International Journal of Agricultural Technology 2018 Vol. 14(7): 1525-1534
Available online http://www.ijat-aatsea.com
ISSN: 2630-0613 (Print) 2630-0192 (Online)

Degradation of troponin-T associated with calpain/ calpastatin genes expression in native Thai beef cattle fed different levels of energy

Non-see, M. [1], Lukkananukool, A. [2], Polyorach, S. [2], Sommart, K. [3], Sazili, A. Q. [4] and Chaosap, C.[1*]

[1]Department of Agricultural Education, Faculty of Industrial Education and Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand; [2]Department of Animal Production Technology and Fisheries, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand; [3]Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand; [4]Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

Non-see, M., Lukkananukool, A., Polyorach, S., Sommart, K., Sazili, A. Q. and Chaosap, C. (2018). Degradation of troponin-T associated with calpain/ calpastatin genes expression in native Thai beef cattle fed different levels of energy. International Journal of Agricultural Technology 14(7): 1525-1534.

Abstract The influence of dietary energy level on the degradation of troponin-T protein and expression of calpain and calpastatin genes in native Thai cattle was determined. Eighteen steers (at 200 days old and average body weight of 100 ± 20 kg) were randomly assigned to 3 dietary treatment groups of different metabolizable energy: Treatment 1 [(8.9 MJ/ kg DM; n=6), Treatment 2 (9.7 MJ/kg DM; n=6) and Treatment 3 (10.5 MJ/kg DM; n=6)]. The diets were in the form of fermented total mixed ration (FTMR). At the end of 520 days of feeding, the steers attained 300 ± 10 kg BW at slaughter. The *Longissimus dorsi* m. samples collected within 2 d postmortem were further aged for 14 d for the troponin-T degradation immunoblot analysis while, the CAPN1, CAPN2, and CAST genes expression analyses only used samples at 2 d postmortem. The relative bands intensities of intact (37 kDa) and degraded troponin-T (30 kDa) were unaffected by the dietary treatments ($p>0.05$). Higher 37 kDa ($p<0.01$) and 30 kDa ($p<0.001$) troponin-T proteins expression were noted at 2 d and 14 d post-mortem, respectively. The CAPN1, CAPN2, and CAST genes expression remained unaffected ($p>0.05$) by the dietary treatment. Nonetheless, significant associations were observed between CAPN2 and CAST genes expression ($r=0.93$; $p<0.0001$), and between CAPN1 gene expression and 30 kDa troponin-T proteins expression at 14 d postmortem ($r=0.58$; $p<0.05$). This study concluded that neither troponin-T proteins nor calpain/ calpastatin genes expression were influenced by dietary energy levels in fattened native Thai cattle. The associations between CAPN2 and CAST genes expression, and between CAPN1 gene expression and 30 kDa troponin-T proteins expression at 14 d postmortem suggest the involvement of CAPN1 in myofibrillar proteolysis during the 14 d postmortem ageing of *Longissimus dorsi* m. in native Thai cattle.

