Talmant 1990] and is considered to be one of the major modulators of the protein turnover. Therefore, CAST may affect proteolysis of myofibrils due to regulation of activity of calpains and is responsible for initiation of *post mortem* degradation of myofibrillar protein [Goll *et al.* 1992].

The bovine CAST gene (*CAST*) has been mapped to BTA7 [Bishop *et al.* 1993], with relative position of 117.8 cM [Kappes *et al.* 1997]. The gene has been sequenced from bovine skeletal muscle with five different domains identified [Killefer and Koohmaraie 1994]. Bishop *et al.* [1993] using *TaqI*, *BamHI* and *EcoRI* endonucleases found RFLP polymorphism of the bovine *CAST* locus, detectable in the region encoding for domains 2 through 4 plus 3'UTR. Chung *et al.* [1999b] noticed genetic variation in the region coding for domains L and I, and identified three different SSCP patterns in the 1C/1D region of the bovine *CAST*. However, Killefer and Koohmaraie [1994] using as a probe the generated fragment from cDNA encoding for domains L and I of bovine *CAST* did not detect the polymorphism. Lonergan *et al.* [1995] reported the *BamHI* and *EcoRI* PCR-RFLP polymorphism using a 2.2 kb cDNA probe encoding for domains 2, 3, 4 and 3' UTR of *CAST* gene. Chung *et al.* [2001] found DNA polymorphism in the intron 6 using PCR-RFLP technique and *XmnI* as the restriction enzyme. Detection of additional polymorphism is necessary to find the relationship between *CAST* polymorphic variants and beef quality indicators. This report presents the results of searching for genetic variation in *CAST* DNA and the PCR-RFLP polymorphism of the bovine gene *CAST* that can affect the quality of beef.

Material and methods

Animals and sampling

Used were Polish Black-and-White (BW, n=84), Polish Red (PR, n=7), Red Angus (RA, n=9), Charolaise (Ch, n=12), Limousine (L, n=10), Simmental (S, n=9), and Hereford (H, n=8) fattened bulls. PR bulls were maintained at the Research Station for Ecological Agriculture and Preserve Animal Breeding, Popielno, Poland, while remaining ones at the Institute Experimental Farm, Jastrzębiec. Bulls were kept tied in a barn and fed *ad lib.* the total mixed ration (TMR) composed of maize silage, hay and concentrate (65, 5, and 30% TMR dry matter, respectively). From each animal 10 ml of venous blood were withdrawn on K₂EDTA, and genomic DNA was isolated from leukocytes according to Kanai *et al.* [1994]. All animals were routinely slaugh-

tered at the age of 15 months and about 360 kg live body weight at the local abattoir. Carcasses of 84 BW bulls were cooled for 24 hours at 4°C. From right carcass-sides samples of *Longissimus dorsi* (LD) muscle were excised and immediately brought to the laboratory of Meat Technology Department, Warsaw Agricultural University for chemical analyses and technological evaluation.

Analytical

Polymerase chain reaction (PCR). The *CAST* gene (exon 1C and 1D – the region coding for protein domain I, and including also the intron F) was screened with primers used for detecting the ovine *CAST* [Palmer 1998]. The 624 bp DNA fragment was PCR-amplified using the following primers:

CAST 1: 5'-TGGGGCCCAATGACGCCATCGATG-3' (exon 1C);

CAST 2: 5'-GGTGGAGCAGCACTTCTGATCACC-3' (exon 1D).

The polymerase chain reactions (PCR) were performed using a PCR-mix with primers (both at concentration 10 pmol/μl), 1U Taq polymerase (SIGMA), 2.5 μl Taq polymerase buffer, four dNTPs (each at final concentration of 2.5 mM/μl), 100 ng of genomic DNA, and H₂O up to a total volume of 25 μl. The following PCR protocol was used: 30 s at 94°C, 45 s at 62°C and 45 s at 72°C – 32 cycles. The amplified PCR product was separated electrophoretically in 2% agarose gel (GIBCO-BRL, England) with ethidium bromide.

