Nutraceuticals & Food
Vol. 7, p.432~436 (2002)

Nutraceuticals and Food

Analysis of Hanwoo Loin Proteome by 2-D Gel Electrophoresis and Peptide Mass Fingerprinting

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Abstract

A proteomic map of Hanwoo loin was obtained using 2-D SDS-PAGE and mass spectrometric analysis: 27 bovine proteins plus 2 proteins having similarities to other mammal proteins out of 52 proteins analyzed. The identified proteins consisted of 50% basic house keeping proteins involved in metabolism, 30% muscle proteins, and other miscellaneous proteins. Many proteins on the 2-D gel with different molecular weights and isoelectric points were identified as same proteins due to posttranslational modification. As many of the identified house keeping proteins showed the high sequence similarities to other mammal equivalent proteins, searching the mammal databases could confirm the annotation. The preliminary identification of the proteome in bovine loin tissue could reveal the functions of proteins at over 50% of chance with high fidelities. Using the established loin proteome map, proteomic difference between 1 yr and 2 yr Hanwoo loin tissues were compared on 2D gel. Regardless of the difficulty normalizing protein concentrations and sample-to-sample variations, three unidentified proteins and myoglobin were selected as up-regulated proteins during the fat deposition period. This study contributes to a more thorough and holistic understanding of beef meat, helping to build the basis for future identification of new markers for good quality meat.

Key words: peptide mass fingerprinting, proteome, Hanwoo, mass spectrometry, 2-D gel, marbling

INTRODUCTION

Korean Hanwoo cattle, *Bos taurus coreanae*, are believed to have originated as a crossbred stain between *Bos taurus* and *Bos indicus* (1). Carcasses produced from the cattle with higher *Bos taurus* breeding tend to have higher marbling scores (2). Increased marbling of intramuscular tissue is associated with tenderness of the meat (3). Furthermore, juiciness and flavor intensity of beef are affected by the intramuscular fat (3,4). Marbling scores are a major determinant of quality grades, such as USDA grade, as well as the tenderness of loin steak (5). It is well known that the quality meat from Japanese Black cattle is very good regarding its palatability and texture. The cattle are characterized a high amount of intramuscular fat, marbling (6). The primary determinants of meat tenderness are amounts of connective tissue, intramuscular fat content, and myofibrillar structure; factors that are largely dependent on the genetic background of cattle (7). Genes of cattle encode the proteins that determine the quality of meat. Thus, as the protein profiles in the tissue vary, so do the meat qualities.

By definition, the entire entity of the expressed proteins in a tissue at any given time is the "proteome" (8) of the tissue. The emerging field of proteomics focuses on the large-scale, comprehensive characterization of the proteins present. Currently, proteomics employs a combination of sophisticated analytical tools. The key technologies are two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). 2-DE can separate a complex mixture of proteins to a high-resolution according to the size and charge of each protein in the sample. Up to several thousand proteins can be separated as spots with various sizes and intensities. Computer-assisted comparison of spot patterns in different 2-D gels can be performed to analyze the changes in protein expression levels that are relevant to disease, stress, aging, palatability, etc. The identification of the specific proteins of interest involves an enzymatic (usually trypsin) digestion of the proteins and mass spectrometry analysis of the peptides obtained from the tryptic digestion. The molecular masses of the peptides comprising a peptide mass fingerprint (PMF) or the amino acid sequences obtained by tandem mass (MS/MS) spectrometry are used to search databases. Proteins with the best match between the experimental and the theoretical *in silico* data are identified.

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So far, proteomic approaches have not been used to analyze bovine loin proteins, partly because there are not enough bovine databases available yet. However, because many of the amino acid sequences of the proteins essential to cellular activity and house keeping of bovine and other mammals are highly conserved and readily available from available databases, it is possible to construct a preliminary bovine proteome map, particularly, on muscle tissue, which provides a relatively simple protein profile.

