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The Differences and Relationship in the Gene Expression of Calpain System and Pork Tenderness between Duroc Purebred and Crossbred Pigs

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Meat tenderness is the most important attribute for consumers' demands which affected by calpain enzyme activity. The objectives of this study were 1) to compare the gene expression of calpain system and meat tenderness of Duroc purebred and crossbred pigs and 2) to investigate the relationship between gene expression of calpain system and pork tenderness. *Longissimus dorsi* (LD) muscle of pigs 1) Duroc purebred (D), 2) two-way crossbred of Large White and Landrace (LWLR), and 3) three-way crossbred of Duroc, Large White, and Landrace (DLWLR) were taken for measuring the gene expression of calpain system by real-time PCR technique and shear force analysis, respectively. The results revealed that gene expression of calpain system of Duroc purebred and crossbred pigs were statistically significant differences ($P < 0.01$). The expression of *CAPN1* of DLWLR was higher than LWLR and D ($P < 0.01$). The expression of *CAPN2* of LWLR and DLWLR pigs were higher than D pigs. Shear force value of LWLR was the highest following by DLWLR and Duroc purebred ($P < 0.01$). There were statistically significant relationship between *CAPN1* and *CAPN2* ($r = 0.71$, $P < 0.01$) and also between *CAST* and *CAPN1* ($r = 0.53$, $P < 0.01$) as well as *CAST* and *CAPN2* ($r = 0.44$, $P < 0.05$).

Keywords: Duroc purebred, crossbred, gene expression of calpain system, meat tenderness

Introduction

The pork quality depends on breed, feed, muscle type, nutrient composition, connective tissue as well as the proteolytic enzyme system especially calpain system which involes in the meat tenderness. Pig breeding technology uses hybrid breeding programs to improve pork production and pork quality. The purebred and crossbred pigs have different performance and pork quality. Three-way crossbred pigs are mainly used for commercial pork production and have more great production efficiency than pure and two-way

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crossbreds (Nelson and Robison, 1976). A white breed such as Landrace, Yorkshire, and Large White is usually included in the crossbreeding system for its maternal traits; highly prolific and good mothering ability. A color breed such as Duroc is normally used for paternal traits; growth performance, carcass, and meat quality (Kim *et al.*, 2002; Kim *et al.*, 2006; Lee *et al.*, 2011). Several studies have demonstrated that meat from pigs sired by Duroc has a higher intramuscular fat content which affects high eating quality especially, juiceness (McGloughlin *et al.*, 1988; Edwards *et al.*, 1992; Oliver *et al.*, 1994). However, in terms of tenderness of crossbred sired by Duroc is still need to investigate. Calpain system is the major system for post-mortem proteolysis which consists of 2 calcium dependent proteases, μ-calpain or calpain 1 and m-calpain or calpain 2 and their endogenous inhibitor calpastatin (Goll *et al.*, 2003; Giusti *et al.*, 2013). Many studies have shown that calpain 1 and its endogenouse inhibitor calpastatin play a major role in meat tenderisation process (Huff-Lonergan *et al.*, 1996; Koohmaraie and Geesink, 2006). *CAPN1* gene encodes the large subunit of calpain 1 and *CAPN2* gene encodes the calpain 2 while *CAST* gene encodes calpastatin (Gandolfi, 2011). Wang *et al.* (2015) reported that three-way crossbred pig (Duroc × Landrace × Yorkshire) showed higher *CAPN1* expression concomitant with higher calpain 1 activity than Meishan pig. Due to the studies of the differences and relationship of calpain enzyme system and meat tenderness in purebred and crossbred pigs are still limit. Therefore, the objectives of this study were 1) to compare the gene expression of calpain system and meat tenderness of Duroc purebred and crossbred pigs and 2) to investigate the relationship between the expression of calpain system and pork tenderness.

Materials and methods

Animals and Muscle samples

There were 3 groups of pigs in this study which were slaughtered at the average weight of 110 kg and their *Longissimus dorsi* (LD) muscle at the 13^{th}-14^{th} ribs were taken for measuring the gene expression of calpain system by real-time PCR method and meat tenderness by Warner Bratzler shear force (SF) method, respectively. There were 10 pigs in each group and pig groups were 1) Duroc purebred (D), 2) two-way crossbred of Large White and Landrace (LWLR), and 3) three-way crossbred of Duroc, Large White, and Landrace (DLWLR). LD muscle samples were cut into 3 pieces. The first ones were snapped in liquid N_2 and then stored at -40°C for RNA extraction and cDNA synthesis which were the process for retrieving calpain gene expression. The

other two pieces were kept in vacuum bags at temp 2 - 4 °C for 1 and 5 day before storing at -20°C until shear force analysis.

