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Proteins in *longissimus* muscle of Korean native cattle and their relationship to meat quality

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ABSTRACT

Proteomic profiling by two-dimensional gel electrophoresis and mass spectrometry of *longissimus dorsi* muscle tissue from Korean native cattle identified seven proteins that are differentially expressed in animals producing low and high quality grade beef. The expression level of alpha actin is increased in high quality grade beef and the expression levels of T-complex protein 1 (TCP-1), heat shock protein beta-1 (HSP27), and inositol 1,4,5-triphosphate receptor type1 (IP3R1), a new protein to be associated with meat quality, are increased in low quality grade beef. In particular, the quantitation of HSP27 and IP3R1 by both silver staining and immunoblotting correlated well with intramuscular fat content, meat tenderness, and free calcium levels. The data suggest that HSP27 and IP3R1 are potential meat quality biomarkers and their identification provides new insight into the molecular mechanisms and pathways associated with overall beef quality.

1. Introduction

Meat quality is determined by various factors such as meat color, fat color, tenderness, and intramuscular fat content (Geay, Bauchart, Hocquette, & Culioli, 2001; Hocquette et al., 2005; Maltin, Balcerzak, Tilley, & Delday, 2003). However, among these factors, intramuscular fat content and tenderness are considered to be the most important factors in determining beef quality and consumers' taste preferences. Therefore, marbling scores for intramuscular fat content and the Warner–Bratzler shear force for meat tenderness are very important factors for determining the dollar value of carcasses in the Korean beef market.

To date, genomic studies for phenotypic profiling involving the use of nucleic acid microarrays and quantitative trait loci (QTL) have identified genes associated with marbling and tenderness in beef (Hocquette, Lehnert, Barendse, Cassar-Malek, & Picard, 2007). However, one problem with genomic studies is that they are not fully competent for understanding the mechanism of marbling and tenderness because of the potential for new proteins expressed through alternative splicing of mRNA. Therefore, proteomic studies can complement genomic studies when analyzing meat quality phenotypes.

Two-dimensional gel electrophoresis (2-DE) is a useful technique for identifying differentially expressed proteins that are associated with meat quality (Bendixen, 2005; Mullen, Stapleton, Corcoran, Hamill, & White, 2006). Such differentially expressed proteins could be implemented as molecular biomarkers for meat quality and may provide new insights into the molecular mechanisms and pathways related to marbling and tenderness (Hollung, Veiseth, Jia, Færgestad, & Hildrum, 2007). Therefore, our 2-DE proteomic approach to identifying differentially expressed skeletal muscle proteins that are associated with low and high grade beef samples might lead to the identification of potential biomarkers for the determination of various meat characteristics and overall beef quality.

The specific objective of this study was to identify proteins that are differentially expressed in *longissimus dorsi* muscle of Korean native cattle (Hanwoo) and to investigate their potential as biomarkers for tenderness, intramuscular fat content, and other characteristics of beef.

2. Materials and methods

2.1. Animals and sample preparation

Five grades of beef (1++, the highest quality, 1+, 1, 2, and 3, the lowest quality) are defined by the Korean beef quality grading

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system based on the marbling score, meat color, fat color, maturity, and texture. Six steers for grade 1++ beef group and six bulls for grade 3 beef group among cattle between 24 and 28 months of age were used. Animals were slaughtered in two groups of six animals over a 2-day period. On each slaughter day, 6 animals were transported to the National Institute of Animal Science (NIAS) abattoir, and fasted for approximately 12 h, but with access to water prior to slaughter. On the following day, the animals were stunned by captive bolt pistol, hung and bled. The carcasses were hung by their Achilles tendon and chilled at 2 °C for 48 h. Samples of *longissimus dorsi* muscle tissue were taken from between the 12th and 13th rib of each carcass, ground to a fine powder under liquid nitrogen with a mortar and pestle, and stored at −80 °C.

2.2. Measurement of meat qualities

The chemical composition of the carcasses including free calcium and intramuscular fat content was analyzed using the methods of the Association of Official Analytical Chemists (AOAC, 1996). The pH was measured using a portable needle-tipped combination electrode (NWKbinar pH-K21, Germany) at approximately 15 min intervals in the centre of the muscle between the 3rd and 4th lumbar vertebrae from approximately 30 min postmortem until the muscle reached ultimate pH. Objective meat color (CIE *L*, *a*, *b*) was determined using a Minolta Chromameter CR300 (Minolta, Japan) on freshly cut surfaces of meat samples after a 30 min blooming at 1 °C. The water-holding capacity (WHC) was determined using the filter paper press method (Kauffman, Eikelenboom, van der Wal, Engel, & Zaar, 1986). Warner–Bratzler (WB) shear force was measured on cooked steaks (2.54 cm thick) in a pre-heated water bath for 60 min or until the core temperature reached 70 °C and subsequently cooled in running water (ca. 18 °C) for 30 min to reach a core temperature below 30 °C. Eight cores of 1.27 cm diameter were made for each sample, and peak force was determined using a V-shaped shear blade with a cross-head speed of 400 mm/min (Wheeler, Shackelford, & Koohmaraie, 2000).

