Cathepsin involvement in muscle proteolysis in meat-type bulls

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ABSTRACT: Measurements were done of some lysosomal proteolytic enzyme activities involved in skeletal muscle proteolysis of the *masculus longissimus lumborum et thoracis* muscle (MLLT) of bulls. Samples from the same region between the 11th and 13th vertebra were taken after slaughter from Limousin (n = 10), Hereford (n = 10), Charolais (n = 10), Angus (n = 11) and Simmental (n = 11) bulls about 15 months old fed complete diet *ad libitum*. The activity of cathepsin D was determined as pepstatin (cathepsin D inhibitor) sensitive activity (PSCatD) towards 1% haemoglobin. Pepstatin-insensitive acid (PIA) and leupeptin-insensitive (thiol proteinases inhibitor) acid (LIA) autolytic activities were measured in the presence of 1mM Mg^{++}. MLLT was also analysed for RNA, DNA and protein variables. The data were processed by analysis of variance. The highest activities in PSCatD ($P \leq 0.05$), AAA ($P \leq 0.01$) and LIA ($P \leq 0.05$) as well as percentage of inhibition by pepstatin in cathepsin D ($P \leq 0.01$) were estimated in Angus bulls, and the lowest in Limousin ones. These breeds differed in the above-mentioned activities by 20.3, 21.1, 31.1 and 13.1%, respectively. RNA/g of tissue was highest in Hereford and lowest in Limousin bulls (by about 15.3%, $P \leq 0.01$). Similar differences (14.3%) were between Charolais and Limousin ($P \leq 0.01$). CPS (10^3 RNA/protein) was higher by 18.3% ($P \leq 0.01$) in Charolais compared to the value in Simmental bulls; similar differences were between Hereford and Simmental (16.4%, $P \leq 0.01$). The DNA concentration was highest in Hereford (by about 30%) compared to Charolais bulls. Protein/10^3DNA ratio (mg/mg) – FCS – was higher by 33.4% in Charolais compared to Hereford; RNA/DNA ratio was higher by 40.2% in Charolais compared to Limousin bulls. These results indicate the fast turnover of proteins in the groups of examined bulls and it can be concluded that in hypertrophic MLLT of bulls an anabolic decrease in degradation occurred.

K**eywords**: lysosomal proteolysis; autolysis; skeletal muscle degradation; DNA; RNA

The current understanding of pathways of protein synthesis and its regulation is far more advanced than that of intracellular protein breakdown, mostly because of technical problems involved in studying degradative processes. The turnover of most functional proteins in the body is a continual process consisting in the destruction of individual protein molecules by proteolysis and their replacement by the processes of protein synthesis. Skeletal muscles contain multiple intracellular proteolytic systems including lysosomal proteases (cathepsins and other hydrolases), calcium dependent proteases (calpains) and ATP-ubiquitin (Ub-dependent) pathway (Thompson and Palmer, 1998). Whereas nonlysosomal degradation occurs at a fairly constant rate of 1–1.5% in most cells, the lysosomal contribution ranges from 0 to 4% of the total protein content of the cell per hour, depending on nutritional and hormonal conditions (Seglen and Bohley, 1992). In many cell types, lysosomal enzymes are thought

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to catalyze the hydrolysis of polypeptides to constituent amino acids although the manner in which specific proteins are targeted for proteolysis has not been fully elucidated yet (Klasing and Calvert, 1987). Under certain conditions the entry of proteins into lysosomes can be an important degradative pathway operating in many cells (Seglen and Bohley, 1992). The lysosomal pathway mainly degrades extracellular proteins which have entered via receptor-mediated endocytosis or pinocytosis and can be activated in some pathological conditions (Gacko et al., 1997). Hayes and Dice (1996) focused on the participation of hsc73 (heat shock protein chaperones) in a selective targeting of proteins (via KFERQ region of certain cytosolic proteins) to the lysosome where they are degraded. The myofibrillar apparatus cannot be enfluged by lysosomes and must be firstly released before undergoing the final stages of proteolysis either by lysosomal (i.e. cathepsins) or nonlysosomal (i.e. proteasome) pathways (Goll et al., 1989). Proteasomes are widely distributed in eukaryotic cells and can account for as much as 0.6–1% of the soluble protein in animal cells (Tanaka et al., 1986). The cathepsin system degrades intra- and extracellular proteins, and actin and myosin are the main myofibrillar proteins degraded by these enzymes. The control of protein degradation 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.