Single strand conformation polymorphism (SSCP). SSCP was determined with the use of a Cooled Mini Vertical Gel Electrophoresis Unit (SIGMA). Ten μl of PCR product were denaturated with formamide and subjected to the electrophoresis in the 8% polyacrylamide gel. The electrophoresis was run at 120 V, 60 mA and 8 W for 20 h at 20°C. Gels were stained using the Silver Staining System (KUCHARCZYK Co., Poland). The SSCP pattern bands were documented with the Molecular Imager System FX (BioRad).

DNA sequencing. PCR products of different variants of the *CAST* gene were purified with a GenElute PCR DNA Purification Kit (SIGMA), and automatically sequenced in an ABJ377 sequencer (APPLIED BIOSYSTEMS, USA).

Restricted fragment length polymorphism (RFLP). PCR-produced amplified DNA product (10 μl) was digested with 10 U of *AfuI* restriction nuclease (BioLabs, New England, USA) at 37°C for 3 h and then subjected to electrophoresis on 2% agarose gel (GIBCO-BRL, England) with ethidium bromide. The gel was visualized under UV light and documented in a Molecular Imager System FX (BioRad).

Meat quality. Associations between meat quality traits and *CAST* genotypes were estimated in 84 BW bulls.

Colour components a*, b* and L* were determined with Minolta CR-200 spectrophotometer.

Texture was measured with ZWICKI type 1120. The slice of meat of about 150 g and 3 cm thick was heated for 2 h at 78°C in 1% solution of table salt and then chilled for 24 h at 4°C. Penetration test was conducted with a plunger 13 mm in diameter,

crosswise the muscle fibres on the 20×40×15 mm sample. The plunger speed was 50 mm/min. The penetration force was measured when the gauge plunger was moved up to 10 mm depth after the initial tension of fibres appeared.

Sensory evaluation was carried according to a 5-point scale. Colour, aroma, taste and tenderness of meat were evaluated as described by Baryłko-Pikielna *et al.* [1964] and Polish Standard PN-ISO6564. The 150 g meat sample was kept first for 72 h at 4°C and next for four days in a 1% table salt solution. Samples were then fried for 8 min on each side at 180°C. Scoring was performed by a group of five experienced people.

Cooking loss was determined on 30 g samples heated for 30 min at 70°C in a beaker. After heating, the sample was chilled for 30 min at a room temperature. The liquid fraction was removed and the beaker weighed. The weight loss was given in per cent as follows:

$$Wt = [(A-B)/(A-C)] \times 100$$

where:

- Wt – liquid outflow after thermal treatment (%);
- A – weight of beaker with sample;
- B – weight of beaker after removing the thermal outflow;
- C – weight of empty beaker.

Water holding capacity was determined with a filter paper method according to Grau and Hamm [1952].

Total content of hem pigment was determined according to Hornsey [1956] after heating of a sample.

Water (105°C, 4 hours), protein (Kjeldahl, factor 6.25) and fat (Soxhlet) were determined according to Polish Standards PN-73/A-82110, PN-75/A-04018 and PN-73/A-82111, respectively.

pH was measured 48 h post-slaughter in a LD muscle homogenates, using the CP-315 pH-meter with the combined glass-calomel electrode, as prescribed by Polish Standard PN-77/A-82058.

Statistical

The data were statistically evaluated with one-way analysis of variance. Student's t-test was used to evaluate the significance of differences between means ($P \leq 0.05$ and 0.01).