Keywords: Dietary energy, troponin-T, calpain, calpastatin, native Thai cattle

[*] **Coressponding Author:** Chaosap, C.; **Email:** chanporn.ch@kmitl.ac.th

Introduction

Native Thai cattle has been classified as *Bos indicus* and previously used as draught animal until recently, being considered for beef production in Thailand. Currently, native Thai cattle are mostly subjected to free grazing on lands containing poor quality native grasses and weeds, without any supplementation except in dry season when the animals are offered with some rice straw. The existing extensive system may only provide at maintenance or probably sub-maintenance dietary energy level to the cattle. Irrespective breeds, it is well established and accepted that feeding management is among major determinants of cattle growth performance and beef quality (Herd and Bish, 2000). Thus, improved feeding system through optimisation of dietary energy levels may be a practical approach for improving the aforementioned traits. Tenderness is the most important and variable meat quality trait particularly in beef (Huffman *et al*., 1996). The influences of calpain system on skeletal muscle growth and postmortem meat tenderisation through proteolysis in beef cattle have been widely documented (Koohmaraie and Geesink, 2006). Available energy in the form of glycogen as stored in the skeletal muscle at slaughter determines the rate and extent of postmortem changes which naturally occur during rigor development (Lomiwes *et al*., 2010). Low energy reserve in skeletal muscles has been implicated in earlier completion of rigor which in turn, result in earlier failure of cellular organelles to maintain their integrity and functions. For example, sarcoplasmic reticulum (calcium ion pump) may not be able to maintain the calcium flux intracellular and extracellular when energy reserve in the form of ATP which depends on the availability of glycogen at death is depleted. As a result, earlier release of calcium will bathe the myofibrillar proteins, and this consequently causes earlier activation of the calpain proteases which has been commonly associated with the early postmortem meat tenderisation through enzymatic myofibrillar proteolysis (Koohmarie, 1992). However, the effects of dietary energy level on the expressions of calpain/ calpastatin genes and troponin-T proteins in native Thai cattle are not yet to be reported. Therefore, this study was conducted with an attempt to determine the influence of dietary energy level on degradation of troponin-T protein and expression of calpain and calapstatin genes in fattening native Thai cattle. We hypothesised that the expression of troponin-T proteins and, calpain/ calpastatin genes are influenced by the different dietary energy levels offered to the animals.

Materials and Methods

Animals and diet

All animal procedures were conducted in accordance with the protocol and guidelines outlined by the Animal Care and Use Committee of

Thailand. Eighteen Thai native steers at the age of 200 days old with body weight of 80 – 120 kg were randomly assigned to 3 dietary treatment groups of different metabolizable energy: Treatment 1 [(8.9 MJ/ kg DM; n=6), Treatment 2 (9.7 MJ/kg DM; n=6) and Treatment 3 (10.5 MJ/kg DM; n=6)]. The diets offered to the steers were in the form of fermented total mixed ration (FTMR) with varying levels of ME as mentioned above. All steers were subjected to 520 days of feeding, whereby at the completion of the fattening period, the steers attained average body weight of 300 ± 10 kg.

Slaughtering and muscle sampling

Prior to slaughter, the animals were held in a lairage for 24 h, restricted from feed with *ad libitum* access to drinking water. Slaughter and processing were carried out in accordance with the procedure following a standard commercial procedure of Halah slaughter without stunning. Immediately following evisceration, all carcasses were longitudinally split to left and right halves and aged at 4°C for 48 h before subsequent samplings of *Longissimus dorsi* muscle from the left side of each carcass. Each sample was initially cut into 2 pieces following which, both sub samples were vacuum packed and subjected to 2 and 14 d of ageing. Samples which have completed their respective ageing period were transferred to a -80°C freezer and stored until subsequent immunoblot and gene expression analyses. Muscle samples aged for 2 and 14 days were assigned for the Troponin-T degradation analysis while, for the CAPN1, CAPN2 and CAST genes expression analyses, only samples taken from 2 days aged LD muscles were used.

Troponin-T immunoblot analysis

The 2 and 14 days aged LD muscle samples were examined for troponin-T protein degradation through immunoblot analysis (Sun *et al*., 2014). The muscle tissue preparation and extraction, SDS-PAGE, Western blotting and immunoprobing procedures were conducted according to the methods as described by Rivero-Gutierrez *et al*. (2014). Briefly, following the extraction, protein concentration of the samples was quantified based on the method described by Lowry *et al*. (1951). Samples were mixed with sample loading/tracking dye (30 mM Tris-HCl, pH 8.0, 3 mM EDTA, 20% [v/v] glycerol, 3% [w/v] SDS, 0.003% [w/v] bromophenol blue) and 100 µg of TnT, before being loaded into 10% TGX Stain-free precast gels (Bio-Rad, USA).