In our experiment we examined the relative importance of changes in the lysosomal proteolytic system – cathepsin activities, in the degradation of the total protein pools in muscle cells in different bovine meat-type breeds. In addition, specific pathways of protein degradation were blocked using inhibitors of lysosomal proteases.

MATERIAL AND METHODS

Animals

All the animals were born on an Experimental Farm in Jastrzębiec. Five different meat breeds were used in an experiment: Limousin (n = 10), Hereford (n = 10), Charolais (n = 10), Angus (n = 11) and Simmental (n = 11). They were reared on the complete diet (maize silage, hay and concentrate up to the age of about 15 months *ad libitum*) (Oprządek et al., 2001). The bulls were slaughtered after 24 hours of fasting and skeletal muscle samples of the *longissimus lumborum et thoracis* (MLLT) were taken from the same region between the 11th and 13th vertebra within 15 minutes after slaughter and frozen at –70°C until required. To perform this experiment, the Animal Care Use Committee of the Institute approved the use of animals in this study according to guidelines established by the World Society for the Protection of Animals.

Biochemical analysis

Enzyme assay. Proteolytic activities were measured according to the procedure of Rosochacki (1985). Briefly, the tissue was homogenized in cold 0.1% Triton X100 in water (all manipulations being done on ice). All enzymatic activities were performed in 500mM formic buffer, pH 3.75 (only for autolytic activity with pepstatin the pH was 3.25) at 45°C during 1 hour. After incubations 3% trichloroacetic acid was added (to remove proteins) and the proteolysis products were measured with Folin reagent (Lowry et al., 1951) in the supernatant, using tyrosine (between 2 and 40 μg/ml) as a standard. Cathepsin D activity was determined as pepstatin sensitive activity (PSCatD) towards 1% haemoglobin as a substrate. Pepstatin-insensitive-acid (PIA) and leupeptin-insensitive-acid (LIA) autolytic activities were measured in the presence of 1mM Mg^{++}. Pepstatin is an inhibitor of cathepsin D while leupeptin inhibits thiol proteinases. The activity of proteinases is defined as μg of tyrosine released per mg of protein.

The extent of total CatD or acid autolytic activity inhibition by pepstatin (300 μg/estimation) was measured to estimate the pepstatin-sensitive CatD (PSCatD) and pepstatin-insensitive acid autolytic activity (PIA). The extent of inhibition of AAA by leupeptin (5.1 μg/estimation) was measured to estimate the leupeptin-insensitive acid autolytic activity (LIA).

Nucleic acids and protein content assays

The nucleic acid content – RNA and DNA – was determined in about 100 mg MLLT samples according to the procedure of Munro and Fleck (1966). The protein level was determined in the alkaline solution of the tissue homogenate by the method of Lowry

et al. (1951) with bovine serum albumin (POCH, Poland) as a standard.

The protein level was also determined in the solution of the tissue homogenate for RNA and DNA samples, in RNA solution – for the correction of RNA estimation, as well as in the proteolysis products dissolved in 3% trichloroacetic acid with Folin reagent (Lowry et al., 1951). From protein, RNA and DNA data the capacity for protein synthesis (CPS) and functional cell size (FCS) were calculated as the ratio of 10^3RNA/protein and protein/10^3DNA, respectively.

Statistics

The data were processed by the analysis of variance (SAS/STAT guide for personal computers, SAS Institute Inc., Cary, NC) according to the following model:

$$y_{ij} = \mu + R_i + e_{ij}$$

where: y_{ij} = observation
μ = overall mean
R_i = fixed effect of i-th breed
e_{ij} = random error

Having found a significant effect of treatment Duncan's test was used to estimate significant differences between the groups. To check statistical differences between the percentages of inhibition, firstly the square root transformation was done, and then analysis of variance was performed. Differences were considered to be significant at $P \leq 0.05$ (single asterisk) and $P \leq 0.01$ (double asterisk).