Results and discussion

PCR-SSCP method was used to identify polymorphism in the fragment including part of the exons 1C and 1D of the bovine *CAST* gene. Specific PCR product obtained of 624 bp was denatured and subjected to polyacrylamide gel electrophoresis to find out the SSCP polymorphism. Three SSCP patterns termed 1, 2 and 3 were found, representing different variants of the bovine *CAST* gene. The DNA samples of different

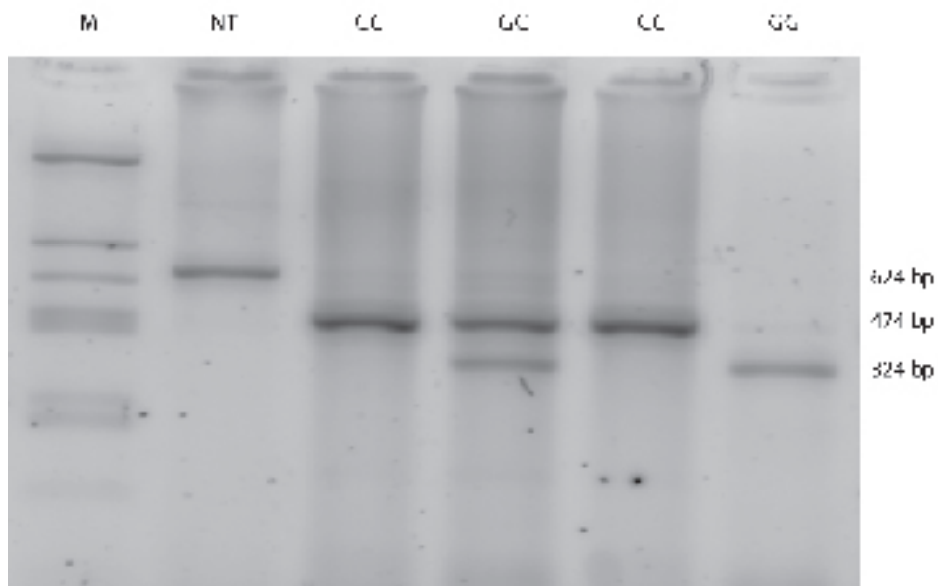


Fig. 2. RFLP-genotyping of the bovine *CAST* gene. Agarose (2%) gel showing *CAST* genotypes after digestion of the 624-bp PCR product with *AhaI* nuclease. Genotypes *CC*, *GC* and *GG* are shown at the top of each line. M – 11-1444 bp DNA marker (*HaeIII* digest of pUC19); NT – non-digested PCR product.

Table 1. The distribution (RFLP) of genotypes and allele frequencies at the *AhaI* site in the bovine *CAST* gene

Breed	n	Number of animals			Allele frequency	
		GC	CC	GG	C	G
Red Angus	9	1	8	–	0.93	0.07
Charolaise	12	2	10	–	0.38	0.42
Limuzin	10	3	6	1	0.75	0.25
Simmental	9	5	3	1	0.61	0.39
Hersford	8	4	4	–	0.75	0.25
Polish Friesian	84	35	31	18	0.38	0.42
Polish Red	7	1	4	2	0.64	0.36
Total mean	138	90	66	22	0.69	0.31

region have also been differentiated by PCR-RFLP. Using RFLP technique, three ovine alleles and six possible genotypes have been found by restriction digestion with *MspI*, *NcoI* and *CfoI* enzymes. Amplimers from cattle (two animals from each of three breeds) were also sequenced using the ovine 1C and 1D exon-derived primer pair (consensus sequence – GenBank AF117813). The polymorphic variation in cattle *CAST* appeared

similar to that in sheep, whereas in the present study the polymorphism in exons 1C and 1D of bovine *CAST* locus digested with *MspI* and *NcoI* was not found.

Chung *et al.* [1999a] observed two alleles (*A* and *B*) and DNA polymorphism by SSCP analysis in the *CAST* gene of Angus cattle using primers based on the sequences of the ovine *CAST* gene. The amplicon sequences of bovine *CAST* gene included also exon 1C and 1D. Allele frequencies for *A* and *B* amounted to 0.29 and 0.71, respectively.

Most research on genetic markers as applied to animal breeding is focused on analysis of mutations identified within economically important structural genes and their linkage to quantitative trait loci (QTLs). The *CAST* gene affecting meat tenderness is considered as a candidate gene for a quantitative marker in cattle breeding [Quali and Talmant 1990].

