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Full Length Research Paper

Comparative proteomic analysis of the effects of dietary mugwort (*Artemisia iwayomogi* Kitamura) on the pig *Longissimus dorsi* muscle

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Proteomic analysis by 2-dimensional electrophoresis (2-DE) of the *longissimus dorsi* muscle in finishing pigs (LY × D) grown on a diet supplemented with mugwort powder found roughly 300 spots on a polyacrylamide gel image. Among them, the expression levels of 12 spots were higher than those of the control group. From the results of peptide mass fingerprinting (PMF) analysis, nine proteins were characterized: myosin light chain 2V, eIF-5A, myosin light chain 1, F1-ATPase chain D, peroxiredoxin-2, Kelch-related protein 1, SLA-7, glycine amidinotransferase, and Tpi 1. The other three spots with increased expression were identified by chemically assisted fragmentation-matrix-assisted laser desorption/ionization (CAF-MALDI) sequencing as serum albumin precursor and two different types of myoglobin. Taken together, these results indicate that mugwort powder has the potential to improve the quality of pork meat.

Key words: 2-DE, *longissimus dorsi* muscle, meat quality, mugwort, pig, proteome.

INTRODUCTION

Because proteins play critical roles in transport, immune responses, storage, and structure, and serve as members of metabolic pathways, they are essential factors for the maintenance of life of all organisms. Thus, proteomic tools were developed to investigate overall protein expression patterns (Anderson and Anderson, 1998; Blackstock and Weir, 1999; Wilkins et al., 1996). From 2-DE-based-proteomic analysis of proteins from white and red pork muscles, about 500 spots were obtained, and five spots were observed with different expression patterns including myoglobin, two slow-twitch isoforms of myosin light chain, and two small heat shock proteins (Kim et al., 2004).

Oriental medicines have antibiotic, anti-cancer, anti-oxidant, and physiological activation properties, and there is a trend towards their increased utilization as feed additives because of the potential for improving intestinal microbiota as well as pig meat quality. In particular, mugwort grows naturally everywhere in South Korea, so it may be an invaluable feed additive. The major components of mugwort are alkaloids, essential oils, vitamins, and various minerals (Kang et al., 1995), and its pharmacological properties include anti-oxidative (Lee et al., 1992; Sim et al., 1992), anti-cancer (Lim and Lee,

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1997; Sun et al., 1992), and liver protective activities (Gilanti et al., 2005). Pigs fed mugwort powder improved their average weight gain, protein content, and the lightness of their meat and fat (Kim et al., 2008).

The improvement of pig meat quality through a mugwort powder diet should be accompanied by altered expression of the relevant genes. Therefore, this study used comparative 2-DE to investigate global changes in protein expression in *longissimus dorsi* muscle associated with dietary mugwort supplementation.

MATERIALS AND METHODS

Experimental animals and sample harvesting

Experimental diets included a control diet (C group), and a diet supplemented with 1.5% (w/w) mugwort powder as a diet additive (T group). Among 20 selected growing landrace (LYxD) pigs of body weight 75 ± 4 kg, half were grown for 50 days on the basal diet, whereas the other half was grown on the mugwort powder diet. *Longissimus dorsi* muscle tissues of the carcass were harvested from four pigs that exhibited good breeding in each group, and these were then used for the experiment.

Protein extraction from *Longissimus dorsi* muscle

The harvested *Longissimus dorsi* muscle tissues were sonicated for 10 s with a Sonoplus (Bandelin electronic, Germany) and were then directly homogenized by a motor-driven homogenizer (PowerGen125, Fisher Scientific) in sample lysis solution composed of 7 M urea, 2 M thiourea, 4% (w/v) 3 - [(3 -cholamidopropy) dimethyammonio]- 1 - propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), 2% (v/v) pharmalyte, and 1 mM benzamidine. After the homogenized product was centrifuged at 15,000 × g for 1 h at 15°C, the precipitated insoluble material was discarded, and a soluble supernatant fraction was collected and used for 2-DE. Protein quantity for sample loading was normalized by Bradford assay (Bradford, 1976).

