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Isolation and Identification of Proteins Increasingly Expressed in Beef Loin on Maturation

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Abstract : Protein profiles of beef loin were constructed by comparing two-dimensional gel electrophoresis patterns of the proteins from different growth stages of Hanwoo, Korean cattle. Proteins from the lean muscle of 0, 6, 12 and 24 months old Hanwoo were separated by isoelectric focusing (IEF) on 16 cm tube gels, and further processed second dimensionally by 12% SDS-polyacrylamide gel electrophoresis (PAGE) using 18×20 cm gel. Proteins with pI values ranging 3.0 to 9.0 and molecular weight of 15 to 100 kDa could be clearly detected on gel by silver staining. Interestingly, many of the proteins significantly increased and decreased during growth happened to be low molecular ones. To isolate the increased proteins, the soluble proteins were obtained from the tissue by 1% Triton X-100 extraction, then, fractionated by 30% and 50% ammonium sulfate. The isolation condition of each particular protein was determined. According to the conditions, two of the increased proteins were isolated, and transferred to PVDF membrane and microsequenced.(Received December 10, 1998; accepted February 5, 1999)

Introduction

The variation in palatability of beef largely depends on animal age, raising and slaughtering conditions, cooking method, and personal preference.[1] It is also true that the characteristics of beef differ from individual genetic trait. Recent studies suggest that genetic differences in beef palatability are associated with variation in the rate of muscle proteolysis, the degree of marbling and other physico-chemical conditions of beef, such as percentage of fat and moisture, color, etc.[2,3] Among these factors, it is well established that proteins in muscle cells are continually synthesized and degraded in both development and maturation.[4] The resulting variation of protein contents in cattle muscle is highly associated with the palatability variation of beef.

In addition, the genetic differences make the protein expression and deposition pattern different in beef. Enzymes, muscle fibers and connective molecules coded from particular genes and deposited in beef are all proteins. Thus, it is quite predictable that the expression level of individual proteins and the expression profile of the proteins differ by the genetic background and on growth phases of cattle.

Isolation and identification of proteins in muscles and other tissues have been successfully done by two-dimensional (2-D) gel electrophoresis.[5] 2-D polyacrylamide gel electrophoresis separates proteins on the basis of their differences in pI in the first dimension and apparent molecular mass in the second dimension.[5] It allows the separation of thousands of proteins, and provides the means for separating interested proteins into homogenous form from a complex sample. Using this technique, we can analyze changes in proteins in response to changing physiological and environmental conditions. This includes analysis of the quantitative changes in protein expression, changes in post-translational modification and, where appropriate, tissue specific expression. Moreover, the 2-D gel electrophoresis based approach allows proteins to be purified without any prior knowledge of their properties.

The objective of the present study was the isolation and the identification of the major proteins significantly increased in marbling phase, which enhances tenderness and taste of beef.[6] To identify any interesting proteins, firstly, the protein profiles on 2-D gel of the lean muscle from different growth phases were compared. The increased proteins at the marbling period, which is about 1 to 2 years of growth, were designated and two of them were isolated to be characterized.

Materials and Methods

Preparation of samples

Loin tissues from 0, 6, 12 and 24 mo old Hanwoo cows were retrieved from the fabrication room and frozen immediately in liquid nitrogen. Four Hanwoo cows at each phase were chosen to evaluate the differences in protein expression. All external fat and peripheral connective tissue was removed

Key words : marbling, Hanwoo, 2-D gel electrophoresis, microsequencing, fractionation
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from each sample. 100 mg of defatted tissue was disrupted and lysed in 1mL lysis solution containing 9.8 M urea, 2% (w/v) NP-40, 2% ampholyte, pH 3~10, 100 mM DTT. The samples were cleared before loading by centrifugation at 10000 g for 10 min at RT.

