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Cloning and Expression Analysis of the α-Subunit of Porcine Prolyl 4-hydroxylase*

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ABSTRACT : Prolyl 4-hydroxylase (P4H) plays a central role in collagen synthesis by catalyzing the hydroxylation of the proline residue in the X-Pro-Gly amino acid sequence, and controls the biosynthesis of collagen that influences overall meat quality. In order to verify expression level of the catalytic α subunit of P4H, a 2.7 kb clone of the α-subunit gene for P4H was selected from a cDNA library prepared from the muscular tissue of *Sancheong berkshire* pigs, and the whole gene sequence was determined. As expression level of the α-subunit of P4H differed between tissues of pigs, we intended to assess more precisely the level of α-subunit expression between tissues of Sancheong Berkshire pigs by using RT-PCR. Muscular and adipose tissues were taken from each pig grouped by growth stage (weighing 60, 80, and 110 kg) of Yorkshire and Sancheong Berkshire pigs, and the expression levels of the α-subunit of P4H were examined. Since there were significant differences in the expression level with respect to variation in growth stage ($p<0.01$), an attempt was made to identify any influences of pig species and tissue variation. The muscular and adipose tissues of pigs weighing 110 kg showed higher expression levels than pigs weighing 60 kg and 80 kg. In general, significantly higher expression levels were found in muscular than in adipose tissue. The expression levels in Sancheong Berkshire were significantly higher than in Yorkshire pigs ($p<0.01$ or $p<0.05$). Since expression level of the α-subunit of P4H affects the activity of P4H and is connected to the biosynthesis of collagen and increased collagen can improve meat texture, this finding may explain why meat quality of the Sancheong Berkshire pig is acclaimed in Korea. Given the higher expression levels of the α-subunit gene in adipose than in muscular tissue, and also in the heavier pigs, more intensive studies are required to assess the correlation between expression level of the α-subunit gene and overall meat quality. (**Key Words :** Prolyl 4-hydroxylase, Meat Quality, Muscle Collagen Synthesis)

INTRODUCTION

Prolyl 4-hydroxylase catalyzes the hydroxylation of proline residues in repeating X-Pro-Gly triplets, thus forming the 4-hydroxyproline required for the correct folding of newly synthesized collagen (Kivirikko and Myllyharju, 1998; Kivirikko and Pihlajaniemi, 1998; Myllyharju, 2003). Also, it has been reported that concentration of the α-subunit of P4H was correlated with cell density and had a positive effect upon collagen formation (Rowe and Schwarz, 1983; Kao et al., 1985). Contrary to several reports showing that mRNA of the α subunit increases in proportion to cell density (Bassuk and Berg, 1991), another report claimed that there was no correlation between the expression of the α subunit and cell density (Lee et al., 2001). Controversy still remains whether this discrepancy may imply complexity in the regulation of the α subunit gene of P4H.

The α-subunit of human P4H consists of 517 amino acid residues and is synthesized in a form containing a signal sequence of 17 residues (Helaakoski, 1989). The α-subunit is believed to be a major component of the catalytic site of the enzyme tetramer. Regulation of the amount of active prolyl 4-hydroxylase tetramer appears to be linked to synthesis of the α-subunit (Kivirkko et al., 1992). Although, the mechanism by which the α-subunit regulates amounts of the holoenzyme is currently unknown, it would be in conjunction with changes in the rate of collagen synthesis. It is known that the core structure of the α-subunit of P4H

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Table 1. Primer sequences for PCR and sequencing

Primer	Sequence	Tm (°C)
P4H-1	5'-CGGCTTGACTGAAGGCGGTCGAG-3'	55
P4H-2	5'-GGGGCAGCCAAAGCTCTGTTGCG-3'	55
P4H-3	5'-GCTGGATCTGCCTGACGAGTAG-3'	55
P4H-4	5'-CACCTCTGCTGTATGCAGCAGG-3'	55
P4H-F	5'-TGGGGAGGGTATCAAAATGA-3'	55
P4H-R	5'-AGCTCCTTGAAAGCATCTGG -3'	55
18S-F	5'-CTCGATGCTCTTAGCTGGAGT-3'	55
18S-R	5'-CTAGTTAGCATGCCGAGAGT-3'	55

plays an important role in collagen synthesis.