In the present study the genetic association of *CAST/AluI* gene polymorphism and selected beef quality traits was searched for in 84 Polish BW bulls. Analysis was conducted to investigate the effects of the genotypes on physical and chemical traits of meat, and its sensory evaluation score.

Differences were found in meat quality indicators between bulls with different SNP/*AluI* genotypes of *CAST* gene (Tab. 2 and 3). The meat of *GC* bulls showed higher thermal cooking loss than that of *GG* ($P \leq 0.01$) and *CC* animals ($P \leq 0.05$). Colour component (part b^*) of meat from *GC* bulls was higher than that estimated in the meat from *GG* ($P \leq 0.01$) and *CC* ($P \leq 0.05$) bulls. This demonstrates that the meat from *GC* bulls was definitely darker in colour. Significant difference appeared in the content of total hem pigment. *GC* bulls showed the highest total content of hem pigment, significantly different ($P \leq 0.05$) from that found in *CC* bulls. The sensory evaluation showed more

Table 2. Least squares means (LSM) and their standard errors (SE) for meat traits of BW bulls across *CAST/AluI* genotypes

Item	Genotype					
	CC		GC		GG	
	LSM	SE	LSM	SE	LSM	SE
Colour components						
a^*	16.71	0.45	16.52	0.44	16.20	0.56
b^*	-0.87 ^a	0.94	1.73 ^{ab}	0.90	-1.52 ^a	1.16
L^*	38.90	1.06	40.48	1.80	38.80	1.30
Penetration force (N/cm ²)	79.34	7.15	63.65	6.78	58.48	8.75
Water content (%)	76.83	0.36	76.45	0.34	76.81	0.44
Protein content (%)	21.32	0.30	20.94	0.28	20.72	0.36
Fat content (%)	1.09	0.16	1.08	0.15	0.92	0.19
Cooking loss (%)	3.19 ^a	1.02	6.88 ^{ab}	0.96	1.77 ^a	1.24
Water holding capacity (cm ³ /g)	30.13	2.05	30.18	1.95	27.65	2.51
Total content of hem pigment (ppm hemin)	128.19 ^a	9.57	157.61 ^a	9.08	137.65	11.73
pH ₁	5.57	0.05	5.53	0.04	5.55	0.06

^aWithin rows means bearing the same superscripts differ significantly: small letters - $P \leq 0.05$; capitals - $P \leq 0.01$.

Table 3. Least squares means (LSM) and their standard errors (SE) for sensory-evaluated traits of meat of FWTbulls scored according to 5 points scale

Trait	Genotype					
	CC		GC		GG	
	LSM	SE	LSM	SE	LSM	SE
Colour	4.77	0.13	4.42	0.12	4.55	0.16
Aroma	4.70	0.13	4.41	0.12	4.40	0.16
Taste	4.60	0.14	4.29	0.13	4.33	0.17
Tenderness	4.07 ^a	0.19	3.88 ^b	0.19	3.85	0.24

^aWithin rows means bearing the same superscripts differ significantly at $P \leq 0.05$.

desirable tenderness of meat in CC than in GC animals ($P \leq 0.05$). Generally, this test showed that meat from GC had lower cooking quality than that from CC bulls.

Cooking losses are negatively associated with tenderness of meat [Daszkiewicz *et al.* 2003]. The highest cooking loss (Tab. 2) was accompanied with medium penetration force in GC bulls.

Very few studies have been performed on the effect of *CAST* gene polymorphism on meat quality traits. Chung *et al.* [1999b] related the presence of *CAST* genotypes determined by PCR-SSCP analysis in Angus bulls to calpastatin activity (CAC), Warner-Bratzler shear force (WBS) and myofibril fragmentation index (MFI) of their meat during tenderization process. A weak positive residual correlation ($r = 0.29$, $P = 0.52$) was found between CAC and WBS, but high and negative between CAC and MFI ($r = -0.74$, $P = 0.05$). The former correlation appeared similar to that reported between CAC and WBS ($r = 0.31$) by Lonergan *et al.* [1995] who, however, found a moderate negative correlation between MFI and WBS ($r = -0.49$, $P = 0.26$).