Two-dimensional gel electrophoresis

Up to 0.4 mg of protein were used in an isoelctric focusing (IEF) first dimension and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis second dimension 5,7). 16 cm long and 1 mm thick IEF tube gels pH ranged 3 to 10 were polymerized for 2 hr at 25°C. For the first dimension, samples were run for 2 hr at 300 V, 2 hr at 500 V, and 18 hr at 800 V 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 dimension separation was run at 6 mA per gel overnight at 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 separate gel. The standard proteins used are 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), soybean trypsin inhibitor (21.5 kD, 4.5 pI) and equine myoglobin (17.5 kD, 7.0 pI).

Fractionation of proteins with ammonium sulfate

For the fractionation, 4 g of frozen loin tissue were weighed and crushed in 1% Triton X-100 in phosphate buffered saline (PBS) using a Dounce homogenizer with 20 strokes on ice. Unsolubilized debris and lipid were pelleted by high-speed centrifugation at 12000 g at 4°C. Proteins in the clear supernatant were fractionated with 30% and 50% ammonium sulfate. The supernatants and pellets dissolved in 1% Triton X-100/PBS were dialyzed against PBS. Appropriate volume of sample containing 0.4 mg of protein in 1.5-m*l* tubes was treated with 10% trichloroacetic acid (TCA) to precipitate proteins to be concentrated. Each sample was dissolved in 10 μL of lysis solution.

Staining gels and N-terminal microsequencing

Silver staining: to visualize proteins, gel was stained by a silver staining method.[8,9] Gel was prefixed for 20 min in fixing solution (50% methanol, 10% acetic acid). The prefixed gel was quickly rinsed with distilled water before it is fixed in 10% glutaraldehyde for 30 min followed by rinsing with plenty of distilled water and reduced with 5 mg/L dithiothreitol (DTT) for 15 min. Silver staining was followed with 0.1% silver nitrate for 15 min. The stained gel was rinsed again with distilled water and developed in 0.019% formaldehyde in 3% sodium carbonate until desired level of stain was obtained. Adding 5 ml of 2.5 M citric acid stopped the reaction.

Coomassie Blue staining: gels were stained for 10 min in 0.25% Coomassie Blue R-250 in 50% methanol to localize the interested proteins before electroblotting them to polyvinylidene difluoride (PVDF) membrane.[10,11] Electroblotting of proteins was carried out on the Bio-Rad Transfer Blot system in transfer buffer containing 48 mM Tris base, 39 mM glycine, 20% methanol (v/v) at a constant 80 voltage for 2h at 4°C. After transfer, the membrane blot was washed in 50% methanol for 5 times at RT.[11]

Microsequencing: the transferred protein on PVDF membrane was destained with 50% methanol and air dried before N-terminal sequencing. The amino terminus sequence of each protein was determined using an automated Edman degradation protein sequencing system[12,13] (G1005A, Hewlett-Packard).

Database searching: the N-terminal sequences data, estimated mass of proteins was matched with the SWISS-PROT database using the AACompID program in ExPASy to identify the proteins using pI and MW windows of ±0.25 and ±30%, respectively.

Results and Discussion

Analysis of loin proteins from different growth phases of Hanwoo cattle by 2-D gel electrophoresis

Duplicated frozen samples from 0, 6, 12 and 24 months Hanwoo cows obtained from Korea Livestock Research Institute, RDA, Suwon, were processed as indicated in "Materials and Methods". Due to the technical difficulty in equalizing the staining intensity, each gel was silver stained until the spot intensity of the arbitrarily designated protein X whose expression is not changed in 2 years period reached a substantial level (Fig. 1). The 2-D gel analyses using the total lysates indicated that the patterns of protein profiles generally are very similar among samples. However, a detailed image analysis could distinguish proteins whose expression was increased (Fig. 1C) or decreased (Fig. 1A) significantly compared to other proteins in 24-months period of growth (Fig. 1, and Table 1).