2.3. Two-dimensional gel electrophoresis and image analysis

Frozen muscle tissue (100 mg) was incubated for 40 min in 1 ml of 8 M urea, 2 M thio-urea, 65 mM dithiothreitol (DTT), 2% CHAPS, 1% bio-lyte ampholytes (pH 3–10, Bio-Rad, Hercules, CA, USA) and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Samples were centrifuged at 40,000*g* for 60 min and the supernatants were used as the protein extract. Protein concentration was determined using the protein assay system (Bio-Rad) with bovine serum albumin (BSA) as a standard.

Samples of approximately 200 μg for analytical gels were applied to 18-cm immobilized pH gradient (IPG) strips (pH 3–10, nonlinear, Bio-Rad). IPG strips were rehydrated overnight with a rehydration solution containing 8 M urea, 0.5% CHAPS, 0.28% DTT, 10% glycerol, 0.5% bio-lyte ampholytes (pH 3–10, Bio-Rad), and bromophenol blue (a few grains). After rehydration, isoelectric focusing (IEF) was performed for a total of 46,000 V h with a PROTEAN IEF Cell unit (Bio-Rad). Voltage levels were stepped-up for 1 h each at 100 V, 200 V, 500 V, and 1000 V and then gradually increased to 8000 V. The current limit was adjusted to 50 mA per strip, and the run was carried out at 20 °C. After IEF, IPG strips were incubated for 20 min with 10 ml of equilibration solution comprised of 50 mM Tris–HCl (pH 8.8), 6 M urea, 2% sodium dodecyl sulfate (SDS), 20% glycerol, bromophenol blue (a few grains), and 5 mM TBP. The IPG strips were transferred to the top of SDS polyacrylamide gels (12.5% T, 2.67% C) for electrophoresis (PAGE) and run at 10 mA per gel for 1 h followed by 20 mA per gel until the dye front reached the bottom of the gel.

Gels were fixed for 1 h in 40% methanol and 10% acetic acid and visualized by silver staining (Hochstrasser, Harrington, Hochstrasser, Miller, & Merril, 1988). Stained gels were matched and analyzed with PDQuest software (Bio-Rad). Three replicate gels obtained for each sample were normalized by the total quantity in valid spots and analyzed. The silver-stained protein spots that demonstrated approximately twofold or greater differences in staining densities between the low (grade 3) and high (grade 1++) beef quality groups were selected and the proteins were identified (see below). The molecular weight (MW) and isoelectric point (p*I*) of each protein was estimated using 2-D SDS–PAGE standards (Bio-Rad).

2.4. Protein identification

The differentially expressed spots were excised from the silver-stained gels and destained with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (1:1). The destained samples were reduced and alkylated in the gel with DTT and iodoacetamide in ammonium bicarbonate solution, and then digested with 20 μl of trypsin solution (7 ng/μl trypsin in 50 mM ammonium bicarbonate) and incubated for 16 h at 37 °C. After enzymatic digestion, spots were extracted twice with 50 μl of 50 mM ammonium bicarbonate and the extracts were treated twice in 50 μl of 0.1% formic acid in 50% acetonitrile (ACN). The extracted solutions were dried in a vacuum centrifuge. The samples were hydrated in 30 μl of 0.5% triflouroacetic acid (TFA), desalted using a C18 resin (ZipTip, Millipore, MA, USA), and eluted with 2 μl of 60% methanol containing 5% formic acid prior to mass analysis. Peptide masses of the samples were obtained using the Applied Biosystems 4700 Proteomics analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) in the positive ion reflector mode. The MS/MS analysis was performed for the five most abundant ions and the proteins identified by searching SWISS-PROT and NCBI databases using the Mascot programs (Matrix Science, London, UK). The mass accuracy was considered to be within 100 ppm for peptide mass analysis and within 150 ppm for MS/MS analysis. Known contaminating peaks such as keratin and products of autoproteolysis were removed and the protein molecular weights, isoelectric points, and protein scores were considered to identify each protein.

2.5. Western blotting

For western blotting, 40 μg of sample proteins were separated on SDS–PAGE according to the method of Laemmli (1970) and gels were transferred to PVDF membranes (Millipore) in ice-cold transfer buffer (25 mM Tris–Cl, pH 8.3, 1.4% glycine, 20% methanol) at 250 mA for 90 min. Membranes were treated with blocking buffer containing 5% non-fat milk (Becton, Dickinson and Company, MD, USA) in TBS/T (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and incubated overnight at 4 °C. Primary goat anti-HSP27 (sc-1048) and goat anti-IP3R1 (sc-6093) antibodies, both from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), were used at 1:200 dilutions in TBS/T with 5% non-fat milk. Following 2 h of incubation with primary antibodies, membranes were washed three times for 10 min each with 10 ml of TBS/T. Horseradish peroxidase-labeled (HRP) anti-goat secondary antibody was diluted 1:5,000 in TBS/T with 5% non-fat milk and incubated for 1 h. After three 10-min washes, membranes were visualized using a chemiluminescent HRP substrate (Millipore) and a luminescent image analyzer (Fujifilm, Tokyo, Japan). The band densities were calculated by Multi Gauge software (Fujifilm, Ver. 3.1) and normalized by density of α-tubulin (sc-12462, Santa Cruz Biotechnology Inc.). All experiments were repeated in triplicate.