Animal Science Papers and Reports vol. 25 (2007) no. 4, 201-209
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Polymorphism in *CTSB* exon 7 as associated with activity of the lysosomal proteases in *longissimus dorsi* muscle of growing Friesian bulls*

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(Received October 5, 2007; accepted November 10, 2007)

Proteolysis regulation is one of the key factors in meat processing. Cathepsins are a group of acid lysosomal proteases involved in *post-mortem* muscle proteolysis and have one of the utmost importance for meat products quality. The aim of the study was to compare activities of lysosomal proteases in skeletal muscle (*longissimus dorsi* – LD) of growing Friesian bulls of different cathepsin B (*CTSB*) *loci* genotypes. Samples from the same region of XIII vertebra of LD were obtained post-slaughter from 157 Friesian bulls aged about 11 months. In relation to proteolytic activity of the LD muscle significant differences were identified between bulls representing different *CTSB* genotypes. In samples from bulls of *CC/CTSB* genotype higher activities were found for acid autolytic activity (AAA, P<0.01), pepstatin-insensitive acid autolytic activity (PIAAA, P<0.01) and leupeptin-insensitive acid autolytic activity (LIAAA, P<0.05) than in samples from *TT/CTSB* bulls. The protein content of LD was higher by 5.0% in muscle samples from bulls of *TT* than of *CT* genotype (P<0.05).

KEY WORDS: cathepsin B gene / cattle / cysteine and aspartic proteases / gene polymorphism / meat quality / proteases / proteolysis

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**Supported by the Ministry of Scientific Research and Informative Technology, grant No. PBZ-KBN-113/P06/2005/02, and the Institute of Genetics and Animal Breeding project S.V.9.

In the meat industry, proteolysis plays a key role both in fresh meat ageing [Uytterhaegen *et al*. 1994] and derived meat products such as dry-cured ham, which is one of the most economically viable products [Sárraga *et al*. 1993, Russo *et al*. 2002]. The control of protein degradation in skeletal muscles *in vivo* is important for energy and protein homeostasis and muscle and body growth. Myofibrillar proteolysis is responsible for the whole body wasting and changes in protein turnover as well as for meat tenderness and quality.

Cathepsins are a group of acidic lysosomal proteases, which are involved in the degradation of a number of proteins in the lysosomes of most somatic cells [Turk *et al*. 2000, Juszczuk-Kubiak and Rosochacki 2002]. In lysosomes the endopeptidases of two classes have been found: aspartic (cathepsin D) and cysteine (cathepsin CatB, H, L and K) [Turk *et al*. 2001].

It is suggested, that these cathepsins are involved in the *post-mortem* degradation of some myofibrillar proteins (myosin and actin). Their activities depend on genetic and non-genetic factors [Armero *et al*. 1999]. Moreover, cathepsin B is thought to be one of the most important enzymes involved also in beef tenderizing [Toldrá and Etherington 1988]. As the ageing of meat proceeds, the lysosomal membrane becomes more permeable, allowing cathepsins to pass from the lysosome to the cytosol. The enzymes are activated at acidic pH caused by the accumulation of lactic acid in the muscle as a result of *post-mortem* anaerobic glycolysis.

The *CTSB* gene encodes for the protease cathepsin B, which plays an important role in lysosmal proteolysis and protein turnover. The bovine *CTSB* gene is mapped to BTA 8 [Sonstegard *et al*. 2000], and it has 9 exons spanning approximately 7.3 kb [Mordier *et al*. 1995]. The last exon is located between Alu-like short interspersed nuclear elements. Studies on cDNAs encoding human, mouse, and bovine cathepsin B have revealed, that cysteine proteinases are synthesized as a preproenzyme that undergoes a post-translational processing during its transport to the lysosmal compartment [Mordier *et al*. 1995]. Alternative polyadenylation site generates three transcripts encoding bovine cathepsin B. The *CTSB* gene is considered as candidate for genetic marker of bovine carcass and meat quality traits [Russo *et al*. 2002].

The aim of this study was to compare activities of some lysosomal enzymes of the skeletal muscle between different *CTSB*/*HpyCH4III* genotypes formerly identified with RFLP method by Juszczuk-Kubiak *et al*. [2007]. In this study the effect of exonic mutation on proteolytic activity was evaluated for the first time.

Materials and methods

Animals

The 157 young Fresian bulls, progeny of 30 Holstein sires, were slaughtered at the age of about 11 months, after 24 h fasting. The samples from the same region of XIII vertebra were excised from the *longissimus dorsi* (LD) muscle within 15 minutes post-slaughter, frozen in liquid nitrogen and stored at -80˚C until required.