Protein separation via 2-DE and analysis of gel images

Immobilized pH gradient (IPG) dry strips were equilibrated for 12 to 16 h with equilibration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, and 1% pharmalyte), and 1.1 mg of each sample was loaded onto a strip fixed in a strip module. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences) according to the manufacturer's instruction. The voltage for IEF was linearly increased from 150 to 3,500 V over 3 h for sample entry into the gel followed by constant 3,500 V for complete focusing until 96 kV/h. Prior to electrophoresis in the second dimension, the treated strips were incubated for 10 min each in equilibration buffer (50 mM Tris-Cl, pH 6.8, 6 M urea, 2% SDS and 30% glycerol) plus 1% DTT and then equilibration buffer plus 2.5% iodoacetamide. The equilibrated strips were inserted into SDS-PAGE gels (20 × 24 cm, 10 - 16%). Protein separation via SDS-PAGE was performed with a Hoefer DALT 2D system (Amersham Biosciences) according to the manufacturer's instructions. 2-DE gels were run for 1,700 V/h at 20°C and then were stained with Coomassie brilliant blue G250 solution as described by Anderson et al. (1991). Colloidal CBB staining of 2-DE gels was used to delicately analyze the protein spots, and the solution was prepared with 177 ml 85% phosphoric acid, 150 g ammonium sulfate, 1. 8 g CBB-G 250, adjusted to 1,200 ml with water, and then finally adjusted to 1,500 ml with methanol.

Quantitative analysis of digitized gel images was carried out using the PDQuest software (version 7.0, Bio-Rad) according to the protocols provided by the manufacturer. The quantity of each spot was normalized by total valid spot intensity. Protein spots showing significant expression variation were selected as spots to exhibit critical changes between the control and treated groups.

Protein identification via PMF and CAF-MALDI sequencing

Protein identification via PMF was done as follows: protein spots were enzymatically digested with modified porcine trypsin in a manner similar to the method previously described by Shevchenko et al. (1996). Target spots were cut from separated gels by 2-DE. The gel pieces were washed with 50% acetonitrile to remove SDS, salt, and staining dye, dried to remove solvent, rehydrated with trypsin solution (8 to 10 ng/µL), and then incubated for 8 to 10 h at 37°C. The proteolytic reaction was terminated by addition of 5 µl 0.5% trifluoroacetic acid (TFA). Tryptic peptides were recovered by combining the aqueous phases obtained from several extractions of gel pieces with 50% aqueous acetonitrile. After concentration, the peptide mixture was desalted using C_{18}ZipTips (Millipore), and peptides were eluted in 1 to 5 µlacetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and 1 µl of the mixture was spotted onto a target plate.

Protein analysis via PMF was performed by an Ettan matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF; Amersham Biosciences). Peptides were evaporated with an N_2 laser at 337 nm, and a delayed extraction approach was used. They were accelerated with a 20-kV injection pulse for time of flight analysis. Each spectrum is the cumulative average of 300 laser shots. The search program profound, which was developed by the Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification by PMF. Spectra were calibrated with trypsin auto-digestion ion peaks m/z 842.510 and 2211.1046 as internal standards.

CAF-MALDI sequencing was done by the following method described here briefly. Protein digestion was performed by the same method as with PMF described above. 4-sulfophenyl-isothiocyanate (SPITC, Aldrich) reaction was done by the procedure of Dongxia et al. (2004). A total of 10 mg/ml (final) SPITC was dissolved in 20 mM $NaHCO_3$, pH 9.5 and then treated with a half volume of SPITC solution versus one volume of trypsin-digested solution. The reaction continued for 30 min at 55°C and was then terminated by adding 1 µl 5% TFA. The subsequent procedures were performed via the same method as was used for PMF.