In this study, it was presumed that the expression of the α-subunit of P4H involved in collagen biosynthesis might be related to growth phase and/or meat quality of pigs. It is known that collagen is a key factor for the elasticity of skin and muscle of mammals, and its adhesive property is responsible for linking of cells, which is related to the firmness of pig muscle. Therefore, we conducted cDNA microarray analysis to examine the global pattern of gene expression in tissues of Sancheong Berkshire pigs (Kim et al., 2005). Then, we attempted to determine differences in expression level of the α-subunit of P4H between each tissue of the pig in order to clarify whether differential expression could be correlated with tissue specificity and sub-speciation of pigs.

MATERIALS AND METHODS

cDNA library construction and microarray analysis

Construction of the cDNA library and analysis of cDNA microarrays were carried out as described in the previous report (Kim et al., 2005). Briefly, we performed microarrays with the slide spotted with total 4,434 ESTs and then scanned by Scanarray 4000XL (Packard Bioscience company, US) with analysis software (ScanArry version 3.1 and QuantArry version 3.0). The microarray analysis was conducted in triplicate with the Cy3-dCTP-labeled probe for fat and the Cy5-dCTP-labeled muscle probe. Then, we selected the clone for P4H from the genes showing more than 2-fold difference in expression between the fat and muscle tissues (mean values of Cy3/Cy5 ratio for the P4H gene = 2.297±0.249; for internal positive control = 1.126±0.049).

Sampling of pig tissues

Sancheong Berkshire (from Sungchuk Farm, the lineage formed with pigs from Kagoshima) and Yorkshire (GaYa Stockbreeding Ltd) female pigs whose body weight reached 60, 80, and 110 kg were butchered, and their tissues were immediately taken, soaked in liquid nitrogen, and kept in a freezer at -80°C until RNA isolation.

Total RNA preparation

Total RNA was extracted with TRIzol reagent according to the manufacturer's protocol (Life Technologies, Invitrogen). Trizol reagent (2 ml) was added to 0.1-0.2 g of tissue, ground and the concentration of total RNA was measured by absorbance, then confirmed by electrophoresis in 1.5% formamide gel.

Cloning of the α-subunit of P4H

The α-subunit of P4H gene was cloned into pBluescript vector using a cDNA library prepared with a cDNA Library Kit (Invitrogen, USA). Transformation of *Escherichia coli* XL1-blue cells was done by the protocol of Mendel and Higa (1970). Plasmid Mini-prep Kit (QIAGEN, USA) was used for isolation of the recombinant plasmid, which was followed by digestion with *Xho* I and *Eco*RI and determination of the expected insert on a 1% agarose gel by electrophoresis.

DNA sequence analysis

The sequence of the cloned DNA was determined using a ABI PRISMR BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit. The primers used for PCR and sequencing the gene are listed in Table 1. The DNA sample for sequencing was purified with DNA Cleanup Kit (Montage^{TM} SEQ_{96}, MILLIPORE, USA). Automated sequencing of the purified PCR products was performed by an Applied Biosystems DNA sequencer (Applied Biosystems, Model 3100, USA). The DNA sequence analysis was done using BioEdit software.