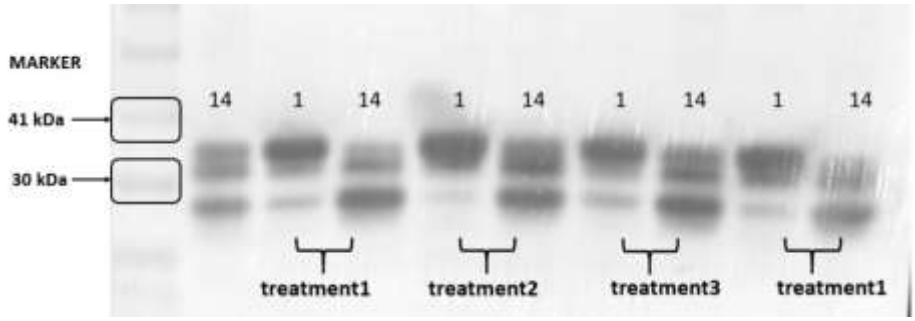

Figure 1. Representative Western blot of whole muscle protein extracted from beef *longissimus dorsi* m. aged for either 2 or 14 days in steers subjected to different dietary energy levels; Treatment 1 = 8.9 MJ/kg DM; Treatment 2 = 9.7 MJ/kg DM; Treatment 3 = 10.5 MJ/kg DM

In the SDS-PAGE, proteins were separated at a constant current (40 mA). The running buffer used contained 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 0.1% SDS (w/v). Following electrophoresis, proteins were transferred onto 0.45 µm, PVDF blotting membranes (Amersham™ Hybond™, Catalogue No. 10600023, Germany) using a Criterion™Blotter (Bio–Rad, USA) at a constant amperage (140 mA) for 1.5 h. The transfer buffer used contained 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 15% (v/ v) methanol. After transfer, a Stain-Free image was taken to verify equal loading. Membranes were blocked with 5% skim (TM Media, Titan Biotech Ltd., India) diluted in 10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween-20 (TBS-T) for 30 min at room temperature to block non-specific antigen binding sites. Troponin-T blots were incubated overnight at 4℃ with a mouse monoclonal anti-troponin T antibody (T6277, Sigma Aldrich, USA) diluted 1:30,000 in 5% skim milk and TBS-T. The blots were incubated subsequently incubated with an anti-mouse IgG whole molecule, horseradish peroxidase-conjugated secondary antibody (A9044, Sigma Aldrich, USA) diluted 1:20,000 in 5% skim milk and TBS-T for 1 h at room temperature. After washing as described above, membranes were developed using a colorimetric kit (Novex® Chromogenic Substrates, Catalogue No. WP20001, Invitrogen Clarity™, USA) and visualized using a ChemiDoc™ Touch Imaging System (Bio-Rad, USA). Band intensities were quantified using Image Lab™ Software (Bio-Rad, USA). Intact and degraded forms of troponin-T measured were immunoreactive bands located at 37 and 30 kDa, respectively (Figure 1).

Calpain and calpastatin gene expression

Muscle samples taken from each cattle were extracted for total RNA using TRIzol® LS reagent in accordance with procedures outlined in the manufacturer's protocol (Catalogue No. 10296010, Invitrogen, USA). The resulted total RNA was treated with deoxyribonuclease as to remove

genomic DNA (gDNA) contamination in the RNA sample prior to cDNA synthesis, whereby cDNA was generated from 0.5 µg of total RNA using random primers, nuclease free water, and transcription mixture (Revert Aid First Strand cDNA Synthesis Kit, Thermo Scientific).

Quantitative Real Time PCR Total cDNA was diluted 1:4, and from this, a pool of cDNA was generated for each sample and a dilution series was made and used as a standard curve. cDNA of individual samples were further diluted to 1:5 for analyzing gene expression. The commercially available primers sets of PrimePCR Assay for the *Bos taurus* : CAPN1 (qBtaCID0005919), CAPN2 (qBtaCID0001849), CAST (qBtaCID0018280) genes used in the amplification of target genes and also housekeeping genes (HKGs), namely Glyceraldehyde-3-phosphate dehydrogenase (G3PDH; qBtaCID0013312) was used to amplify the reference genes (Biorad, USA).

The real-time PCR reaction used the mixture consisted of 3.5 µl cDNA, 0.4 µl of forward and reverse primers, and 5 µl SYBR Green Universal PCR Master mix (SensiFastTM SYBR, BIOLINE). Reactions were carried out in duplicates on a 96-well plate read on a Bio-Rad CFX96 system (Bio-Rad, USA), at 95 ℃ for 2 min, and then 40 cycles of 95 ℃ for 5 s and 55 ℃ for 15 s, through which, the resulted fluorescence was detected in real time. Following the PCR reactions, the melting curve was analyzed using CFX Manager™ Software (Bio-Rad, USA) to ascertain the specificity of the amplication. The calculated average mRNA expression of CAPN1, CAPN2, and CAST genes were normalized relative to the expression of reference gene GAPDH (Chaosap *et al*., 2011).