Statistical analysis

Statistical analysis was done by general linear model (GLM) method of the SAS Program (Statistics Analytical System, USA, 1999). Duncan's multiple range tests was used to compare the differences between the means of the investigated samples. The results of the statistical analysis are presented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Changes in protein expression patterns by mugwort powder

Mugwort powder contains various physiologically active materials, and the tissue of animals fed with the powder

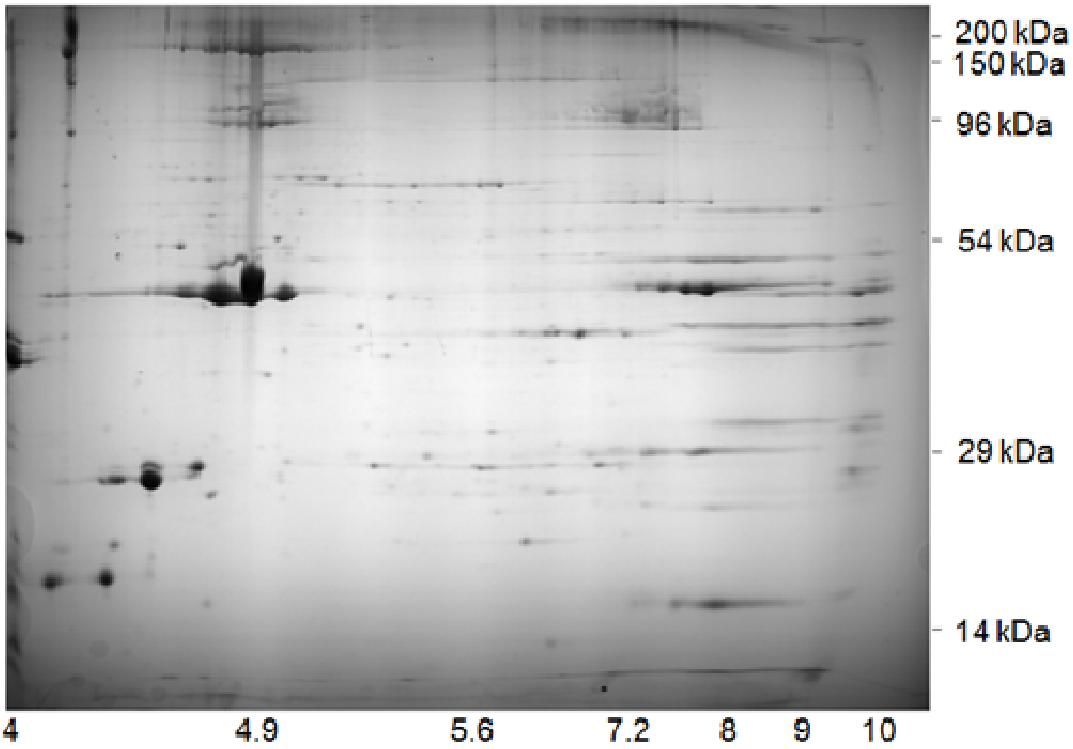

Figure 1. Full 2-DE gel image of proteins from the *Longissimus dorsi* muscle of pigs fed with mugwort powder. The 1st and 2nd gel runs were done by isoelectric focusing and SDS-PAGE, respectively. Numerals at the bottom indicate pI values, and kDa values at right side indicate the molecular weights of the separated proteins.

is capable of undergoing changes in the expression of various proteins in response to the materials. Therefore, in order to investigate the patterns of protein expression associated with a mugwort powder diet, 2-DE analysis was performed using 1.1 mg protein extracted from pig *l. dorsi* muscle, which is one of the most important meat cuts for human beings. After 2-DE separation of the proteins, quantitative Colloidal CBB staining revealed roughly 300 spots (Figure 1). The 2-DEs originating from the two different groups (C group, a basal diet and T group; a basal diet supplemented with 1.5% mugwort powder) were used to analyze the protein expression patterns associated with mugwort. A spot with different densities was selected through analysis of the gel images as a protein to show expression changes between groups. However, the spot selected as a protein showing an expression change was used only to show regular density changes of 2 to 3 spots among the four spots obtained per group.