Reverse transcription (RT) PCR

For RT-PCR, first strand cDNA was synthesized using Superscript II Reverse Transcriptase, according to the manufacturer's protocol (Invitrogen, USA). In brief, 1 μl of reverse transcription mixture (cDNA) was added to the PCR mixture consisting of 1.5 μl 10×PCR-buffer, 1 μl 15 mM $MgCl_2$, 1.2 μl 2.5 mM dNTPs (Promega, USA), 1 μl of each primer (10 pmol), 0.2 μl Taq-DNA-Polymerase (Promega, USA), and 9.2 μl DEPC-H_2O. PCR was conducted under the following conditions: the first segment at 95°C for 3 min, 30 cycles (94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min), and the final segment at 72°C for 10 min. A set of negative controls was also included except for

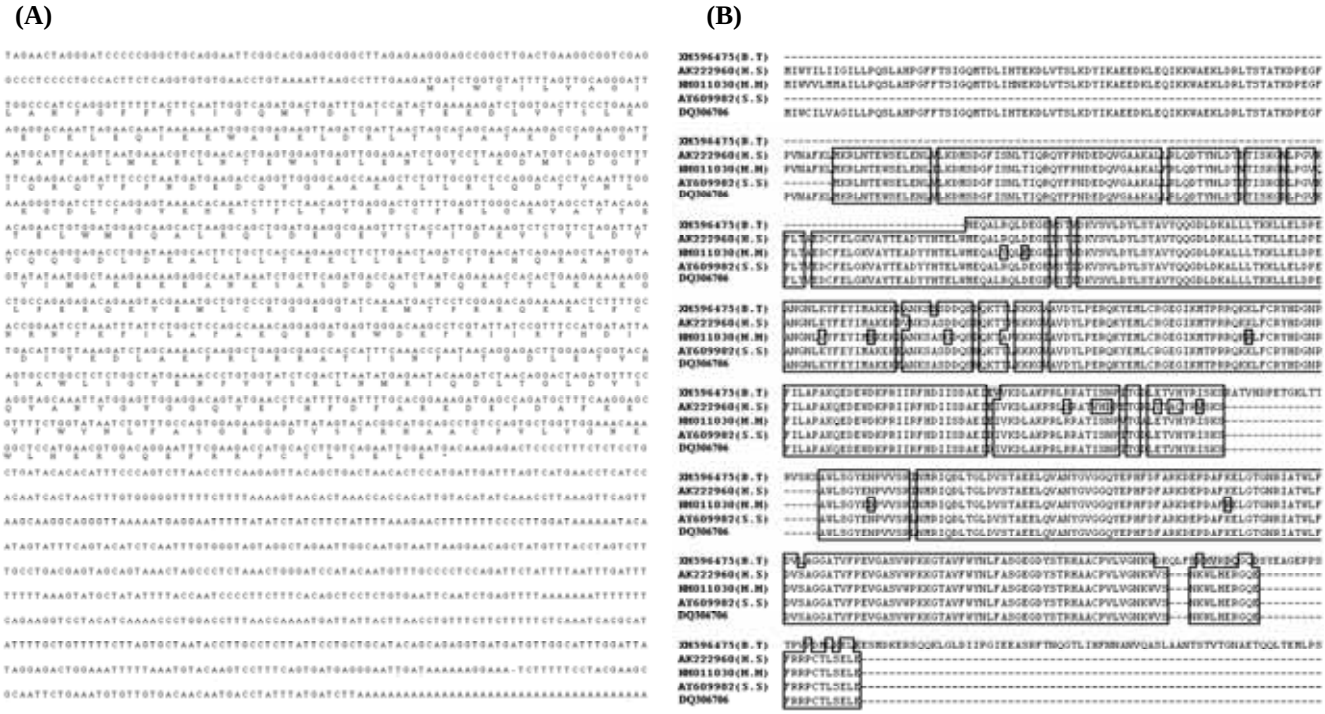

Figure 1. (A) Nucleotide and deduced amino acid sequence of the α-subunit of the P4H gene. The nucleotide sequence of the 2,755 bp cDNA clone contained a coding region (532 amino acid) and 3'-untranslated region. (B) Alignment of the deduced amino acid sequence of the α-subunit of P4H with different species of mammals. XM_596475 (Acc. no. of *Bos taurus*), AY609982 (Acc. no. of *Sus scrofa*), AK222960 (Acc. no. of *Homo sapiens*), NM_011030 (Acc. no. of *Mus musculus*), and DQ306706 (the α-subunit of P4H of the Sancheong Berkshire pig). The deduced amino acid sequence of DQ306706 (the α-subunit of P4H of the Sancheong Berkshire pig) shared 88% identify with those of the human and rat.