Statistical Analysis

Analyses of variance for immunorreactivities of troponin T was generated by using the GLM procedure (SAS Inst. Inc., Cary, NC) with dietary energy level and ageing period as the main effect with interaction. For gene expression of calpain system, ANOVA was generated by using the GLM procedure with dietary energy level as the main effect. Least squares means were separated using the probability of difference option (PDIFF), and the results were considered significant difference when $P<0.05$. The relationships between study traits were evaluated by Pearson correlation coefficients.

Results

The intensities of 30 kDa (degraded) and 37 kDa (intact) troponin-T protein bands were determined colorimetrically using the ChemiDoc^{TM} Touch Imaging System (Bio Rad, USA). The results of 30 kDa and 37 kDa are expressed as optical densities relative to total proteins (Table 1). In this study, the relative band intensities of intact (37 kDa) and degraded troponin-T (30 kDa) were unaffected by the dietary energy levels ($p>0.05$). As for the

37 kDa troponin-T, the intensities of the intact protein bands were recorded as 0.29, 0.29 and 0.22 in the LD samples obtained from the steers subjected to Treatment 1, Treatment 2 and Treatment 3, respectively (Table 1). Meanwhile, the intensities of 30 kDa troponin-T in LD muscles from Treatment 1, Treatment 2 and Treatment 3 steers, are presented as 0.14, 0.17 and 0.16, respectively (Table 1).

Table 1. Immunorreactivities of 37 kDa and 30 kDa troponin-T proteins of *longissimus dorsi* m. aged for either 2 or 14 days in steers subjected to different dietary energy levels

Trait[1#]	**Treatment (T)**			**Ageing (A)**		**RMSE**	**P-value**		
	1	2	3	2	14		T	A	TxA
37 kDa	0.29	0.29	0.22	0.32	0.21	0.10	0.173	0.003	0.766
30 kDa	0.14	0.17	0.16	0.11	0.20	0.06	0.418	0.0002	0.943

Treatment 1 = 8.9 MJ/kg DM); Treatment 2 = 9.7 MJ/kg DM; Treatment 3 = 10.5 MJ/kg DM;
kDa = Kilodalton; ME = Metabolizable energy; DM = dry matter ;
[1] 37 kDa = intact troponin-T; 30 kDa = degraded product of troponin-T ;
[#]Equal amount of total protein (100 µg) was loaded into each lane;
The means presented above are the ratio between relative quantity of target protein and relative quantity of total protein in each lane. The values are expressed as optical density (OD).

Table 2. CAPN1, CAPN2 and CAST genes expression of *Longissimus dorsi* m. in steers subjected to different dietary energy levels

Gene	**Treatment**			**RMSE**	**P-value**
expression[1]	1	2	3		
CAPN1	0.61	0.66	0.79	0.22	0.479
CAPN2	0.60	0.79	0.90	0.29	0.339
CAST	0.76	0.90	0.99	0.23	0.358

Treatment 1 = 8.9 MJ/kg DM); Treatment 2 = 9.7 MJ/kg DM; Treatment 3 = 10.5 MJ/kg DM;
ME – Metabolizable energy; MJ – megajoule; DM – dry matter;
CAPN1 encodes µ-calpain; CAPN2 encodes m-calpain; CAST encodes calpastatin;
[1]Gene expression of target gene: GAPDH.