Gene expression of Calpain system

RNA Extraction and cDNA Synthesis

Muscle samples taken from each pig were extracted for total RNA using Trizol reagent according to the protocol of the manufacturer (Invitrogen, Paisley, UK). Total RNA was treated with deoxyribonuclease for removing genomic DNA (gDNA) contamination in the RNA sample prior to cDNA synthesis. cDNA was generated from 0.5 µg of total RNA by using random primers, nuclease free water, and transcription mixture (RevertAid 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 made and used as a standard curve. cDNA of Individual samples were further diluted 1 : 5 for analyzing gene expression. The real-time PCR reaction used the mix 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 duplicate on a 96-well plate run on a Bio-Rad CFX96 system (Bio-Rad, USA): 95℃ for 2 min, and then 40 cycles of 95℃ for 5 s and 55℃ for 15 s; fluorescence was detected in real time. After PCR reactions, the melting curve was analyzed to guarantee the specificity of the amplication. The calculated average mRNA expression of *CAPN1*, *CAPN2*, and *CAST* genes were normalized by the expression of reference gene *GAPDH*.

Table 1 List of primers used to quantify gene expression

Gene	Primer sequence (5'to3')	GenBank accession no.
CAPN1	Forward: GACACCCTCCTGCACCGA	AF263610
	Reverse: TCCACCCACTCCCCAAACT	
CAPN2	Forward: ACATGCACACCATCGGCTTT	U01181
	Reverse: CGCTCTGTGCGTCAGGAAG	
CAST	Forward: AGGCTGTAAAAACAGAACCTG	M20160
	Reverse: ATTTCTCTGATGTTGGCTGCTC	
GAPDH	Forward: GCGTGAACCATGAGAAGTATGA	AF017079
	Reverse: GGTAGAAGCAGGGATGATGTTC	

Lindholm-Perry *et al.* (2009), Chaosap *et al.* (2011)

Warner–Bratzler shear force

After thawing at 1-4°C for 24 h, samples were cooked in vacuum bags in a 80°C water bath for 30 min or internal temperature at 70°C and then cooled for 30 min. Eight pieces of 1×3×1 cm^3 (width × length × height) were removed from each sample parallel to longitudinal orientation of muscle fibers. Samples were sheared perpendicular to long axis using an Instron model 1011 (Instron, U.S.A.).

Statistical analysis

Statistical analysis was performed using the Proc GLM offered in SAS software (SAS Institute Inc., Cary, NC, USA). The model used in this procedure includes animal groups as a treatment factor. Least square means were separated by using PDIFF option ($P < 0.05$). Pearson correlation was used to find out the relationship between gene expression of calpain system and shear force value.

Results and Discussion

Gene expression of calpain system

Gene expression of calpain system and SF value of three different pig groups were shown in Table 2. *CAPN1* from DLWLR pig expressed higher than the other two groups ($P < 0.01$). Three-way crossbred pig had higher *CAPN1* than two-way crossbred and purebred pig. While two-way and three-way crossbred had higher *CAPN2* expression than purebred Duroc. This corresponds with the finding of Wang *et al.* (2015) research reported that three-way crossbred had higher *CAPN1* expression and also calpain activity than commercial Meishan pigs. *CAPN1* and *CAPN2* encoded calpain 1 and calpain 2 emzymes which is known to be associated with post-mortem proteolysis. Higher *CAPN1* expression could correspond to higher expression of calpain 1 enzyme which is reflected in greater calpain 1 activity (Wang *et al.*, 2015). Calpain 1 and 2 are calcium dependent proteases and responsible for the myofibrillar degradation observed during post-mortem ageing (Livisay *et al.* 1996). Calpain 1 is activated first (at pH 6.3), when the calcium levels are still low. As the calcium levels increase calpain 2 is activated (Dransfield, 1994). Calpain 1 rather than calpain 2 enzyme is the major post-mortem proteolytic enzyme (Kemp *et al.*, 2010; Geesink *et al.*, 2006). *CAST* encoded calpastatin which is calpain inhibitor. Calpastatin has positive correlation with meat

toughness (Parr *et al.*, 1999). However, in this study the *CAST* from three pig groups were not significant differences. Thus, the tenderization process in pork could be affected by the differences in the expression of calpain encoded genes.