the reverse transcription reaction. For internal control when assessing relative amounts of target gene from different tissue samples, we used 18S rRNA gene (acc. no. AF102857) for the comparison (Table 1). RT-PCR products (649 bp) were separated on a 1% TAE agarose gel and visualized by UV after ethidium bromide staining. Primer design was based on the sequence obtained from Sancheong Berkshire pig used in this study (GenBank accession no. DQ306706). The gene amplification for the α subunit of P4H was achieved by using PCR with specific primers, P4H-1 and P4H-2 (Table 1).

Statistical analysis

The data are presented as the mean±SEM of 18 animals (Sancheong Berkshire and Yorkshire pigs at 60, 80, and 110 kg body weight, respectively) prepared by the same protocol. With each tissue, the assessment of expression level was conducted by RT-PCR. In order to identify significance of the difference in expression levels, ANOVA analysis of variance was performed by means of the SAS program, and the significance was verified with Duncan's multiple range test. The linear model used was as follows: $Y_{ijkl} = \mu+G_i+Tj+Sk+GT_ij+GS_ik+TSjk+GTS_ijk+\varepsilon_ijkl$, where μ = Overall mean; G_i = Effect of Growth-stage; i = 60, 80, 110 kg; T_j = Effect of Tissues; j = muscle, fat; S_k = Effect of Species; k = Yorkshire, Berkshire; GT_{ij} = Effect of i^{th} Growth-stage and j^{th} Tissue; GS_{ik} = Effect of i^{th} Growth-stage and k^{th} Species; TS_{jk} = Effect of j^{th} Tissue and k^{th} Species; GTS_{ijk} = Effect of i^{th} Growth-stage, j^{th} Tissue, and k^{th} Species; E_{ijkl} = The random residual effect.

RESULTS

The 2.7 kb full-length sequence of the gene encoding the α-subunit of P4H was determined using the primers listed in Table 1 (Figure 1A). The determined sequence from Sancheong Berkshire pigs (accession no. DQ306706) was nearly identical to the previously reported sequence (accession no. AY609982) of the α-subunit of P4H, except for some variations in the UTR region in which there were 7 nucleotide changes as shown in Table 2. These sequence variations could reflect sub-species differences of the pigs used. In comparison with other mammals, relatively low amino acid sequence homology (46%) was found with *Bos taurus* (accession no. XM596475). However, the homology was high in comparison with the human (accession no. AK222960, 96%) and rat (accession no. NM_011030, 94%) (Figure 1B).

Table 2. Nucleotide changes in the DNA sequence of the cloned α-subunit gene of P4H from Sancheong Berkshire pigs (accession no. DQ306706), compared with the previously reported gene of *Sus scrofa* (acc. no. AY609982)

Nucleotide no.[Δ]	DQ306706	AY609982	Change
212	-	G	Deletion
230	T	G	Transition
1838	-	T	Deletion
2389	-	G	Deletion
2392	-	G	Deletion
2393	A	G	Transition
2394	A	G	Transition

[Δ] denotes the number of nucleotides in Sancheong Berkshire pigs (accession no. DQ306706).

The expression levels of the α-subunit of P4H were determined with the muscular and adipose tissues at each growth stage (weighing 60, 80, and 110 kg) obtained from Sancheong Berkshire pigs (Figure 2). It was assumed that the content of α-subunit of P4H increases in proportion to physical activity and muscular mass. As revealed in this experiment, the highest expression level was observed in pigs weighing 110 kg, while the lowest expression level was observed in pigs weighing 80 kg rather than in pigs weighing 60 kg. This relationship was also seen in both muscular and adipose tissues.