Currently, the Hanwoo cattle industry is focused on improving beef quality. Few studies have evaluated the protein profiles expressed in muscle tissue and their effect on beef quality. Therefore, the purposes of this study were to find proteins specifically expressed during the marbling period and to establish a bovine loin proteomic map. To construct the proteomic maps and to identify proteins associated with the accumulation of fat in muscular tissue, we analyzed the proteins of the loin tissues from 1 and 2 yr old Hanwoo cattle by 2-DE and Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometric analysis. The proteomic maps of the tissues were established and the comparison between the two stages enabled us to select the marbling-stage specific proteins.

MATERIALS AND METHODS

Preparation of samples

Loin tissues from 1 and 2 yr old Hanwoo cows were retrieved from a slaughter house and immediately frozen in liquid nitrogen. Four Hanwoo cows at each age-related marbling phase, were chosen to evaluate the differences in protein expression. All external fat and peripheral connective tissue was removed using a scalpel from each sample. For each 100 mg of defatted tissue, 1 mL of extraction solution containing 1% Triton X-100 and protease inhibitors in PBS was used to disrupt and lyse the sample in a Dounce homogenizer. The lysed samples were clarified by centrifugation at 10,000×g for 10 min at room temperature. Protein concentrations were determined by the Bradford method (9) using bovine serum albumin (BSA) as a standard. Appropriate volumes of samples containing 0.4 mg of protein in 1.5 mL tubes were treated with 10% trichloroacetic acid (TCA) to precipitate proteins (10). The precipitated proteins were washed 10 times with absolute ethanol to remove salts and lipids. Each sample was dissolved in 100 μL of lysis solution containing 9.8 M urea, 2% (w/v) NP-40, 2% ampholyte, pH 3-10, and 100 mM dithiothreitol (DTT). Samples were clarified by centrifugation at 10,000×g for 10 min at room temperature.

Two-dimensional gel electrophoresis

Up to 0.4 mg of protein was used for isoelectric focusing (IEF); for the first dimension IPG strip (Bio-Rad, USA) was used and 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was used for the second dimension (11). The pH of the IEF gels ranged from 3 to 10, and were passively rehydrated with the sample dissolved in rehydration buffer for 1 hr at 15°C. For the first dimension, samples were run for 2 hrs at 300V, 2 hrs at 500V, and 18 hrs at 800V at 4°C. The gels were extruded from the tubes and laid on top of the second dimension SDS polyacrylamide vertical slab gel. Each second dimensional separation was run overnight at 6 mA and 4°C. At the end of the run, the separating gel was subjected to silver staining. The molecular weight and the pI were determined by using 2-D SDS-PAGE Standards (Bio-Rad, USA) run on a separating gel. The standard proteins used were hen egg white conalbumin type I (76 kD, 6.0~6.6 pI), bovine serum albumin (66.2 kD, 5.0~5.18 pI), bovine muscle actin (43 kD, 5.47~5.53 pI), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36 kD, 8.3~8.5 pI), bovine carbonic anhydrase (31 kD, 5.9~6.0 pI), soybean trypsin inhibitor (21.5 kD, 4.5 pI) and equine myoglobin (17.5 kD, 7.0 pI).

In-gel digestion of proteins

Proteins resolved on 12% SDS-polyacrylamide gels were visualized by staining with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid. The proteins in the gel were picked using wide-orifice pipet tips, and transferred to reaction tubes containing distilled water. Washed gel slices were broken into fine particles with a blade and pestle. The gel particles were incubated for 20 min in wash solution (50% acetonitrile in 50 mM NH_4HCO_3) to remove the staining dye and then dried in a vacuum centrifuge for 40 min. Subsequently 20 μL of trypsin solution (10 ng/μL in 50 mM NH_4HCO_3) was added to the tubes containing the gel particles and incubated at 37°C for 16~20 hrs. Following digestion, peptides were extracted by vortexing in extraction buffer (5% trifluoroacetic acid in 60% acetonitrile). The extracted peptides were concentrated by a Speed-vac centrifugation for 3 hrs, resolubilized in a resuspension solution (0.1% trifluoroacetic acid in 50% acetonitrile), sonicated in a sonicator bath for 5 min, and then centrifuged for 2 min.