Meat tenderness

There was statistically significant difference in shear force value at day 1 ($P < 0.01$) which the lowest was from Duroc purebred pig. Whereas, the SF value at day 5 were not statistically significant difference ($P > 0.05$). Duroc purebred pig had the lowest SF value at day 1 or most tender which due to Duroc breed having superior meat quality. Various studies reported that meat from Duroc sired genotype pigs has a higher intramuscular fat (IMF) level (McGloughlin *et al.*, 1988; Edwards *et al.*, 1992; Oliver *et al.*, 1994) which affects eating quality, in particular, juiciness (Wood *et al.*, 1986). Jelen ková *et al.* (2008) reported that intramuscular fat had a negative correlation with shear force value while had a positive correlation with tenderness and juiceness. In this study, the three-way crossbred sired by Duroc had lower SF value than two-way crossbred (5.33 kg vs 5.92 kg) at day 1 post-mortem that might be due to no influence of Duroc breed on intramuscular fat content in two-way crossbred. Correspondance finding with Poldvere *et al.* (2015) who reported that three-way crossbred sired by Duroc (D × LW × LR) had higher intramuscular fat than two-way crossbred (LW × LR), 2.7 % and 1.71 %, respectively.

Table 2 Least square means of meat tenderness and gene expression of calpain system

Traits	Groups			P-value
	D	LWLR	DLWLR	
CAPN1	0.62^{b}	0.79^{b}	1.08^{a}	0.0001
CAPN2	0.87^{b}	1.24^{a}	1.49^{a}	0.0009
CAST	0.82	1.20	1.45	0.141
SF day 1	4.56^{c}	5.92^{a}	5.33^{b}	0.0003
SF day 5	4.48	4.40	4.15	0.347

$^{a, b, c}$ least square mean in row with a different letter differ ($P<0.05$)

SF; Warner Bratzler shear force at day 1 and day 5 post-mortem

D; Duroc purebred, LWLR; Large White×Landrace, and DLWLR; Duroc×Large White×Landrace

Reletionship between gene expression of calpain system and meat tenderness in purebred and crossbred pigs

Table 3 Correlation of meat tenderness and gene expression of calpain system

Traits	*CAPN2*	*CAST*	SF day 1	SF day 5
CAPN1	0.71**	0.53**	0.37	-0.29
	(<.0001)	(0.003)	(0.055)	(0.121)
CAPN2		0.44*	0.39*	-0.27
		(0.016)	(0.041)	(0.148)
CAST			0.22	0.07
			(0.263)	(0.723)
SF day 1				0.19
				(0.342)

*P<0.05, **P<0.01

There were statistically significant positive relationship between *CAPN1* and *CAST* (r = 0.53, P = 0.003), *CAPN2* and *CAST* (r = 0.44, P = 0.016), *CAPN1* and *CAPN2* (r = 0.71, P < .0001). SF value at day 1 had a positive correlation with the expression of *CAPN1* (r = 0.37, P = 0.055), *CAPN2* (r = 0.39, P = 0.041), and *CAST* (r = 0.22, P = 0.263), respectively. While SF value at day 5 had a negative correlation with the expression of *CAPN1* (r = -0.29, P = 0.121) and *CAPN2* (r = -0.27, P = 0.148), respectively. Gandolfi *et al.* (2011) reported that *CAPN1* and *CAST* expression have relationship with calpain activity and SF value. Higher *CAPN1* expression could correspond to higher calpain 1 protein expression which is reflected in greater calpain 1 activity. According to the positive correlate between *CAST* and SF value at day 1, in agreement with Gandolfi *et al.* (2011) reported that *CAST* expression was 29% higher in muscle with high SF value.

Conclusion

From this study can be concluded that the purebred and crossbred pigs have differences in gene expression of calpain system. Both *CAPN1* and *CAPN2* expression from three-way crossbred pigs sire by Duroc pig were higher than two-way crossbreds. *CAPN1* and *CAPN2* encoded calpain 1 and 2 which play a major role in muscle proteolysis during post-mortem aging leading to meat tenderness. This information demonstrated that three-way crossbreds terminal sired by Duroc breed would have more tender meat due to the higher expression of calpain genes.

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