RESULTS

Table 1 shows the content of protein in MLLT in the examined bulls. The content of protein was the highest in Charolais but the lowest in Angus bulls (being higher by about 21.7%, $P \leq 0.05$), and was statistically different from Hereford, Angus and Limousin; some statistical differences were also found between Hereford and Simmental bulls ($P \leq 0.01$).

All measured enzymatic activities were statistically different between the breeds of examined bulls (Table 1). Cathepsin D activity was the highest in Simmental bulls and the lowest in Charolais by 16.7% ($P \leq 0.05$). The highest activities in PSCatD, AAA and LIA as well as the percentage of inhibition by pepstatin in cathepsin D were estimated in Angus bulls, and the lowest in Limousin ones (statistical differences). These breeds differed in the above-mentioned activities by 20.3, 21.1, 31.1 and 13.1%, respectively. All these values reflect the activity and amount of cathepsin D in the examined muscle. PIA – reflecting thiol proteinases – was also highest in Angus bulls. Most statistical differences in enzymatic activities were found out between Limousin (14) or Simmental (11) and other examined bulls, but between Hereford and other bulls only 5 statistical differences were found out.

The composition of bull's MLLT is shown in Table 2. RNA/g of tissue was highest in Hereford and lowest in Limousin bulls. This difference was about 15.3%. Similar differences (14.3%) were between Charolais and Limousin ($P \leq 0.01$). CPS (10^3 RNA/protein) was higher in Charolais by 18.3% ($P \leq 0.01$) compared to the lowest value in Simmental bulls; similar differences were between Hereford and Simmental (16.4%, $P \leq 0.01$). The DNA concentration was the highest in Hereford (267.01 μg/g of tissue) bulls compared to the lowest (205.94 μg/g of tissue) in Charolais ones (by about 30%; $P \leq 0.01$). The protein/10^3 DNA ratio (mg/mg) – FCS – was higher by 33.4% in Charolais compared to Hereford ($P \leq 0.01$). The RNA/DNA ratio was much higher (by 40.2%) in Charolais than in Limousin bulls ($P \leq 0.01$).

DISCUSSION

A muscle hypertrophy condition was identified and selected for in cattle and is called double muscling. This double muscling in cattle is mostly due to an increase in the number of muscle fibres during prenatal growth and development. It is believed that it is controlled by a single dominant-recessive gene (e.g. Belgian Blue and Piemontese) or by a multiple gene complex with additive effects (e.g. Limousin). Double muscling, which has been observed in many cattle breeds for the last 190 years, appears to be inherited as a single major autosomal locus with several modifiers of phenotypic expression, resulting in incomplete penetrance. Similarly like in Belgian Blue cattle, the double muscling phenotype segregates as a single genetic locus designated muscular hypertrophy (mh) in Piemontese with extremely high frequency, suggesting that

Table 1. Enzymatic activity in MLLT of bulls (in μg of tyrosine per mg of protein) and inhibition (in %) of cathepsin D by pepstatin and acid autolytic activity by pepstatin and leupeptin (mean ± SD)