Furthermore, Koohmaraie [1994] reported that the coefficient of genetic correlation (r_G) between postrigour CAC and WBS exceeds 0.5, demonstrating that selection leading to decrease the CAC activity could improve the tenderness of meat. Wulf *et al.* [1996] found residual correlation coefficient of 0.42 between WBS and CAC in Limousine and Charolaise cattle. The same authors noticed for the same breeds the r_G of -0.75 between CAC and meat marbling, and -0.53 between CAC and WBS. Shackelford *et al.* [1994] reported r_G of -0.34 between CAC and intramuscular fat content, as well as between the latter and shear force ($r = -0.57$) in Charolaise and Limousine cattle. These results indicate that the rate of genetic progress in meat tenderness would be greater if selection goes towards the CAC activity on hour 24, rather than towards an increased meat marbling. Chung *et al.* [2001] analysed the correlation between *CAST/XmnI* polymorphic variants and beef quality traits. Analysis of variance was conducted to investigate the effects of the CAC genotype on carcass traits and meat tenderness in Angus bulls. *CAST* genotypes affected CAC ($P \leq 0.05$), as well as fat per cent of kidney, pelvic and heart ($P \leq 0.01$). *CAST/XmnI* genotypes did not, however, correlate with meat tenderness. Recently, Chung *et al.* [2002] reported the effect of SSCP/*TaqI* and RFLP/

XmnI *CAST* genotypes on growth and carcass traits in Korean bulls. They described a correlation between SSCP/*TaqI* polymorphic variants and fat thickness on the back, the latter appearing greatest in *BB* bulls.

In the present study a novel mutation coding for the protein domain I was found in exon 1C of the bovine *CAST* gene. The mutation can be used in further search for associations between gene polymorphism and beef quality traits. For the first time the association of genotype SNP/*AluI* with meat quality was shown, significant differences being found between bulls of different *CAST* genetic variants. The important message from these results is that the mutation can be used in further research seeking the associations between gene polymorphism and meat quality traits in cattle.

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Nowy polimorfizm RFLP/*AluI* w genie *CAST* bydła i jego związek z jakością wołowiny

Streszczenie

Opisano nowy polimorfizm w eksonie 1C genu *CAST* bydła. Polimorfizm analizowano metodą PCR-SSCP, a następnie poddawano sekwencjonowaniu próbki DNA odbiegające od siebie wzorcem SSCP. Analiza sekwencyjna wykazała istnienie SNP polegającego na transwersji G→C w pozycji 61 nt (GenBank nr. AF117813). Substytucja ta znajduje się w kodonie seryny (AGC) i wywołuje zamianę na kodon treoniny (ACC). Mutację można rozpoznać metodą RFLP stosując enzym restrykcyjny *AluI*. W grupie 138 opasanych buhajów siedmiu ras stwierdzono występowanie dwóch alleli – C i G – i trzech genotypów – CC, GC i GG. Genotyp GG nie występował u buhajów angus, charolaise i hereford. Częstość występowania alleli C i G wynosiła odpowiednio 0,69 i 0,31.

Związek między polimorfizmem genu *CAST* a jakością wołowiny (mięsień najdłuższy grzbietu) badano na 84 buhajach rasy cb. Mięso zwierząt o genotypie GC okazało się najmniej wartościowe pod względem kulinarnym. Charakteryzowało się ono większym wyciekami termicznymi niż stwierdzony w mięsie buhajów CC (P<0,05) i GG (P<0,01). Mięso buhajów GC było ciemniejsze (P<0,01) niż mięso zwierząt o genotypie GG. Ogólna zawartość barwników hemowych oznaczona metodą Hornsey'a była najwyższa w mięsie buhajów o genotypie GC i różniła się (P<0,05) od stwierdzonej w mięsie zwierząt CC.

Wnioskuje się, że *CAST* można traktować jako gen kandydujący na marker jakości wołowiny.