Sample preparation for MALDI-TOF MS

CHCA (α-cyano-4-hydroxy-*trans*-cinnamic acid, 40 mg/mL) and nitrocellulose (20 mg/mL) were separately dissolved in acetone and mixed with isopropanol at a ratio of 2 : 1 : 1 v/v, respectively. The matrix solution was mixed with the sample at a ratio of 1:1 and 1 uL was spotted onto the target and dried. The immobilized samples were washed with 5% formic acid and then water by reverse pipetting of 5 uL of each solution on the surface

of the sample spots. The samples were then dried for a second time before MALDI-TOF mass spectrometry analysis and each sample repeated 5 times. The extracted peptides were spotted onto the MALDI plate over 4 positions and each crystallized spot was analyzed over three times. Peptide peak intensities of the samples were averaged and converted into percentage. Bradykinin, angiotensin I, and neurotensin were used as internal and/or external calibrants for spectra calibration.

MALDI-TOF mass spectrometry

Samples were analyzed on a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, USA). The spectra were acquired in the delayed extraction, reflector mode and standard conditions of 20,000 V acceleration voltage and 150 ns delay time. Peptides were selected in the mass range of 700~3500 daltons. Spectra obtained by averaging 50~200 individual laser shots were calibrated internally and/or externally with the calibrants and trypsin autolytic products, m/z 842.5, 1045.6 and 2211.1.

Protein mass fingerprinting

For the identification of the protein fragments, MS-Fit program in Protein Prospector of UCSF (http://prospector.ucsf.edu/) was used.

RESULTS AND DISCUSSION

Analysis of the beef loin proteome by 2-DE

The beef loin proteome was characterized by making protein maps of longissimus steaks of four 1 yr and four 2 yr cows obtained immediately following slaughter. Fig. 1 shows the Coomassie-stained 2-DE map of bovine loin tissue proteins. As shown in Fig. 1, electrophoretic analysis of bovine loin tissue was problematic because of dragging of the proteins on the first dimension. These dragging phenomena could not be resolved, even by an extensive desalting and delipidification with trichloroacetic acid (TCA)-precipitation and ethanol washing. In addition, these phenomena varied between individuals and gel-to-gel running. Regardless of a consequence of individual and gel-to-gel variations, an average of 90% of the protein spots were found to be reproducible between gels. The resulting representative images of the 1 and 2 yr loin proteins were established and compared with each other to identify proteins whose expressions were increased or decreased during the fat deposition period (Fig. 2).

To probe proteome changes during the fat deposition into the tissue, randomly selected clearly visible Coomassie Blue stained spots were excised from the gels shown in Fig. 1 and digested with trypsin and analyzed by MALDI-TOF. Peptide mass data were used to search sequence databases via a search engine, MS-Fit at UCSF (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm), for protein identification. All searches were performed with a mass tolerance of 0.005% error (50 ppm). Proteins identified and the supporting identification data are listed in Table 1. Twenty-five among the fifty randomly selected proteins were annotated with twenty-two bovine and three similar mammal proteins. The success rate for the annotation was about 50%. Considering the fact that, at present (December, 2002), the NCBInr database contains 6389 entries for *Bos taurus* proteins, compared with 63762 and 11630 for *Mus musculus* and *Homo sapiens* out of total 1217808 entries, respectively, this preliminary establishment of bovine loin tissue proteomic map is satisfactory. To accomplish a complete analysis of bovine loin tissue proteome, it is necessary