All experimental procedures involving animals were approved by a Local Ethics Commission (permission No. 67/2001).

Determination of cathepsins activity

In LD muscle samples the following determinations were made:
– acid autolytic activity (AAA) towards natural proteins extracted from tissue as a substrate;
– extent of inhibition of AAA by pepstatin (3.0 µg per determination) to estimate the pepstatin-insensitive acid autolytic activity (PIAAA);
– extent of inhibition of AAA by leupeptin (5.1 µg per determination) to estimate the leupeptin-insensitive acid autolytic activity (LIAAA);
– content of protein (%).

Proteolytic activity was determined according to Rosochacki [1985]. Briefly, the tissues were homogenized in cold 0.1% Triton x 100 in water (all manipulations being done on ice). All enzymatic activities were tested in 500 mM formic buffer, pH 3.75 (only for autolytic activity with pepstatin – PIAAA – the pH was 3.25) at 45ºC during 1 hour. The activity of cysteine cathepsins (catB, L and H) was assessed after inhibition of total acid autolytic activity by pepstatin (PIAAA). The aspartic cathepsin (catD) activity was assessed using leupeptin which inhibited proteolytic activity of thiol cathepsins (LIAAA). The activity of proteinases was defined as µg of tyrosine released per mg of protein. Pepstatin is an inhibitor of cathepsin D, while leupeptin inhibits thiol proteinases. Both PIAAA and LIAAA were measured in the presence of 1mM Mg^{2+}. The activity of proteinases was defined as µg of tyrosine released per mg of protein.

The protein content was determined in an alkaline solution of homogenates dissolved in 3% trichloroacetic acid with Folin reagent [Lowry *et al*. 1951] against albumin as a standard.

***CTSB* polymorphism**

Three *CTSB* genotypes (*CC*, *CT* and *TT*) conditioned by two alleles (*C* and *T*) were identified according to Juszczuk-Kubiak *et al*. [2007] using restriction enzyme *HpyCH4*III. Single SNP was found within exon 7 in the third position of the Ser codon and did not modify the amino acid sequence of the CatB protein.

Statistical

Association between *CTSB/HpyCH4*III gene polymorphism and activity of the lysosomal proteolytic enzymes was analysed in 157 Fresian bulls. Differences in the frequencies of *CTSB* genotypes (*CC*, *CT* and *TT*) were assessed with the chi-square test. The relationship between *CTSB* genotypes and the activity of the lysosomal proteolytic enzymes was estimated with the General Linear Model (GLM) from SAS statistical package [SAS/STAT 1999]. The model used was as follows:

$$y_{ijklm} = \alpha + G_i + R_j + S_k + o_l + \beta(x_{ijkl} - \bar{x}) + e_{ijklm}$$

where:

y_{ijklm} – trait studied;

α – the intercept;

G_i – fixed effect of *i*-th genotype of *CTSB* gene (i = 1, 2, 3);

R_j – fixed effect of *j*-th year at the start of fattening (2002, 2003, 2004, 2005; j = 1, 2, 3, 4);

S_k – fixed effect of *k*-th season at the start of fattening (January-March, April-June, July-September, October-December; k = 1, 2, 3, 4);

o_l – random effect of *l*-th sire;

$\beta(x_{ijkl} - \bar{x})$ – regression of the trait analysed on carcass weight;

e_{ijklm} – random error.

Results and discussion

The SNP at the *CTSB* gene had a significant effect on the content of protein (%), and AAA, PIAAA and LIAAA of LD of bulls. The relationship between *CTSB* genotype and enzymatic activity of the lysosomal proteolytic enzymes in the LD muscle of bulls is presented in Table 1.

The protein content of LD in *TT* and *CT* bulls was 18.05 and 17.14%, respectively ($P<0.05$), the difference amounting to approx. 5%.

The significant differences between the *CTSB* genotypes in relation to enzymatic activities in the LD were also found. Total lysosomal AAA was higher by about 12.5% in *CC* than in *TT* bulls (27.58 *vs* 24.14 μg tyrosine/mg protein/h, $P<0.01$). The activity of the most thiol cathepsins, assessed after inhibition of total AAA by pepstatin (PIAAA), was also higher by 17.11% in bulls bearing the *CC* genotype (14.89 *vs* 12.31 μg tyrosine/mg protein/h, $P<0.01$). The higher activity of LIA (reflecting mostly activity of non-cysteine cathepsin, eg. cathepsin D – LIAAA) was also estimated in *CC* (by 14.5%) than in *TT* bulls (16.16 *vs* 13.78 μg tyrosine, $P<0.05$).