Group	% of protein	CatD	CatD + Pep	PSCatD	% of inhibition	AAA	PIA	% of inhibition	LIA	% of inhibition	PSCatD /PIA
Angus *n* = 11	14.84 ± 1.83 (9)	37.30 ± 5.79 (9)	5.16 ± 0.83 (9)	32.14 ± 5.49 (9)	85.98 ± 2.49 (9)	22.69 ± 2.42 (9)	6.63 ± 0.76 (9)	70.70 ± 3.33 (9)	16.66 ± 1.88 (9)	26.33± 6.33 (9)	4.85
Hereford *n* = 10	15.81 ± 0.93 (9)	34.33 ± 5.71 (9)	5.64 ± 1.16 (9)	28.68 ± 6.17 (9)	83.02 ± 4.97 (9)	20.27 ± 3.73 (9)	6.36 ± 1.00 (8)	68.92 ± 5.18 (8)	15.66 ± 3.43 (8)	24.76 ± 8.54 (8)	4.51
Charolais *n* = 10	18.06 ± 1.04 (10)	32.72 ± 5.68 (10)	5.59 ± 1.63 (10)	27.13 ± 4.96 (10)	82.90 ± 4.96 (10)	20.72 ± 2.59 (10)	6.51 ± 1.19 (10)	68.67 ± 3.50 (10)	14.37 ± 2.06 (10)	30.35 ± 7.70 (10)	4.17
Limousin *n* = 10	16.89 ± 1.30 (10)	34.37 ± 4.57 (10)	8.15 ± 1.54 (10)	26.72 ± 3.64 (10)	76.03 ± 4.98 (10)	18.73 ± 2.79 (10)	5.87 ± 1.45 (10)	68.82 ± 5.34 (10)	12.71 ± 1.91 (10)	31.68 ± 6.08 (10)	4.55
Simmental *n* = 11	17.70 ± 0.95 (10)	38.20 ± 2.92 (10)	5.94 ± 1.49 (9)	31.63 ± 2.53 (9)	83.71 ± 2.53 (9)	20.22 ± 1.88 (10)	5.22 ± 0.55 (10)	74.00 ± 3.65 (10)	14.50 ± 2.39 (10)	28.73 ± 11.93 (10)	6.06
Statistical differences	H–CH, S** CH–A, L*	CH–S*	L–A, H, S, CH**	CH–A, S* L–A, S*	L–A, H, S, CH**	A–L**	S–A, CH** H–S*	S–L, H, CH*	A–CH* L–A, H*		

L – Limousin, H – Hereford, CH – Charolais, A – Angus, S – Simmental

CatD – cathepsin D, PSCatD – pepstatin sensitive cathepsin D (measured towards 1% haemoglobin); AAA – acid autolytic activity, PIA – pepstatin insensitive acid autolytic activity, LIA – leupeptin insensitive acid autolytic activity, PEP – pepstatin; in parenthesis – number of animals

*$P \leq 0.05$, **$P \leq 0.01$

Table 2. Composition of MLLT of bulls (mean ± SD)

Group	*n*	µg RNA/g tissue	10^3 RNA/protein (CPS) (mg/mg)	µg DNA/g tissue	Protein/10^3 DNA (FCS) (mg/mg)	RNA/DNA
Angus	11	329.22 ± 21.58	1.11 ± 0.07	219.24 ± 24.00	1360.89 ± 171.08	1.51 ± 0.15
Hereford	10	355.06 ± 25.19	1.21 ± 0.11	267.01 ± 17.42	1104.28 ± 93.84	1.33 ± 0.13
Charolais	9	351.96 ± 39.68	1.23 ± 0.07	205.94 ± 33.99	1472.78 ± 183.51	1.71 ± 0.19
Limousin	10	308.00 ± 53.55	1.06 ± 0.16	251.60 ± 25.66	1190.60 ± 146.40	1.22 ± 0.21
Simmental	11	320.12 ± 45.40	1.04 ± 0.12	233.17 ± 25.73	1318.65 ± 174.49	1.38 ± 0.24
Statistical differences		H–S* L–H, CH**	H–L, S** CH–L, S**A–CH*	A–H, L** H–CH, S**, CH–L**	A–H** H–CH, S** A–L* CH–S*	A–CH, L** A–H* CH–H, L, S**

L – Limousin, H – Hereford, CH – Charolais, A – Angus, S – Simmental
CPS – capacity for protein synthesis; FCS – functional cell size; *n* – number of animals
*$P \leq 0.05$, **$P \leq 0.01$

these mutations (in the myostatin gene) are responsible for the double muscling phenotype. The mechanism of this phenotype is different in these two breeds – in Belgian Blue it is 11-nucleotide deletion in the third exon of myostatin gene, but in Piemontese it is a missense mutation in exon 3. The analysis performed by McPherron and Lee (1997) revealed the absence of these mutations in any of Angus, Charolais, Simmental, Limousin, Holstein and some other 11 breeds. These genes cause an increase in birth weights and muscle mass (about 20%) (Hendricks et al., 1973; Hanset and Michaux, 1985a,b; Hanset et al., 1987). Although much is known about the effect of double muscling gene(s) on carcass composition, very little is known about its mode of action and its effect on muscle growth and metabolism. Some results indicate that all major muscles of bulls are heavier in all examined meat-type breeds compared to milk-type ones (Szucs et al., 1995; Chambaz et al., 2003).