Although most spots were of similar density between the C and T groups, 12 spots showed different expression levels, and all the spots were of greater density in the T group (Figure 2). The spots with increased expression were described by mean intensity, as shown in Table 1. The density of spot 7002 dramatically increased by 4.66-fold, while spots 1004, 5001 and 8002 increased more moderately by 2.51, 2.73 and 2.57 fold, respectively. The densities of spots 1106, 2001, 5002, 5602 and 7102 were marginally increased by 1.35, 1.41, 1.64, 1.69 and 1.60 fold, respectively.

Properties of the proteins identified by PMF

The spots with higher expression levels identified by PMF are shown in Table 2. Myosin light chain 2V and eukaryotic translation initiator factor 5A (eIF-5A) were obtained with 61 and 58% sequence coverage, respectively. Myosin light polypeptide 3, F1-ATPase, peroxiredoxin-2, Kelch-related protein 1, MHC class 1 antigen 7 (SLA-7), glycine amidinotransferase, and triosephosphate isomerase (Tpi 1; http://www.ncbi.nlm.nih.gov/protein /38512111) were obtained with sequence coverage below 44%. When compared with known proteins, the proteins discovered by PMF were highly likely to be correctly allocated because of their isoelectric point (pI value), their 2-DE position, and their molecular weight.

Myosin is the most abundant contraction molecule in mammalian skeletal muscle, and it relates directly to the quality of pork meat. Although the heavy chain of myosin has been studied with respect to skeletal pork meat

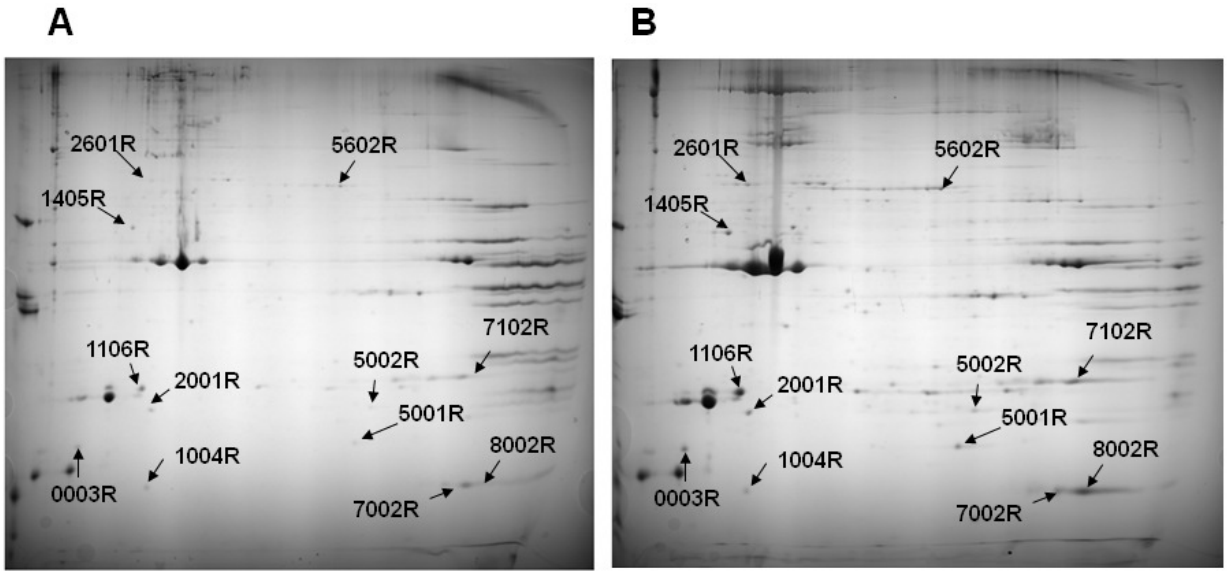

Figure 2. Comparative analysis of protein expression patterns with and without dietary mugwort powder. (A) and (B) indicate protein patterns of pig *longissimus dorsi* muscle grown on diets with and without mugwort. The enhanced spots are marked by numerals.