The results of CAPN1, CAPN2 and CAST genes expression (Table 2) are presented as the ratios between gene expression of the target genes and GAPDH. It is clearly demonstrated that neither CAPN1 and CAPN2, nor CAST genes expression was affected ($p>0.05$) by the different dietary energy levels. In the present study, significant positive correlations (Table 3) were observed between CAPN2 and CAST genes expression (r=0.93;

p<0.0001), and between CAPN1 gene expression and 30 kDa troponin-T protein bands intensities at 14 d postmortem (r=0.58; p<0.05). Nonetheless, the associations between CAPN1 and CAPN2 (r=0.40; p>0.05), CAPN1 and CAST (r=0.48; p=0.084), and CAST and 30 kDa troponin-T at 14 d postmortem (r=0.42; p>0.05) were not significant.

In this study, CAST gene expression was found to be positively and significantly associated with the expression of CAPN2 gene. The observed association suggests possible regulation of postmortem proteolysis by CAPN2 through the specific inhibition by calpastatin as indicated by increased expression of CAST gene. Meanwhile, CAPN1 gene expression was found to be positively associated with the expression of the degraded product of troponin-T (30 kDa) protein at 14 d postmortem.

Table 3. Correlation coefficients of CAPN1, CAPN2, CAST gene expressions, and 37 kDa and 30 kDa troponin-T proteins expression in *Longissimus dorsi* m. of native Thai cattle

Trait	**CAPN2**	**CAST**	**37 kDa TnT_2**	**30 kDa TnT_2**	**37 kDa TnT_{14}**	**30 kDa TnT_{14}**
CAPN1	0.40	0.48	-0.64	0.04	-0.21	0.58
	0.157	0.084	0.014	0.901	0.476	0.029
CAPN2		0.93	-0.20	0.01	0.11	0.33
		<0.0001	0.494	0.983	0.703	0.243
CAST			-0.17	0.01	0.14	0.42
			0.565	0.978	0.619	0.139
37 kDa TnT_2				0.41	0.17	-0.14
				0.118	0.524	0.604
30 kDa TnT_2					-0.39	0.33
					0.139	0.216
37 kDa TnT_{14}						0.25
						0.348

TnT = troponin-T , [2] 2 days postmortem ageing, [14] 14 days postmortem ageing

Discussion

The degradation of troponin-T, especially the 30 kDa polypeptide, is considered to be an indicator of meat tenderization due to its good relationship with meat tenderness (Harris *et al*., 2001). The intensities of 37 kDa and 30 kDa troponin-T were found to be significantly affected by the ageing period in the present study. The intact troponin-T (37 kDa) protein bands intensities were found to be higher (p<0.05) at day 2 (0.32) than those quantified at day 14 (0.21) postmortem. Inversely, in the case of degraded

troponin-T (30 kDa), higher ($p<0.0001$) protein bands intensities were noted in LD samples aged for 14 d (0.20) than its counterpart which was aged for 2 d (0.11) postmortem. In this study, there was no interaction between the dietary energy level and ageing period and this was consistently observed in both intact (37 kDa) and degraded (30 kDa) troponin-T proteins, suggesting that the effects of ageing on their bands intensities were independent of the dietary energy levels.

The dietary energy level had no significant effect on the degradation of troponin-T in the current study. In agreement with Xiong *et al*. (1996) who found the similar proteolysis pattern between steers fed with grain on grass which had higher dietary energy level and grass-fed steers. Unlike the dietary energy level, the intensities of 37 kDa and 30 kDa troponin-T were found to be significantly affected by the ageing period. The present results of troponin-T as affected by the ageing period are in agreement with the previous report in beef (Steen *et al*., 1997). The increase in 30 kDa troponin-T with decreasing 37 kDa troponin-T proteins during the progress of postmortem ageing process has been well documented (Ho *et al*., 1994).

The relationship between 30 kDa troponin-T and meat tenderization asscociated with postmortem myofibrillar proteolysis has been well established and documented (Huff-Lonergan *et al*., 1996). Thus, the increased intensities of 30 kDa troponin-T observed in the present study, further support the role and involvement of μ-calpain (as indicated by the increased expression of CAPN1 gene) on proteolysis during the 14 d postmortem ageing of *Longissimus dorsi* m. in native Thai steers. The association between CAPN1 gene expressions at 2 d postmortem and 30 kDa troponin-T protein bands intensities also highlight its potential as a molecular marker for predicting meat tenderisation through the rate and extent of proteolysis at 14 d postmortem.