Regarding the constant intensity of protein X and the reference protein spots (arrow head), the protein profiles of molecular weight 31 to 66 kDa on 2-D gel showed that the relative expression level of many proteins increased as cattles grew up (Fig. 1). There was no significant or at best only minor difference between 12 and 24 month samples. Thus, through out the experiments samples from 0, 6, and

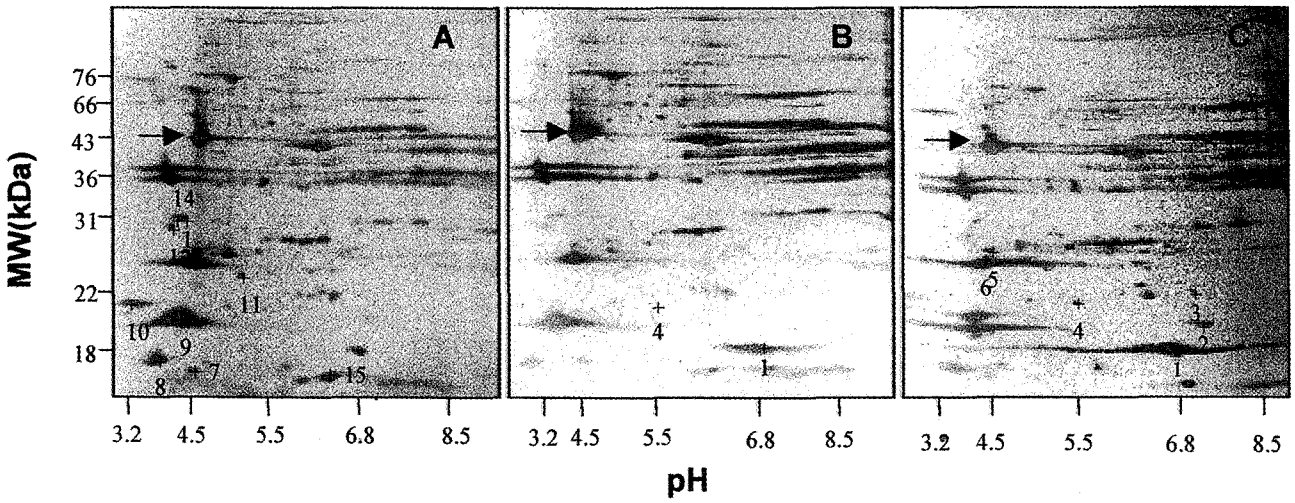

Fig. 1. Protein profiles of the lean muscle from 0, 6 and 24-month Hanwoo cattle. Proteins separated on gel were visualized by silver staining. Developing time for the staining was adjusted until the band intensity of the protein X (→) reached to an appropriate level. The increased and decreased proteins were determined by comparing the intensity of the protein spots at the same position on the other gels as the reference protein (arrow head) spot remained constant. The decreased proteins are indicated by + and numbers in panel A (0-month) and the increased are indicated the same way in panel C (24-month). Panel B shows 6-month sample.

Table 1. Variations in protein expression during the growth of Hanwoo

	Protein ID	pI	MW (kDa)	Solubility in 1% TritonX-100	Precipitated with $(NH_4)_2SO_4$
Increased	1	6.4	18	Soluble	>50%
	2	7.0	20	Soluble	>50%
	3	6.8	22	Soluble	>50%
	4	5.6	22	Soluble	50%
	5	4.3	27	Insoluble	NA
	6	4.3	28	Insoluble	NA
Decreased	7	4.6	16		
	8	3.8	17		
	9	4.4	19		
	10	3.2	21		
	11	5.1	24		
	12	4.3	29		
	13	4.4	30		
	14	4.35	31		
	15	6.3	12		

24 months cows were compared.

The 2-D gel system could resolve proteins with molecular weights between 10 kDa and 80 kDa, and pIs between 3.0 to 9.0. The localization of the interested proteins on 2-D gel was determined if there is significant difference in the intensity of the staining of proteins at the same molecular weight and isoelectric point (pI). Interestingly, most of the proteins whose expressions were decreased or increased were found to be under 30 kDa low molecular weight proteins. Although there were other proteins increased in tiny amount, we eliminated them in this study because we could not identify them by N-terminal microsequencing.

