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Skeletal Ryanodine Receptor 1-Heterozygous PSE (Pale, Soft and Exudative) Meat Contains a Higher Concentration of Myoglobin than Genetically Normal PSE Meat in Pigs

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ABSTRACT : Comparisons of properties between skeletal ryanodine receptor 1 (*sRyR1*)-heterozygous-mutated and normal types of meat were carried out in pigs using PSE (pale, soft and exudative) meat found during the butchering process. All samples considered to be PSE meat showed irregular running and disorder of the muscle fibers and a wider inter-fiber space upon light microscopic observation. Electron microscopy revealed disintegration, twisting, and disorder of the myofibril arrangement and elimination of the Z line in PSE meat, compared with normal meat. Meat property tests demonstrated greater decreases in water holding capacity, moisture and sarcoplasmic protein, and higher L* values for the meat color index in PSE meat than in normal meat, but there were no differences in these factors between genetically normal and *sRyR1*-heterozygous PSE meat. On the other hand, higher a* and b* values were observed in *sRyR1*-heterozygous than in normal PSE meat, and similar alterations to the a* value were observed in terms of the amount of myoglobin and density of the 17-kDa protein band, corresponding to the molecular mass of myoglobin, on SDS-PAGE gels. These results suggest that *sRyR1*-heterozygous PSE pork contains much more myoglobin than genetically normal PSE meat. (**Key Words :** Meat Color, Myoglobin, PSE Pork Meat, Skeletal Ryanodine Receptor 1-heterozygote)

INTRODUCTION

The incidence of PSE (pale, soft and exudative) pork meat, which has a reduced commercial value, is dependent on various genetic and environmental factors and their possible interactions (Rosenvold and Anderse, 2003). Among the genetic factors, the halothane gene is well known to play a role in the occurrence of PSE pork. The halothane gene is related to porcine stress syndrome (PSS), which develops in genetically predisposed individuals upon exposure to halogenated anesthetics. In 1991, it was clarified that PSS resulted from a single nucleotide substitution in the gene encoding the skeletal ryanodine receptor 1 (*sRyR1*), and this finding allowed accurate diagnosis on the basis of three types: normal, *heterozygote* and *homozygote* (Fujii et al., 1991). Since the results of the halothane challenge test have suggested that the inheritance of PSS is recessive (Mabry et al., 1981), pigs with the *sRyR1* heterozygote have been regarded as merely carriers of the mutated gene (Sellier and Monin, 1994). However, it has been reported recently that heterozygous pigs have meat quality intermediate between the dominant and recessive homozygotes (Rosenvold and Andersen, 2003). On the other hand, studies of the *sRyR1* heterozygote in relation to the properties of PSE pork have been limited (Pommier and Houde, 1993; Horiuchi et al., 1996).

PSE meat results from rapid postmortem muscle degeneration due to excessive glycolysis in combination with high temperature (Solomon et al., 1998). Precipitation of sarcoplasmic proteins has been thought to be one of the causes of paleness (lightness) of PSE meat (Lopez-Bote et al., 1989; Joo et al., 1999). Meat color has been quantified by the CIE L* (lightness), a* (redness) and b* (yellowness) color space index. PSE meat has higher L* and b* values than normal meat, whereas the reported changes in the a* value for PSE meat have differed among researchers (Pommier and Houde, 1993; Klont et al., 1993;1994; Klont and Lambooy, 1995). The a* value of meat color is correlated with the concentration of myoglobin (heme

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pigment) in skeletal muscle (Beriain et al., 2000; Latorre et al., 2004). Although the ammount of myoglobin in normal pork meat in relation to *sRyR1* mutation has been determined (Wykle et al., 1978; Klont and Lambooy, 1995; Tam et al., 1998), hardly any attempts have been made to assay myoglobin in PSE meat.

In this study, using pork meat showing actual signs of PSE, we compared the amounts of myoglobin and other meat properties between genetically normal PSE meat and *sRyR1*-heterozygous PSE meat.