Table 1. Proteins from pig *l. dorsi* muscle exhibiting changes in expression due to dietary mugwort powder.

SSP[1)]	MW[2)]	PI[3)]	Mean intensity of C[4)]	Mean intensity of T[5)]	Fold variation: T versus C
3	19.69	4.34	540 ± 235[6)]	1055 ± 519	1.95
1004	15.58	4.68	309 ± 116	774 ± 366	2.51
1106	27.06	4.64	3292 ± 733	4442 ± 1773	1.35
1405	55.91	4.58	569 ± 161	1020 ± 765	1.79
2001	24.12	4.69	817 ± 128	1153 ± 521	1.41
2601	78.37	4.69	295 ± 153	574 ± 450	1.95
5001	19.98	6.23	357 ± 331	973 ± 825	2.73
5002	24.42	6.48	309 ± 417	508 ± 256	1.64
5602	76.14	5.98	551 ± 256	933 ± 530	1.69
7002	15.67	7.69	316 ± 65	1472 ± 1475	4.66
7102	28.79	7.95	2930 ± 73	4692 ± 889	1.60
8002	15.53	8.10	2550 ± 44	6559 ± 2333	2.57

[1)]SSP; standard spot protein, [2)]MW; molecular weight in kDa, [3)]PI; isoelectric point, [4)]Pig fed basal diet without mugwort, [5)]Basal diet + mugwort powder 1.5%, [6)]standard deviation.

muscle by various methods (Bee et al., 1999; Gunawan et al., 2007; Lin and Hsu, 2005), studies involving other tissue types and myosin light chain have yet to be conducted. The protein eIF-5A has been widely observed in all eukaryotic cells, and disruption of the gene encoding this protein causes the arrest of cell growth, cell death, and/or differentiation of cancer cells (Jao and Yu Chen, 2002). F1-ATPase mediates ATP production in mitochondria (Boyer, 1997). Although F1-ATPase chain D subunit d (Zhang et al., 2000), is one of the components of this complex, we do not know the reason why only this subunit shows increased expression. If F1-ATPase chain D acts to recruit the F1-ATPase complex or enhance its activity, it is a reasonable hypothesis that chain D would have an effect on meat quality. Muscle fiber is divided into types 1, 2a, and 2b. Type 1 maintains a high number of mitochondria, whereas type 2b has fewer of this organelle (Cieslak et al., 2000; Kim et al., 2009). Since F1-ATPase is one of the major components of mitochondria, an increase of F1-ATPase might lead to an increase of type 1 fiber containing abundant mitochondria. Therefore, it was assumed that an increase in F1-ATPase

Table 2. Proteins identified by PMF.

Spot	Predicted protein	Accession number (source)	Sequence coverage (%)	pI/MW (kDa)
3	myosin light chain 2V	NP_998956	61	4.8/18.87
1004	eukaryotic translation initiation factor 5A	NP_001003658	58	5.1/17.04
1106	similar to Myosin light polypeptide 3 (Myosin light chain 1, slow-twitch muscle B/ventricular isoform) (MLC1SB) (Ventricular/slow twitch myosin alkali light chain) (Cardiac myosin light chain-1) (CMLC1) isoform 1	XP_533849	43	5.0/22.52
1405	Chain D, The Structure of Bovine F1-Atpase Covalently Inhibited With 4-Chloro-7-Nitrobenzofurazan	1NBM_D	38	5.0/51.46
2001	Peroxiredoxin-2 (Thioredoxin peroxidase 1) (Thioredoxin-dependent peroxide reductase 1) (Thiol-specific antioxidant protein) (TSA)	P52552	34	4.7/13.82
2601	similar to Kelch repeat and BTB domain containing protein 10 (Kelch-related protein 1) (Kel-like protein 23) (Sarcosin) isoform 2	XP_535949	21	5.1/68.88
5001	MHC class I antigen 7	NP_998933	21	9.0/46.05
5002	Glycine amidinotransferase (L-arginine:glycine amidinotransferase) (Transamidinase) (AT)	P50441	14	6.2/44.63
7102	Tpi 1 protein	AAH61781	44	7.1/ 27.21