In two years growth period, the relative expression of protein #1 was most significantly increased among the designated proteins. In addition, protein #4 could be detected by silver staining after 12 months. This is a particularly interesting protein because its appearance coincides with the maturation period of the marbling in the loin tissue.[6]

Fractionation of proteins with ammonium sulfate to enrich the interested proteins.

Considering the cost for microsequencing, the feasibility of separation of the proteins in a demanded amount, and the development of a method identifying proteins, we decided to focused on proteins #1 and #4 for this study.

To enrich the protein #1 and #4 up to an amount enough for microsequencing, we have tried many methods including electroelution, gel filtration, electrotransfer from 2-D gel onto membrane, and ammonium sulfate fractionation in conjunction with 2-D gel analysis followed by electrotransfer onto PVDF

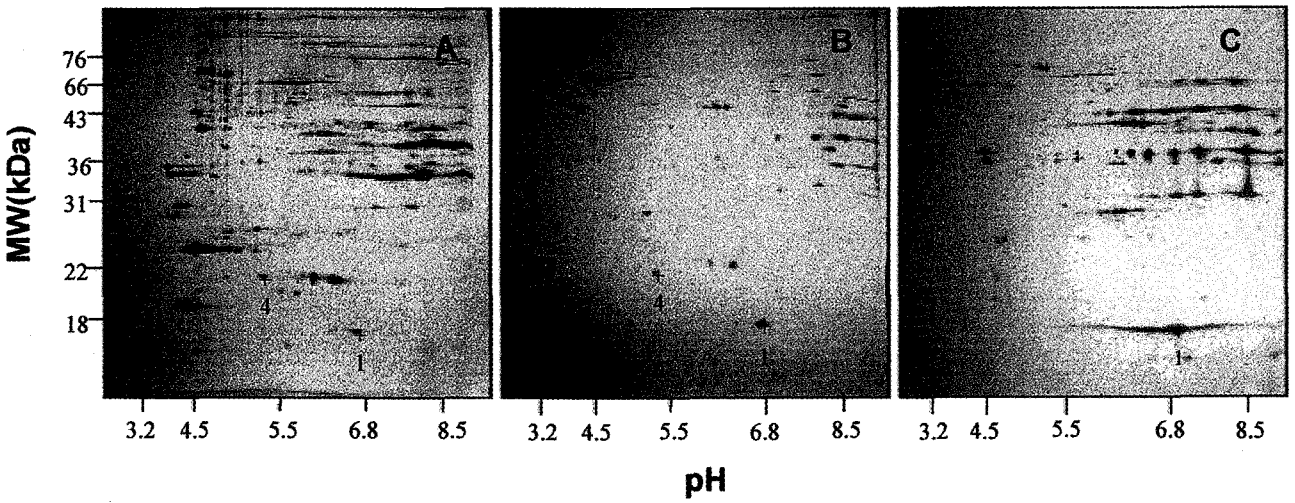

Fig. 2. The lean muscle proteins fractionated with ammonium sulfate and separated on 2-D gel. Proteins solubilized in 1% Triton X-100 in PBS were sequentially fractionated with 30% (A) and 50% (B) ammonium sulfate. Majority of protein #1 and #4 were enriched in the supernatant (C) and 50% pellet (B), respectively. Proteins were visualized by silver staining.

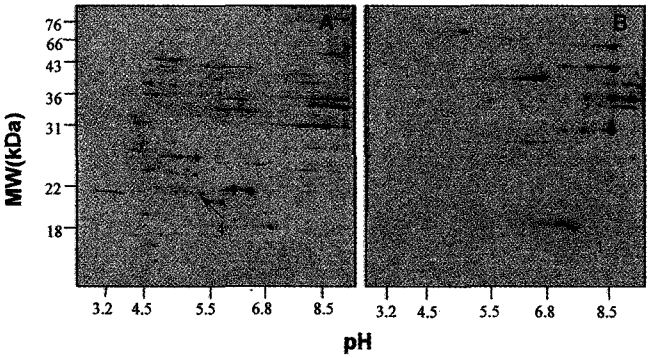

Fig. 3. Coomassie Blue R-250 staining of the fractionated proteins separated on 2-D gel by 50% ammonium sulfate. Protein #1 and #4 were cut from the gel stained with Coomassie Blue for visualization. Proteins in three cut gels were electroblotted onto PVDF membrane.