MATERIALS AND METHODS

PSE meat and sample collection

Commercially raised pigs, which were crossbred (Duroc×(Yorkshire×Landrace)), slaughtered by electrical stunning and severing of the carotid arteries at a commercial abattoir were used. The last rib regions of longissimus muscle (LM) samples were collected during butchering of the carcasses at about 24 hours postmortem. Meat for which the cut surface appeared pale macroscopically was selected, and the meat colors were measured using a colorimeter (Minolta Camera, Osaka, Japan). The CIE L*, a* and b* color space index was used for quantification. Samples were then collected for light microscopic observation and investigation of the *sRyR1* genotype. Simultaneously, apparently normal meat was also sampled from different animals as a control. Each meat sample was determined as a PSE meat when having an L* value of >50 (Joo et al., 1999; Ryu and Kim, 2006) and microscopically irregular running and disorder of the muscle fibers. Forty-three samples of PSE meat and 17 samples of normal meat were collected. Water holding capacity (WHC), moisture, electron microscopic observation, assay of heme pigment (myoglobin concentration) and myoglobin analysis using SDS-PAGE were carried out using a portion of all samples.

Genotyping of sRyR1 mutation in meat samples

The s*RyR1* genotype was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Genomic DNA was isolated from skeletal muscle, using digestion with proteinase K and extraction with phenol-chloroform-isoamylalcohol. PCR-RFLP was carried out in 25 μl of a mixture described previously by Fujii et al. (1991). The PCR product was digested with the restriction enzyme Hha I at 37°C for 3 h. Restriction fragments were electrophoretically separated in 2.0% agarose gel and stained with ethidium bromide solution. The s*RyR1* genotypes were classified into the normal (*NN*) type, in which separated fragments of 493 bp and 166 bp were observed, the recessive homozygous (*nn*) type, in which an undigested fragment of 659 bp was observed, and the heterozygous (*Nn*) type, in which both undigested and separated fragments of 659 bp, 493 bp and 166 bp were observed.

Histological examinations

For light microscopy, each meat sample was fixed in 10% buffered formalin, embedded in paraffin wax, and sectioned at a thickness of 3 μm. Dewaxed sections were stained with hematoxylin and eosin. For electron microscopy, small fragment of each meat was immediately fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer for 2 h at 4°C, post-fixed in 1% osmium tetroxide in the same buffer for 2 h at 4°C, and routinely embedded in Epon812. Ultrathin sections were then cut and stained with uranyl acetate and lead citrate and observed with H-7000 KU transmission electron microscope at 75 kV.

Meat quality measurements

WHCs were determined by the centrifugation method. A fresh sample of LM was weighed (0.5000±0.0500 g), placed in a test tube including glass beads, and centrifuged at 2,100×g for 30 min. After centrifugation, the samples were weighed, and the post-centrifugation weight was divided by the pre-centrifugation weight to calculate the percentage WHC. Moisture (water content) was determined by the heating method. A fresh sample was weighed (2.000±0.200 g) on aluminum dish, then heated (120°C, 90 min.) in a dry heat chamber. After heating, the samples were weighed. Moisture was calculated as a percentage by following formula: moisture (%) = (1-(post-heated weight/ pre-heating weight))×100. The heme pigment (myoglobin) content of the LM was measured in two minced slices from each sample using a spectrometer according to Hornsey (1956) as modified by Boccard et al. (1981). Briefly, a 5-g sample of minced LM was placed into an extraction vessel with 20 ml of acetone and 1 ml of water, and stirred for 30 s. Afterward, 0.5 ml of 12 N HCl was added, then the suspension was kept in a sealed vessel overnight in the dark, filtered, and the absorbance of the filtrate was measured at 510 nm. Each slice was evaluated twice, and the average was determined. The concentration of myoglobin (milligrams per gram fresh muscle weight) was obtained by multiplying the absorbance reading by a factor of 8.816 obtained by calibration (Boccard et al., 1981).

Determination of protein concentration and SDS-PAGE

Pieces of muscle weighing 1.0 g were added to 9.0 ml of ice-cold 20 mM Tris buffer including 1mM phenylmethane sulfonyl fluoride, 3 μg/ml leupeptin and 1 mM EDTA, and homogenized with a Polytron on ice for 30 s two times with a 30-s interval. After centrifugation at 12,000 rpm at 4°C for 10 min, filtered supernatants were used for determination of protein concentration and for SDS-PAGE. Protein concentrations were measured by the

method of Bradford (1976). SDS-PAGE was carried out in 12% gels as described by Laemmli (1970) with 2-mercaptoethanol. Each protein sample was attenuated by three-fold dilution with sample buffer, heated at 100°C for 2 min, and separated at a constant current of 20 mA. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. Quantitative analysis of 17-kDa bands (Figure 1), corresponding to the molecular mass of myoglobin (Okumura et al., 2005), was performed using an image scanner. In one lane on each gel, 0.5 μg of bovine serum albumin was applied as a standard for analysis.