The aim of this study was to compare some protein variables and proteolytic enzyme activities in the *musculus longissimus lumborum et thoracis* in five different meat breeds bulls.

The capacity for protein synthesis – CPS, measured as 10^3 RNA/protein ratio, was the highest in MLTL of Charolais and Hereford bulls and similar to that of Piemontese ones, but higher than in Black-and-White (B-W) animals (Table 2) (Rosochacki et al., 2004). This value obtained in the bull muscle is much lower than in younger bulls (Rosochacki et al., 1990) or in other muscles of mouse (Rosochacki and Piekarzewska, 1988) and chickens (Rosochacki, 1985). The CPS value estimated in bulls is also very similar to that found out in 6-month-old Duroc (1.96) and Pietrain (1.60) pigs (Rosochacki et al., 2000). The measured RNA is mostly of ribosomal origin, so CPS was mostly attributed to a rise in rRNA transcription in the examined hypertrophic muscles compared to B-W ones.

Investigated muscles from different breeds of bulls have a lower muscle DNA content, from 31.6% in Hereford to 47.2% in Charolais (Table 2) compared to B-W bulls (Rosochacki et al., 2004). This indicates a smaller increase in the satellite cell activity. Total DNA/mg of tissue (DNA content) is an acceptable measure of myotube number (but not total DNA) and allows to compare nucleus contents between different populations of bulls. This difference in DNA content, together with higher RNA content (by 20.9% in Limousin to 39.3% in Hereford), suggests that the increased capacity for protein synthesis in Charolais and Hereford bulls, compared to Black-and White (Rosochacki et al., 2004), may be associated with greater muscle mass. According to Muir et al. (2000), Hereford steers had higher carcass weight, higher dressing percentage and were significantly fatter than B-W or crossbred steers B-W × Hereford. However, they reported that meat colour, total weight of significant meat cuts and meat tenderness were similar in all breeds. In the experiment performed on the same bulls (Oprządek et al., 2001) the higher weight of entrecote, round, neck, fore quarter of the carcass side and the weight of valuable cuts were also found in all examined breeds of bulls, compared to B-W. The

other measured traits of meat quality (body weight, percentage of valuable cuts, weight of valuable lean cuts, weight of: carcass side, shoulder, sirloin, hind quarter of the left and right carcass side, fore quarter of the left carcass side) were always higher in Charolais and Limousin bulls compared to B-W ones (Oprządek et al., 2001).

Muscle hypertrophy is associated with higher functional cell size (FCS), expressed as protein/10^3DNA ratio, in the muscle of examined bulls (Table 2) by 56.6% in Hereford up to 108.8% in Charolais compared to B-W (Rosochacki et al., 2004). Cheek et al. (1971) proposed the name of FCS as an imaginary volume of cytoplasm controlled by a single nucleus and they assumed that any changes in DNA content were proportional inside and outside in muscle fibrils. The data from this experiment are remarkably different between FCS in B-W muscle compared to the highest values in Charolais (by 108.8%) and the lowest ones in Hereford, but being still higher by 56.6%. This is also supported by RNA/DNA ratio – the concentration of ribosomes per nucleus, these values were much higher than those of B-W (Table 2) (Rosochacki et al., 2004). This also supports the higher muscle as well as physiological maturity in meat-type bulls compared to B-W ones.