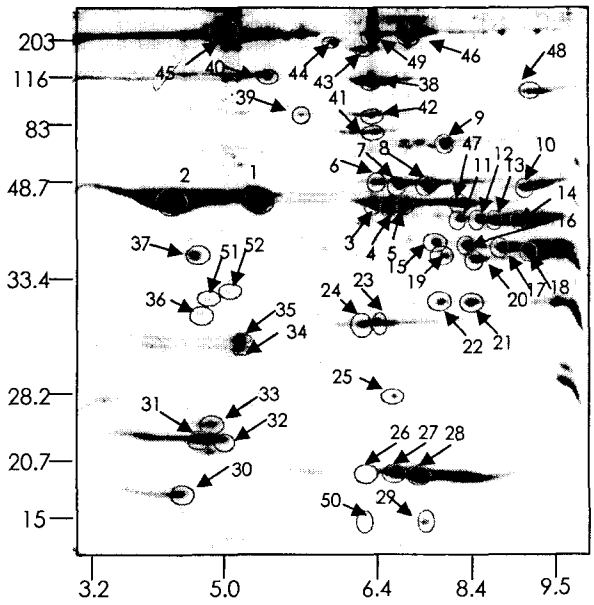

Fig. 1. Proteomic map of Hanwoo loin tissue. Total proteins were extracted from the loin tissue using 1% Triton X-100 in PBS in the presence of protease inhibitor cocktail. Proteins were separated on IPG strip with a pH range 3-10 first-dimensionally and further separated on 12% SDS-PAGE. Protein spots were stained with Coomassie Brilliant Blue R-250.

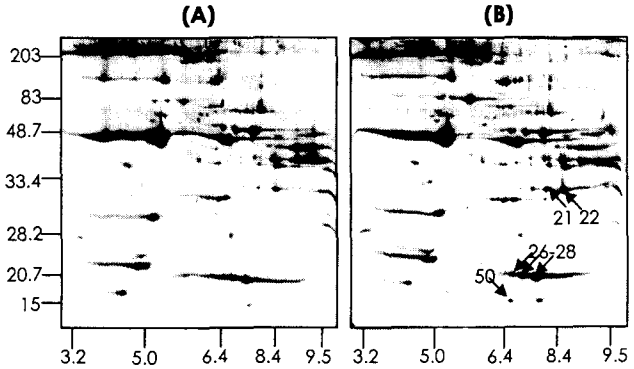

Fig. 2. Comparison of proteomes of bovine loin at 1 yr (A) and 2 yr (B). Gel images from four replicated runs were compiled for each stage of loin sample.

Table 1. Proteins identified in bovine loin tissue

Spot no.	Protein	Identification criteria			On gel		Theoretical	
		Accession no.	Peptide match	Coverage, %	MW (kDa)	pI	MW (kDa)	pI
1	α-skeletal actin precursor	409694	10	40	45000	5.5	42136.5	5.3
4	Creatin kinase, M chain (M-CK)	13629372	15	45	43000	6.6	42971.3	6.6
5	Creatin kinase, M chain (M-CK)	13629372	11	34	43000	6.5	42971.3	6.6
6	α-enolase	13124248	5	21	48000	6.2	47277.4	6.4
7	α-enolase	13124248	6	24	48000	6.7	47277.4	6.4
8	α-enolase	13124248	8	28	48000	7.5	47277.4	6.4
9	Glutamate dehydrogenase	118533	6	27	55000	7.8	55561.8	8.3
10	Elongation factor EF-Tu precursor	2119917	5	12	49000	8.7	49408	6.7
14	D-aspartate oxidase	346505	4	14	38000	8.8	37329	8.8
15	Glyceraldehyde-phosphate dehydrogenase	1841758	4	18	35500	7.7	33524.6	8.5
16	Glyceraldehyde-3-phosphate dehydrogenase	2506439	13	57	35000	8.3	34382.6	8.7
17	Glyceraldehyde-phosphate dehydrogenase	1841758	4	25	34500	8.6	33524.6	8.5
18	Glyceraldehyde-3-phosphate dehydrogenase	2506439	4	20	34000	8.8	34382.6	8.7
19	L-lactate dehydrogenase A chain	126045	22	68	34000	7.8	36598	8.1
25	α-crystallin chain B	71482	11	50	28000	6.6	20036.8	6.8
26	Myoglobin	363848	10	46	18000	6.4	17077.7	6.9
27	Myoglobin	363848	10	46	18000	6.7	17077.7	6.9
28	Myoglobin	363848	10	46	18000	7.1	17077.7	6.9
29	Hemoglobin β-chain	223864	5	25	15200	7.2	15964.5	6.4
30	Myosin regulatory light chain 2	21326174	10	73	17000	5.0	19015	4.9
33	Similar to human Myosin light chain 2	4557775	8	66	23500	4.8	18775	4.9
36	40S ribosomal protein P40	125968	6	25	30500	4.8	32897	4.8
37	Annexin IV	1063258	8	35	35000	4.7	35874.1	5.9
39	Heat shock protein, 70 kDa	Q27965	7	16	84000	5.8	70229.0	5.7
40	A-kinase anchor protein 3	Q77797	6	9	105000	5.4	94217.6	6.3
41	Similar to rabbit phosphoglucomutase	P00949	12	37	65000	6.5	61558.9	6.6
42	Albumin	162648	12	25	84000	6.5	69294.1	5.8
45	Myosin heavy chain	13560273	4	7.3	220000	5.1	223231	5.6
51	Protein kinase C inhibitor protein-1	108451	9	28	32000	4.8	28121	4.8
52	Protein kinase C inhibitor protein-1	108451	5	14	32500	5.0	28121	4.8