Statistical analysis

Results are expressed as the mean±SEM. Statistical analyses were performed by LSD test after one-way analysis of variance. Significance was established when the probability level was equal to, or less than 5%.

RESULTS AND DISCUSSION

As a result of genomic diagnosis for *sRyR1* mutation, the *Nn* genotype was not detected in normal meat, but was detected at a rate of 9.3% in PSE meat (Table 1). The *nn* genotype was not observed in this study, perhaps due to the low prevalence of *sRyR1* mutation in reproductive pigs. We had previously investigated the *sRyR1*-mutation in reproductive pigs, and predicted a very low level of expression of the *nn* genotype in fattening pigs because of the absence of *sRyR1* mutation in Duroc boars (Kitsutaka et al., 2008).

Table 1. Number of *sRyR1* genotypes in normal meat and PSE meat

Meat	*sRyR1* genotype		
	NN	*Nn*	*nn*
Normal (19)	19	0	0
PSE (43)	39	4	0

NN = Dominant homozygote; *Nn* = Recessive heterozygote; *nn* = Recessive homozygote.
Figure in parenthesis is the number of each sample.

It has been reported that pigs with the *Nn* genotype more frequently produce PSE meat than pigs with a normal genotype (Pommier and Houde, 1993; Cheah et al., 1995; Horiuchi et al., 1996). Our results also indicated that pigs with the *Nn* genotypes (9.3%) more frequently showed PSE meat than those with the *NN* genotype (0%), although this rate was much lower than the figure of 30% reported by Pommier and Houde (1993) and 20% reported by Horiuchi et al. (1996). This low rate was also thought to be due to the low prevalence of *sRyR1* mutation in the colony of pigs used in the present study.

Light microscopic observation demonstrated that PSE meats had irregular running and disorders of the muscle fibers, and that the inter-fiber width tended to be larger than in normal meats (Figure 2). Electron microscopy revealed disintegration, twisting, and disorder of the myofibril arrangement with elimination of the Z line in PSE meat, compared with normal meat (Figure 3). These findings were

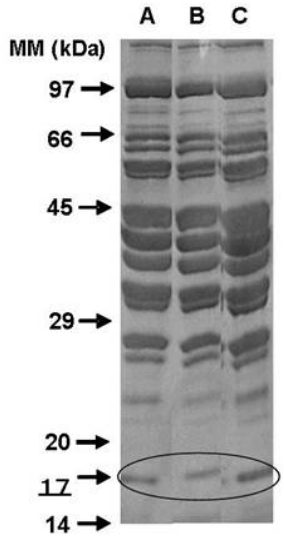

Figure 1. Myoglobin band (17 kDa) on SDS-PAGE gel in sarcoplasmic protein extracts from longissimus muscle. Supernatants after centrifugation of homogenates were applied in normal pork meat with dominant homozygote (lane A) for skeletal ryanodine receptor 1, PSE meat with dominant homozygote (Lane B) and PSE meat with heterozygote (Lane C). The band on the gel was visualized by CBB R-250. MM represents molecular mass.

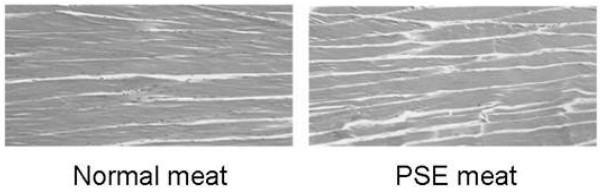

Figure 2. Light micrographs of porcine normal (left) and PSE (right) longissimus muscle stained with hematoxylin-eosin. PSE meat shows irregular running and disorder of the muscle fibers (×100).