Lysosomal proteinases that are active in low pH are believed to play an important role in the skeletal muscle protein metabolism (Goll et al., 1983; Calkins and Seideman, 1988). Some changes in the enzymatic activities measured in the present experiment are shown in Table 1. The average activities of PSCatD, AAA, PIA and LIA were the highest in the MLLT of Angus bulls and the lowest in Limousin ones, by 20.3%, 21.1% 13.0% and 31.1%, respectively (some statistical differences, Table 1). LIA supports strongly PSCatD activity in both examined strains of bulls, both reflecting the activity of cathepsin D. The ratio of PSCatD/PIA was the highest in Simmental bulls (6.06) compared to the average of 4.52 for the other examined muscles. This ratio indicates higher participation of CatD in the process of proteolysis in Simmental muscle compared to the other MLLT muscles examined – by 34.1%, but much lower participation in MLLT muscles of Piemontese – by 21% (6.0 vs. 3.57), and B-W bulls – by 30% (6.0 vs. 3.17) (Rosochacki et al., 2004). These differences support very strongly the hypothesis concerning a different pattern of the protein turnover in meat-type and B-W MLLT muscles, and probably in the overall protein metabolism in the skeletal muscle of bulls with genetic hypertrophy compared with control ones. The highest enzymatic activities (PSCatD, AAA, PIA and LIA and less total protein in the muscle) in Angus MLLT were found out among the other examined bulls in this experiment, which is also in agreement with the data obtained in other examined muscles in previous experiments (Rosochacki et al., 2004). Chambaz et al. (2003) reported that the Angus bulls showed a growth rate similar to Simmental (and Charolais) while Limousin grew more slowly and that Angus carcasses showed the lowest weight but the highest fatness score. Indeed, in our study we did not find any statistical differences between Angus and Simmental bulls in DNA and RNA variables or in enzymatic activities (except PIA, Table 1). From the data obtained here – showing the increase in FCS and decrease in proteolytic activities, and from the data reported by Rosochacki et al. (2004) and Oprządek et al. (2001) – as far as some variables in the weight of meat-type bulls are compared to B-W, it can be concluded that in hypertrophic MLLT of meat-type bulls an anabolic decrease in degradation occurred.

For agricultural applications, there are some disadvantages of double-muscled cattle (e.g. Piemontese), namely the reduction in female fertility, lower viability of offspring and delay in sexual maturation. Muscle characterization of examined bulls by some lysosomal proteolytic enzymes and DNA and RNA variables is associated with lower rate and extent of post-mortem proteolysis compared to B-W, and thus probably with lower meat tenderization. Differences in tenderness, immediately after slaughter, are associated with the higher calpastatin to μ-calpain ratio in the meat of steers which were slaughtered in muscle biological maturity (Muir et al., 2000). In our previous study (Sakowski et al., 2001) we showed some differences in the quality traits of meat. However, the physicochemical analysis of the same meat obtained from investigated bulls did not indicate any breed as a leader in beef quality (Sakowski et al., 2001). We concluded that the meat of one breed from the examined ones was better for processing, but another had some better cooking properties. The best technological properties were observed for the meat of Piemontese crosses compared to the other examined breeds. However, the meat obtained from Charolais bulls was the best as the colour, taste and aroma were regarded, but the meat from Angus bulls showed the best consistency measured by the panel and some instrumental tests

(Sakowski et al., 2001) and it might be associated with the highest activities of proteolytic enzymes measured (Table 1). The genetic differences in the quality of meat between breeds must be related to genetic differences in connective tissue or fibre type or to genetic differences in tenderness change prior to early postmortem structural changes in the myofibril or early postmortem proteolytic activity. We therefore suggest that a reduced rate of muscle protein degradation may also contribute to greater muscle protein accretion in hypertrophic muscles as well as to its final quality.

CONCLUSION

These results demonstrate that the muscle-type phenotype of bulls is manifested by lower measured proteolytic activities compared to B-W. Among the examined MLLT the highest proteolytic activities were in Angus and the lowest in Limousin bulls. In Simmental bulls the cathepsin D way of proteolysis was much more important compared to the other bulls. In addition, the much larger FCS in MLLT of meat-type bulls (the highest values in Charolais, Angus and Simmental) indicates a higher anabolic increase in degradation compared to B-W bulls (Rosochacki et al., 2004). This also indicates a different (slower) turnover of proteins in the examined groups of bulls and much higher meat quality traits (specially in Limousin and Charolaise bulls, both originated in France, Oprządek et al., 2001). Lysosomal proteinases play a major role in the turnover of muscle proteins in bulls and can characterize also the muscle physiology together with DNA concentration and protein/DNA ratio (e.g. muscle maturity and the apparatus for meat tenderization post mortem). These results suggest that both reduced rate of protein degradation and higher capacity for protein synthesis are consequences of the hypertrophic condition. However, further work will be required to investigate this possibility as well as the details how degradation is going on in the muscle tissue by lysosomes.

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