o analyze the proteome using narrow pI, high resolution SDS-PAGE and tandem mass spectrometry (MS/MS) (12).

Interestingly, several proteins, such as creatin kinase, enolase, glyceraldehyde-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and myoglobin, appeared at multiple positions on the gels. The long-range spread protein spots mean that during the period of 1~2 yr, which is an active fat deposition period in the muscle tissue, posttranslational modifications should be actively occurring (13,14). It remains to be determined whether the mass and charge alterations observed on 2D-gel are related to the fat deposition process.

When no convincing hits were obtained from protein databases searches, we utilized the peptide masses of the loin proteins to search the mammal databases (223457 entries at present). Two proteins, #33 and #41, showed a high-level of hits and sequence similarities to myosin light chain-2 and phosphoglucomuatse, respectively, of other mammals, such as mouse, rat and rabbit (Table 1). To assign the predicted functions of the proteins, the protein sequences were compared with those of other species (15). The sequence identities of theses proteins to those of other species were >90% (data not shown). Likewise, a mammalian database search with the peptide masses of many of the bovine house-keeping proteins, whose sequences are highly conserved between mammals, extracted the same functional proteins. The results of homologous search help confirm the bovine database searching results. But to make sure the real function of the protein, it is necessary to sequence the protein spots using tandem MS/MS or N-terminal sequencing (16).

Proteome changes during marbling period

To detect protein changes in the tissue during the fat deposition period, computer assisted image analysis (PDQUEST, Bio-Rad, USA) was used to align gel patterns and spot intensities. Four gels on each tissue sample were matched and the relative amounts of proteins were compared between 1 yr and 2 yr tissues. As previously reported (17), the gel patterns of the 1 yr and 2 yr loin tissue were very similar (Fig. 2). Despite the limitation of quantitation of the relative protein amounts, there were potentially significant differences in protein expression between 1yr and 2 yr loin tissues (Fig. 2) with significantly increased proteins found in the 2nd year cattle. Unfortunately, the sel-

ected spots, #21, #22 and #50 (Fig. 2), did not match well with any known bovine or mammal proteins. The peptide mass values of spot #21 (31 kDa, pI 8.4) was best matched by human 3'-phosphoadenosine 5'-phosphosulfate synthetase 2 (PAPSS 2) at a low sequence coverage (8%) and different molecular weight (69 kDa). Spot #22 (31 kDa, pI 7.8) showed an identity to bovine mitochondrial aconitate precursor protein at 4% sequence coverage with molecular weight of 85 kDa. Neither bovine nor mammal databases had proteins that matched spot #50. With such a low level of matches, it was impossible to assign protein functions to the differentially expressed proteins, #21, #22, and #50. The unidentified proteins need to be identified by amino acid sequencing by tandem MS/MS analysis, and matching the sequences with those in NCBInr using BLSAT-P program. Myoglobin, spot# 26-28, was already identified from a previous study (17). Myoglobin is involved in meat color development as cows are mature.

As a preliminary study, we used IPG strips with pH range 3~10 and total proteins without fractionation. It is necessary to fractionate the proteins and use narrow pH range-IPG strips to enrich the low abundance proteins (12). To overcome the limitation of the bovine database, bovine EST databases and tandem MS/MS spectrometric analysis should be employed (15).

ACKNOWLEDGMENTS

This work was supported by a grant from KISTEP at the Ministry of Science and Technology of Korea.

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(Received October 18, 2002; Accepted December 6, 2002)