The effects of year and season at start of fattening on enzymatic activity of LD proteases were not found significant.

Most of the traits of economic importance in farm animals are polygenic (quantitative traits *loci* – QTLs) and affected by non-genetic environmental factors. Initial molecular genetic studies detecting OTLs for carcass and meat quality have used either whole genomic scan or candidate gene approach methods. Candidate genes are selected on the basis of known relationship between physiology and production trait(s) and are tested as putative QTLs. The following genes are considered as candidate genes for carcass and meat quality in domestic animals: *GH* [Ge *et al*. 2003], *GHR*

[Maj *et al*. 2004], *IGF-1* [Crown *et al*. 2000], *LEP* [Kennes *et al*. 2001] or *myostatin* gene [McPherron and Lee 1997].

Cathepsins B, L and D, are the most abundant lysosomal proteinases in animal tissues. *In vivo* they degrade proteins mainly inside lysosomes and are involved in the processing of several pro-enzymes as well as in antigen presentation [Turk *et al*. 2000]. The rate and extent of skeletal muscle growth ultimately depends on three main factors: rate of muscle protein synthesis, rate of muscle protein degradation, and the number and size of skeletal muscle cells. The cathepsin system degrades intra- and extracellular proteins, and actin and myosin are the main myofibrillar proteins degraded [Thompson and Palmer 1998].

The control of degradation of proteins in skeletal muscles is important for energy and protein homeostasis and muscle and body growth. Increased myofibrillar proteolysis is responsible for the whole body wasting and changes in protein turnover as well as for meat tenderness and quality [Parolari *et al*. 1994, Russo *et al*. 2002].

In the present study, significant effect was observed of the *C*540*T* transition within exon 7 of the *CTSB* gene on the proteolytic activity of cathepsins in the LD of the growing bulls. It was shown that bulls with the *CC* variant of the *CTSB* gene had the highest AAA, PIAAA (activity of thiol cathepsins) and LIAAA (mostly activity of cathepsin D) in the LD muscle (Tab. 1).

Table 1. Enzymatic activity in LD muscle samples across *CTSB/HpyCH4*III genotypes of bulls

Trait		*CTSB* genotype		
		CC (n=77)	*CT* (n=49)	*TT* (n=31)
Protein (%)	mean	17.65	17.14^{a}	18.05^{b}
	SD	0.55	0.56	0.60
AAA[1] (μg tyrosine/mg protein/h)	mean	27.58^{A}	25.27	24.14^{B}
	SD	1.75	1.79	1.91
PIAAA[2] (μg tyrosine/mg protein/h)	mean	14.89^{A}	13.01	12.31^{B}
	SD	1.35	1.38	1.47
LIAAA[3] (μg tyrosine/mg protein/h)	mean	16.16^{a}	14.70	13.78^{b}
	SD	1.16	1.19	1.27

$^{aA...}$Means within rows bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.
[1]AAA – total lysosomal acid autolytic activity.
[2]PIAAA – cysteine cathepsins (Cat. B, L, H) activity.
[3]LIAAA – aspartic cathepsine (Cat. D) activity.

The protein determination in LD muscle showed (Tab. 1) that in bulls of *TT* genotype the level of LD protein was the highest. It may mean that proteolytic enzymes are less active in *TT* bulls (more total protein remained in the muscle). An increase in the proteolytic activity of enzymes, e.g. cathepsin, could result in changes

in cathepsin-cystatin interactions rather than in the gene expression. It was shown by Hasselgren [1999] that an increased amount of mRNA was not directly correlated with the increase in protein breakdown rate. Therefore, it is not possible to explain muscle degradation only by an increase or decrease in mRNA concentration. The changes in the protein content and the measured enzyme activities were connected with the estimated genotypes of the bulls. By measuring the inhibition of activity of proteolytic enzymes (reflecting the amount of proteases) and the activity of the protein degradation system (cathepsins activity) is much easier to observe the changes in the concentration of proteinases in muscle and their effect on protein degradation [Millward *et al*. 1981, Jank *et al*. 2001]. Recently, Rosochacki *et al*. [2004, 2005] found significant differences in the LD enzymatic activities of cysteine cathepsins and cathepsin D between bulls of different breeds. The AAA, PIAAA and LIAAA were highest in the Angus and lowest in the Limousine LD muscle. However, the activity of aspartic and cysteine enzymes was higher in the muscle of Friesian than of Piedmontese bulls.