According to a previous report regarding the quality of meat (Tadayosi, 2003), Sancheong Berkshire pigs used in this study have been recognized for their better meat quality in Korea. To clarify the correlation between the expression level of the α-subunit gene of P4H and pig speciation, the expression level in Yorkshire pigs was analyzed using the same procedure as mentioned earlier. Results with muscular tissue showed that there was no distinctive variation in expression levels between the samples taken from pigs weighing 60 kg and 110 kg. However, unlike Sancheong Berkshire pigs, the highest expression level was observed in pigs weighing 60 kg, whereas the lowest expression level was seen in pigs weighing 80 kg which was similar to that of Sancheong Berkshire pigs (Figure 3). With adipose tissue, the lowest expression level was observed in pigs weighing 80 kg, and the highest expression level was observed in pigs weighing 110 kg, similarly to that of Sancheong Berkshire pigs.

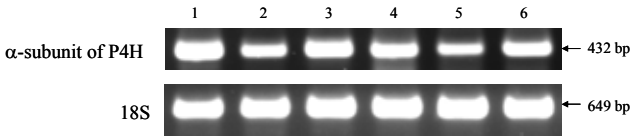

Figure 2. Expression levels of the α-subunit of P4H mRNA in muscle and fat tissues of Sancheong Berkshire pigs at various body weights. Lanes 1; 60 kg muscle, 2; 80 kg muscle, 3; 110 kg muscle, 4; 60 kg fat, 5; 80 kg fat, 6; 110 kg fat.

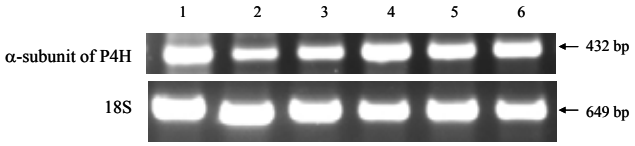

Figure 3. Expression levels of the α-subunit of P4H mRNA in muscle and fat tissues of Yorkshire pigs at various body weights. Lanes 1; 60 kg muscle, 2; 80 kg muscle, 3; 110 kg muscle, 4; 60 kg fat, 5; 80 kg fat, 6; 110 kg fat.

With respect to the sub-speciation of pigs, it was found that the expression levels of the α-subunit of P4H were higher in adipose than in muscular tissue. The results showed a higher level of expression in the adipose tissue of pigs weighing 110 kg, which suggests that level of expression of the α-subunit of P4H is closely associated with the growth stage of pigs, regardless of choice of tissues.

A statistical analysis was carried out to clarify the relationship between level of expression and variations in the species, growth stage and tissue parameters (Table 3), with an attempt to verify the significance of the interaction between two or more variations in the sample. Accordingly, the results in Figure 4 suggested statistical significance for the interaction between growth stage and type of tissue sample ($p<0.01$). The highest expression level appeared in adipose tissue of pigs weighing 110 kg, whereas much lower level of expression was observed in muscular tissue, regardless of growth stage.

The result shown in Figure 4(B) suggests that the influence of pig species is a more significant determinant of the level of expression than the other variables ($p<0.01$).

Table 3. Statistical analysis of gene expression level and source of samples (growth-stage, tissue, species)

Source (variable)	DF	Anova SS	Mean square	F value
Growth-stage	2	3.89	1.94	60.74**
Tissue	1	17.50	17.50	546.85**
Species	1	5.31	5.31	166.06**
Growth-stage×tissue	2	0.91	0.45	14.17**
Growth-stage×species	2	0.45	0.22	6.99**
Tissue×species	1	0.16	0.16	5.07*
Growth-stage×tissue×species	2	0.07	0.04	1.14
Error	24	0.77	0.03	
Corrected total	35	29.057		

* $p<0.05$, ** $p<0.01$.