PI, isoelectric point; MW, molecular weight.

might induce a change in the quality of pig meat. Since peroxiredoxin-2 exists in mitochondria and plays a role in electron transport (Gromer et al., 2004), an increase in this protein could correlate with mitochondrial number, similarly to F1-ATPase.

Kelch-related protein 1 has been reported to play a critical role in the maintenance of an ordered cyto-skeleton (Jiang et al., 2005). MHC class I immune responses react against foreign pathogens and transplanted tissue. MHC class I molecules are divided into classical and non-classical types, and SLA-7 is included in the non-classical type (Joyce et al., 2008). Although this protein is induced as a result of immune responses due to physiological activating materials in mugwort, a definite function of the protein remains to be discovered. Since glycine amidinotransferase relates to the biosynthesis of creatine in the liver and pancreas (Engelke et al., 2009), we do not know the reason why this protein's expression is higher in this tissue. Tpi protein is an enzyme that mediates the reaction from dihydroxyacetone phosphate (DHAP) to glycerol-aldehyde-3-phosphate, and it plays an essential role in the Embden-Meyerhof pathway (EMP). Since increases in this enzyme were assumed to correlate with mitochondrial numbers as mentioned previously, the increased amount of Tpi would lead to increased sugar utilization by promoting the complete oxidation of sugar. Therefore, the increased Tpi protein might be involved in energy utilization owing to the increased growth rate and maintenance energy requirements.

Properties of proteins identified by CAF-MALDI sequencing

The protein spots that could not be identified via PMF were analyzed by CAF-MALDI sequencing. As shown in Table 3, serum albumin precursor and two types of myoglobins were identified by CAF-MALDI sequencing. Generally, serum albumin, which has the signal peptide from serum albumin precursor consisting of 18 amino acids removed, plays an essential role in maintaining osmotic pressure for the approximate distribution of cellular fluidity in the extracellular matrix and between cells. Serum albumin also plays other roles as a serum carrier via nonspecific interactions with hydrophobic steroid hormones as well as a transport protein for hemin and fatty acids (Roche et al., 2008). Therefore, the increase of this precursor might be involved in promoting physiological activation in pigs due to mugwort. Myoglobin transfers oxygen to muscle cells. Since the mitochondrial numbers in pigs fed with mugwort were assumed to indirectly increase as described in the cases of F1-ATPase and peroxiredoxin-2, following the increase in the overall activation of oxidative electron transport, it was predicted that myoglobin in muscle helps to promote the supply of oxygen, which is the final electron acceptor. Two myoglobin spots might have been obtained as derivatives originating from the chemical treatment process.

In summary, the effects of mugwort treatment potentially include increases in growth, ordered arrangement of

Table 3. Proteins identified by CAF-MALDI sequencing.

Spot	Identified protein	Accession number (source)	Identified amino acid sequence	pI/MW (kDa)
5602	Serum albumin precursor	P07724	DVFLGTFLYEYSR	5.98/76.14
7002	Myoglobin 1	1606205A	HGBTVLTALGGILK	7.69/15.67
8002	Myoglobin 2	1606205A	HGBTVLTALGGILK	8.10/15.53

the cytoskeleton, immune responses, aerobic respiration, oxygen transport, and type 1 muscle fiber, which contains the highest amount of mitochondria.

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