The variation in the *CTSB locus* in cattle was a subject of several studies. Sonstegard *et al*. [2000] found one substitution at position 212 (A/G) in intron 8 and substitution (A/G) at position 563 within the 3'UTR of the gene. Two SNPs in the 3'UTR of the *CATB* were identified by Mordier *et al.* [1995] – a transition T/C at position 637 and a transversion A/T at position 657 after stop translation codon. All polymorphisms were detected by sequencing the amplified gene fragments. In the earlier authors' study [Juszczuk-Kubiak *et al*. 2007] identified were three new polymorphisms within the *CTSB* gene sequence. A substitution C/T was found in exon 7 and two SNPs – G/C at position 309 and C/T at position 390 within introns 8 and 9 – respectively. All SNPs are deposited in the GenBank database under accession numbers DQ978217S1 (for SNP in exon 7, DQ978217S2 (for SNP in intron 8), and AY639598 (for SNP in intron 9).

This is the first report indicating an association of the *CTSB* gene polymorphism with proteolytic activity of the cysteine (PIAAA) and mostly aspartic – CatD – (LIAAA) cathepsins in cattle. The SNP in exon 7 is supposed to be important for cathepsin B function, because it is localized in the region coding two hairpin loops, including the occluding loop that accounts for CatB peptidyl-dipeptidase activity [Mordier *et al*. 1995]. The functional significance of this SNP cannot be ignored, even as it does not modify the amino acid sequence of the CatB protein. It has been reported that synonymous SNPs in coding regions may influence mRNA stability and translation process [Duan *et al*. 2003], and/or protein folding [Kimchi-Sarfaty *et al*. 2007], thereby resulting in altered function. Additionally the SNP may be linked to another mutation in the coding or regulatory regions of the gene possibly having impact on function, level, or time of expression and thereby being a causative one.

Lysosomal cysteine proteinases appear also to be the main factors responsible for proteolysis in the curing process of ham [Toldrá and Etherington 1988; Russo *et al* 2002]. A high proteolysis level throughout the processing period has been associated with excessive softness of dry-cured hams that usually goes together with other defects

like stickiness on chewing, dark colour, astringent or metallic aftertastes, depots of tyrosine crystals and formation of white films on the cut surface [Virgili *et al*. 1998]. Parolari *et al*. [1994] reported that the residual activity of cathepsin B in dry-cured hams 12-13 month old is associated with the protein cleavage level. Virgili *et al*. [1995] showed, that the proteinase activity in fresh pork muscles a few days after slaughtering is directly related to the proteolysis level reached in the same muscles at the end of ham ageing. These results may suggest genetic variation to exist in the lysosomal proteinases and their inhibitors in pigs, and show that selection based on mentioned parametres could improve meat quality for dry-cured ham production.

In this study we have examined mutation within the bovine *CTSB* gene which can be considered a candidate gene for the proteolysis activity in dry-cured hams and tenderness of beef. The discovered mutation in exon 7 of the bovine *CTSB* gene was associated not only with activity of the cysteine cathepsins, but also of the other lysosomal proteinases. It is concluded that *CTSB* may be used as genetic marker for meat quality traits to identify genomic regions associated with QTLs in cattle.

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Związek mutacji w eksonie 7 genu *CTSB* z aktywnością enzymów proteolitycznych w mięśniu najdłuższym grzbietu rosnących buhajów rasy fryzyjskiej

S t r e s z c z e n i e

Oznaczono aktywność autolityczną ogólną w środowisku kwaśnym (AAA) w homogenatach mięśnia najdłuższego grzbietu (LD) 11-miesięcznych buhajów rasy fryzyjskiej. Dodatek poszczególnych inhibitorów: pepstatyny i leupeptyny pozwolił na ocenę udziału katepsyn siarczkowych (PIAAA) oraz katepsyn asparaginianowych (LIAAA) w autolizie. Wykorzystano próbki mięśni pobrane po uboju od 157 buhajów, zróżnicowanych pod względem genotypu *CTSB*. Udowodniono istotne różnice między genotypami w zakresie AAA, PIAAA i LIAAA oraz zawartości białka w mięśniu.

Największą aktywnością autolityczną ogólną (AAA) oraz aktywnością katepsyn siarczkowych (PIAAA) i asparaginowych (LIAAA) charakteryzowały się osobniki o genotypie *CC*. Wskaźniki AAA, PIAAA i LIAAA były wyższe odpowiednio o 12,5%, 17,11% i 14.5% w mięśniu buhajów o genotypie *CC* w porównaniu z mięśniem buhajów o genotypie *TT* (odpowiednio $P<0,01$, $P<0,01$ i $P<0,05$).

Zawartość białka mierzona z użyciem odczynnika Folina była w LD buhajów o genotypie *TT* o 5% wyższa niż w LD buhajów o genotypie *CT* ($P<0,05$).