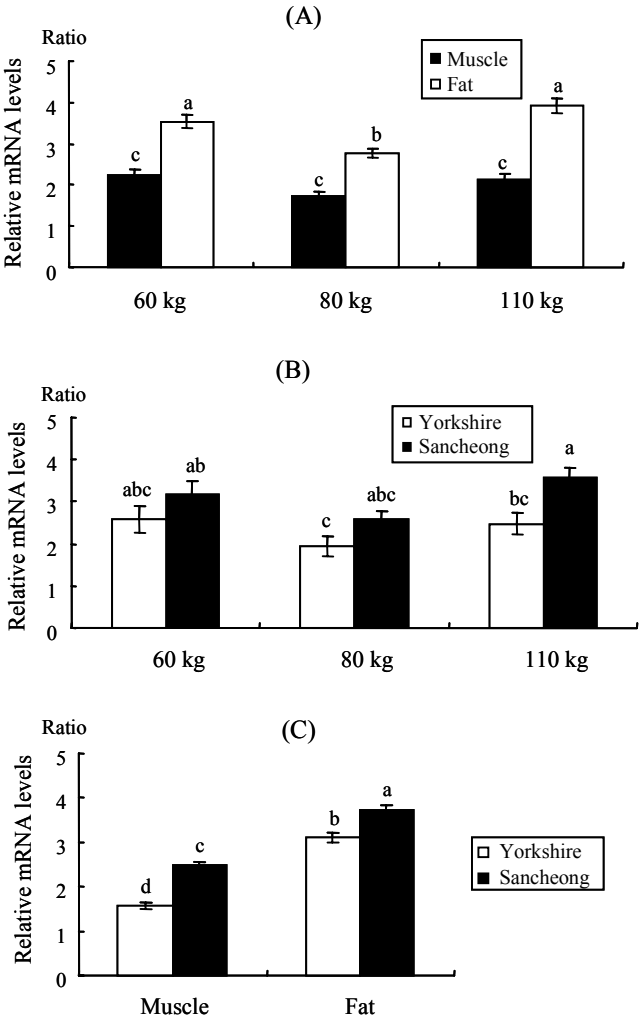

Figure 4. The relative mRNA levels of the α-subunit genes of P4H in considering interactions between variables of pig samples. (A) growth-stage and tissues, (B) growth-stage and species, and (C) species and tissues. Data were expressed as mean±SE. The lower case letters above the same bars indicate statistical significance (A and B; $p<0.01$, C; $p<0.05$).

Sancheong Berkshire pigs exhibited much higher expression levels in tissues than Yorkshire pigs, but differences in the expression levels according to growth stage of the two species were not significant. However, it was noted that the expression level in the Yorkshire pigs weighing 80 kg was significantly lower than at the other weights. The result shown in Figure 4(C) indicated that there was a significant correlation between pig species and tissue sample ($p<0.05$).

From the pigs weighing 110 kg, the growth stage at which expression level of the α-subunit gene of P4H reached the highest point, brain, fat, muscle, bonded rib, heart, and liver tissues were taken for examination of expression level (Figure 5). The results showed that the highest expression level was observed in fat, followed by boned rib where muscular and adipose tissues coexist, then

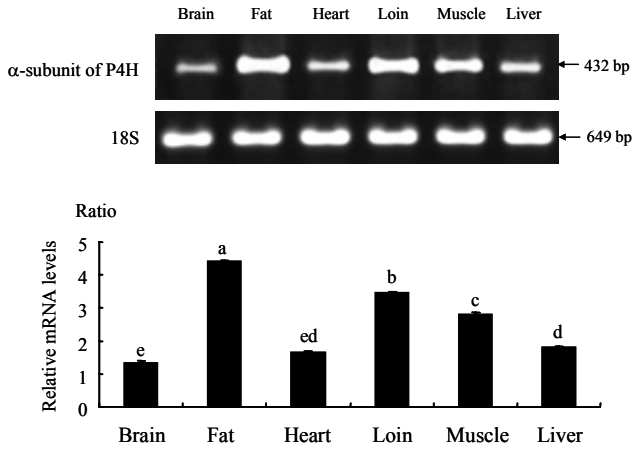

Figure 5. The relative mRNA levels of the α-subunit genes of P4H in various tissues of Sancheong Berkshire pigs at the growth stage of 110 kg body weight. [a] P-[e] P indicate significant difference compared with control at $p<0.01$.

in muscle, liver, heart, and brain, in order.