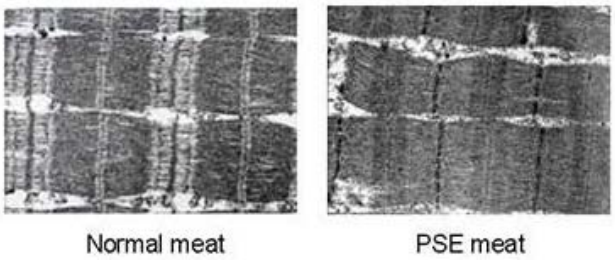

Figure 3. Electron micrographs of porcine normal (left) and PSE (right) longissimus muscle. PSE meat shows disintegration, twisting and disorder of the myofibril arrangement with elimination of the Z line (×12,500).

Table 2. Meat quality measurements on normal meat and PSE meat according to sRyR1 genotypes

Measurements	Normal meat (*NN*)[1]	PSE meat	
		NN	*Nn*
Water holding capacity (%)	79.3±1.3 (13)	74.3*±1.3 (30)	72.6*±2.0 (3)
Moisture (%)	72.3±1.3 (17)	70.5*±0.4 (36)	69.2*±0.4 (3)
Lightness (L*)	47.4±0.6 (17)	58.7*±0.7 (39)	59.3*±1.8 (4)
Redness (a*)	10.1±1.0 (17)	7.6*±0.3 (39)	12.0*±.9 (4)
Yellowness (b*)	10.1±0.4 (17)	11.8*±0.2 (39)	13.5**±0.6 (4)
Sarcoplasmic protein (mg/g)	65.3±3.8 (17)	40.7*±1.9 (39)	39.6*±7.5 (4)

Mean±SEM.

[1] *NN* = Normal genotype; *Nn* = Heterozygous genotype.

* Compared to normal meat ($p<0.05$). ** Compared to normal genotype (*NN*) ($p<0.05$).

Figure in parenthesis is the number of each sample.

similar to those described by Mircheva and Vitanov (1987). However, no remarkable differences were observed between the *NN* and *Nn* genotypes (data not shown).

Moisture and WHC were significantly lower in PSE meat than in normal meat (Table 2), reflecting the exudative condition that is one of the characteristics of PSE. However, the moisture and WHC values of PSE meat were not significantly different between the the *Nn* and the *NN* genotypes. The L* and b* values were significantly higher in PSE meat than in normal meat (Table 2), indicating the pale condition of the former. Meat with an L* value of >50 has previously been considered to have PSE (Joo et al., 1999; Ryu and Kim, 2006). All of our PSE meat samples also had an L* value of >50, but there were no significant differences between the *NN* and *Nn* genotypes (Table 2). On the other hand, the b* value was significantly higher in *Nn* genotype than in the *NN* genotype (Table 2). The a* value of PSE meat with the *NN* genotype was significantly lower than that of normal meat, as reported by Pommier and Houde (1993), whereas the a* value of PSE meat with the *Nn* genotype was significantly higher than that with the *NN* genotype, and that of PSE meat with the *Nn* genotype tended to be higher than that of normal meat with the *NN* genotype (Table 2).

The concentration of myoglobin in normal meat with the *NN* genotype, PSE meat with the *NN* genotype and PSE meat with the *Nn* genotype was 318.7±20.8 (mean±SEM, μg/g wet tissue), 247.3±5.3, and 398.0±63.5 (Figure 4A), respectively, thus reflecting the alteration of the a* value we observed (Table 2). These results suggest that the higher amount of myoglobin in skeletal muscle influences the higher a* value of PSE meat with the *Nn* genotype.

Skeletal muscle protein consists of sarcoplasmic (soluble) and myofibrillar (insoluble) proteins, and a lower concentration of sarcoplasmic proteins has been observed in PSE meat than in normal meat (Joo et al., 1999). In the present study, a lower protein concentration in the supernatant of muscle homogenate, mostly attributable to sarcoplasmic protein (Joo et al., 1999), was observed in PSE meat than in normal meat (Table 2). However, the amount of sarcoplasmic protein in the PSE meat was not affected by heterozygosity for the *sRyR1* (Table 2). On the