It concluded that dietary energy levels did not affect degradation of troponin-T protein and expression of calpain/ calpastatin genes in *Longissimus dorsi* muscle of native Thai cattle. However, the relationship observed between CAPN2 and CAST genes expression which suggests a possible inhibition of m-calpain activities by calpastatin, further supported by the association between CAPN1 gene expression and 30 kDa troponin-T protein expression highlight a possiblility that the enzymatic myofibrillar proteolysis of *Longissimus dorsi* m. at 14 d postmortem was mainly mediated by μ-calpain.

Acknowledgments

The authors would like to thank The Thailand Research Fund (TRF) for financial support and Faculty of Industrial Education and Technology, King Mongkut's Institute of Technology Ladkrabang for research facility supports.

References

Chaosap, C., Parr, T. and Wiseman, J. (2011). Effect of compensatory growth on forms of glycogen, post-mortem proteolysis, and meat quality in pigs. Journal of Animal Science. 89:2231-2242.

Harris, S. E., Huff-Lonergan, E., Lonergan, S. M., Jones, W. R. and Rankins, D. (2001). Antioxidant status affects color stability and tenderness of calcium chlorideinjected beef. Journal of Animal Science. 79:666-677.

Herd, R. M. and Bisho, S. C. (2000). Genetic variation in residual feed intake and its association with other production traits in british hereford cattle .Livestock Production Science. 63:111-119.

Ho, C. Y., Stromer, M. H. and Robson, R. M. (1994). Identification of 30 kDa polypeptide in postmortem skeletal muscle as a degradation product of troponin–T. Biochimie. 76:369-375.

Huffman, K. L., Miller, M. F., Hoover, L. C., Wu, C. K., Brittin, H. C. and Ramsey, C. B. (1996). Effect of beef tenderness on consumer satisfaction with steaks consumed in the home and restaurant .Journal of Animal Scicence. 74:91-97.

Huff-Lonergan, E., Mitsuhashi, T., Beekman, D. D., Parrish F. C. Jr., Olson, D. G. and Robson, R. M. (1996). Proteolysis of specific muscle structural proteins by mu-calpain at low ph and temperature is similar to degradation in postmortem bovine muscle. Journal of Animal Scicence. 74:993-1008.

Koohmaraie, M. (1992). The role of Ca^{2+}-dependent proteasesin(calpain)postmortem proteolysis and meat tenderness. Biochimie. 74:239-245.

Koohmaraie, M. and Geesink, G. H. (2006). Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain syste. Meat Science. 74: 34-43.

Lomiwes, D., Reis, M. M., Wiklund, E., Young, O. A. and North, M. (2010). Near infrared spectroscopy as an on-line method to quantitatively determine glycogen and predict ultimate ph in pre rigor bovine M. longissimus dorsi. Meat Science. 86:999-1004.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin fhenol reagent. Journal of Biological Chemistry. 193:265-275.

Rivero-Gutierrez, B., Anzola, A., Martinez-Augustin, O. and Sanchez de Medina, F. (2014). Stain-free detection as loading control alternative to Ponceau and housekeeping protein immunodetection in Western blotting. Analytical Biochemistry. 467:1-3.

SAS Institute Inc. 2003. SAS/STAT User's Guide: Version 9.1.SAS Institute Inc,. Cary, NC, USA.

Steen, D., Claeys, E., Uytterhaegen, L., De Smetb, S. and Demeyer, D. (1997). Early post-mortem conditions and the calpain/calpastatin system in relation to tenderness of double-muscled beef .Meat Science. 45:307-319.

Sun, X., Chen, K. J., Berg, E. P., Newman, D. J., Schwartz, C. A., Keller, W. L. and Maddock Carlin, K. R. (2014). Prediction of troponin-t degradation using color image texture features in 10 d aged beef longissimus steaks. Meat Science. 96:837-842.

Xiong, Y. L., Moody, W. G., Blanchard, S.P., Liu, G. and Burris, W.R. (1996). Postmortem proteolytic and organoleptic changes in hot-boned muscle from grass and grain-fed zeranol-implanted cattle. Food Research International. 29:27-34.

(Received: 15 September 2018, accepted: 6 November 2018)