DISCUSSION

We cloned the gene encoding the α-subunit of P4H from the cDNA library of Sancheong Berkshire pigs and examined differential expression of the α subunit of P4H in various tissues using microarray analysis (Kim et al., 2005). It is known that the α subunit of P4H contributes to collagen synthesis and supports muscular growth and regeneration after trauma (Mayne, 1982; Han, 1999). In this study, we used an approach to analyze expression pattern of the target gene in pig tissues to clarify the correlation between level of expression of the α subunit of P4H and meat quality.

For the comparison of the cloned α-subunit of P4H with the human counterpart (Helaakoski, 1989), a complete amino acid sequence was determined. Also, the pig clone was compared to that of a previous report (Jorgensen, 2005). However, we attempted to expand our study into expression level of the gene in the various pig tissues and tried to correlate the level of expression with overall meat quality. Because there were variations for the sample preparation, which were attributed to the growth stages, choice of tissues and pig species, the levels of expression of the α-subunit gene of the P4H enzyme were compared with each other to verify any possible correlations between them (Table 3; Figure 4).

The level of expression of the α-subunit of P4H was significantly higher when the pig weight reached 110 kg, indicating that the collagen content would be higher at this growth stage. At the growth stage corresponding with 110 kg body weight, the increase of muscle mass is evident along with the higher mRNA transcription of the α-subunit,

as suggested in this study. Therefore, it is suggested that pigs weighing 110 kg through 130 kg might produce the best meat quality possible. Also, the expression level (α-subunit of P4H) was shown to be higher in adipose than muscular tissue, giving an indication that the presence of an appropriate amount of adipose tissue in the meat will contribute to overall meat quality.

Since the collagen-mediated adhesion involves linking of cells, water holding capacity and affording bony tissue flexibility (Kivirikko, 1992 and 1998; Johnstone, 2000), the amount of collagen expressed in the tissue is highly correlated with the firmness of meat. In this regard, the level of expression of the α-subunit is an important determinant in the initiation of collagen biosynthesis. To clarify the difference in the expression levels of the α-subunit gene from Sancheong Berkshire pigs, Yorkshire pigs were used for comparison of the expression profiles. Collectively, the expression levels were higher in the adipose tissue than those in the muscular tissue, and were higher in Sancheong Berkshire than in Yorkshire pigs. With respect to meat quality, Sancheong Berkshire pigs have been acclaimed in Korea, which might be related to relatively high expression level of the α-subunit gene. In addition, the adipose tissue taken from pigs weighing 110 kg showed the highest expression level, compared with variations in the growth stage and tissues. However, we cannot rule out possible environmental influence on differences in expression level of the α-subunit gene, because the two pig species were housed in different farms (Sancheong Berkshire: Sungchuk Farm, Yorkshire: GaYa Stockbreeding Ltd), although the breeding condition of the two pig species was similar.

The lower the content of α-subunit of P4H, the lower are the activities of the entire enzymes of P4H. In this respect, it is considered that the α-subunit of P4H plays an important role in activating the P4H holoenzyme. According to the results of the study with mice performed by Han et al. (1989), the synthesis of fibrillar collagen-which is a fibrous protein belonging to proteins forming the muscular tissue-decreased with reduced physical activity of mice. As pigs with high physical activity require much higher content of fibrillar collagen, it is expected that a higher level of α-subunit expression would occur in pigs at the growth stage showing increased physical activity. Therefore, it can be concluded that collagen biosynthesis increases in pigs weighing 110 kg which showed the highest expression level of α-subunit of P4H.

We attempted to assess the differential level of α-subunit expression in pigs and also tried to relate the level of expression of the α-subunit with overall meat quality. In addition, we would like to continue to analyze genes related to the meat quality of pigs using various approaches, by which we want to establish a relationship between gene expression and pork quality in order to produce better quality pigs.

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