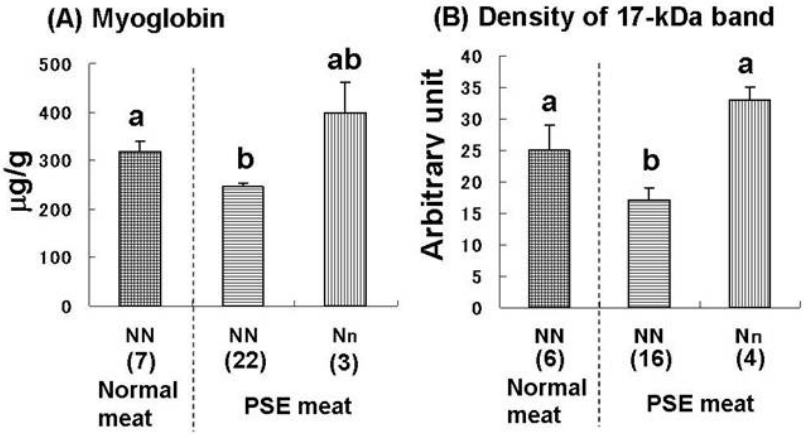

Figure 4. Concentration of myoglobin (A) and density of 17-kDa band (B) on SDS-PAGE gels in normal and PSE meat. NN and Nn indicate *sRyR1*-homozygote and heterozygote, respectively. Vertical bars represent the mean (±SEM) obtained from meat of different pigs (indicated by numbers in parenthesis). Means with different superscripts significantly ($p<0.05$) differ from each other.

other hand, the density of the 17-kDa protein (myoglobin) band on SDS-PAGE gels (Okumura et al., 2005) was 25.0±4.0 (mean±SEM, arbitrary unit) in normal meat with the *NN* genotype, 17.0±2.0 in PSE meat with the *NN* genotype, and 33.0±2.0 in PSE meat with *Nn* genotype (Figure 4B), indicating an alteration similar to that of the a* value (Table 2), as well as the amount of myoglobin (Figure 4A). These results have strongly suggested that the amount of myoglobin is larger in PSE meat with the *Nn* genotype than the *NN* genotype.

At a low pH, the heme group of myoglobin becomes labile and will dissociate into heme and globin (Antonini and Brunori, 1971). Klont and Lambooy (1995) found a higher myoglobin concentration in *nn* pigs than in *NN* pigs, but no significant difference between *NN* and *Nn* pigs. Wykle et al. (1978) observed that no differences in the banding patterns obtained by isoelectric focusing for porcine skeletal muscle myoglobin extracted from pigs with different halothane sensitivity, and Tam et al. (1998) reported that the amount of myoglobin in the *Nn* genotype did not differ from that in the *NN* genotype. However, those studies were done using apparently normal meat. On the basis of their results plus the present ones obtained using the PSE meat, it is thought that the degradation of sarcoplasmic proteins in both the *NN* and *Nn* genotypes is equally affected by progression of PSE after slaughter, whereas in the *Nn* genotype degradation of myoglobin is hardly affected. Therefore certain factor(s) might inhibit the degradation of myoglobin in the *Nn* genotype postmortem. It has been reported that the onset of PSE in meat is caused by excessive anaerobic glycolysis (excessive accumulation of lactic acid) (Solomon et al., 1998). However, there have been few studies of the *sRyR1* mutation in relation to myoglobin, which is a transporter of oxygen (Wykle et al., 1978; Tam et al., 1998). Further study of *sRyR1* mutation in relation to postmortem degradations of sarcoplasmic proteins including myoglobin will be required.

As described above, most researchers tend to agree that heterozygous pigs have intermediate meat quality between dominant and recessive homozygotes (Rosenvold and Andersen, 2003). However, biochemical or physiological reports about the differentiations between *sRyR1* heterozygote and homozygote have been limited (Weaver et al., 2000; Shen et al., 2007). It has been reported that pigs with *sRyR1* heterozygote were different from genetically normal pigs in hypothalamic-pituitary-adrenal axis function (Weaver et al., 2000) and adenosine monophosphate-activated protein kinase activity (Shen et al., 2007), of which high activity is thought to be the cause of incidence of PSE (Barbut et al., 2008). It seems important to investigate the relation between high myoglobin content in this study and above reported biochemical studies.

In conclusion, comparison of the quality of porcine PSE meat between the *NN* and *Nn* genotypes for *sRyR1* revealed higher values of redness (a*) and yellowness (b*) in the *Nn* genotype than in the *NN* genotype, and no differences in WHC, moisture or lightness (L*) of meat color. The higher a* value in the *Nn* genotype was dependent on the amount of myoglobin. These results suggest that *sRyR1*-heterozygous pork meat showing PSE contains much more myoglobin than genetically normal PSE meat. These data might contribute to understanding of the genesis of PSE meat.

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