(A)

#1

X-L-X-D-X-E-X-Q-L-V-L-N-A-W(?)-*G-K-V-E-A-D-V-A-G-H-X-Q-E-V-L-I-R-*L(?)-F

#4

N-terminal Blocked

(B)

Fig. 4. Microsequencing of the proteins on PVDF membrane by Edman degradation method. The N-terminal sequence of protein #1 was obtained from the protein transferred onto PVDF membrane (A). The sequence was used for database (SWISS-PROT) searching using the BLAST-P engine. The N-terminal of protein #4 was found blocked.

membrane. We figured that it was most convenient and efficient to obtain the proteins using ammonium sulfate fractionation prior to 2-D gel separation. After 2-D gel separation, proteins were stained with Coomassie Blue R-250 in 50% methanol for short period of time to prevent any possible N-terminal blocking[14] (Fig. 3). The stained proteins were transferred to PVDF membrane and washed with sufficient amount of 50% methanol to remove glycine and destain the blot15).

For the fractionation, proteins should firstly be solubilized in an appropriate buffer without liberating DNA from the cells. Thus, we extracted proteins from the muscle tissue without breaking the cells by using a homogenization in 1% Triton X-100 in PBS. The proteins chosen in Fig. 1 were categorized into soluble and insoluble proteins with a careful 2-D gel analysis using the soluble and insoluble proteins from the Triton X-100 extraction (Table 1).

The fractionation with ammonium sulfate could efficiently enrich the interested proteins and help remove unwanted proteins from the interested spots on 2-D gel (Fig. 2). The #1 and #4 proteins were fractionated in the supernatant and in the pellet of 50% saturation of ammonium sulfate, respectively. The relative protein amount of the #1 and #4 proteins in the sample increased so efficiently after the fractionation that these proteins could be visualized by Coomassie Blue R-250 staining. This means that those proteins were enriched up to near a microgram quantity in a spot and could be transferred to membrane for microsequencing

(Fig. 3 and 4A).

Amino acid analysis and database searching

Amino acid analysis of protein #1 went well and could read 36 residues (Fig. 4B). Database matching with the combination of the protein amino acid composition, pI, and MW indicated that this protein is a bovine myoglobin. But protein #4 could not be read to a single residue (Fig. 4B). As the amount of protein #4 transferred on PVDF membrane was comparable to that of #1 (Fig. 4A), we tentatively concluded that the N-terminus of protein #4 was blocked by some reason. Protein #4 in gel will be treated with pepsin to be partially digested.[16] Then, the internal sequence of the protein can be read.

Myoglobin is a relatively small (MW=16,700), oxygen-binding protein of muscle cells.[17] The heme group in the molecule is responsible for the deep red-brown color of meat. Generally a progressive change in the color of the lean occurs as the animal matures. Veal lean is characterized by a pale pink color; whereas yearling cattle produce lean with a bright cherry-red color and mature cow lean is usually a deep, dark red color.[18] According to the fact that the protein expression profiles of the 24 months cow shown in Fig. 1C and that of the 12 months cow are similar, the muscle development seems to be almost completed within one year of cow maturation. It is interesting to notice that the marbling in bovine longissimus muscle is rapidly increased from one year of maturation.

In this study, the myoglobin spot from the gel of the mature lean was selected because its expression level was high and it could be isolated easily. The protein could be visualized even by Coomassie Blue staining. Transferring the stained protein to PVDF membrane made it easy to determine the position and the approximate amount of the protein. For the weakly expressed proteins, in many cases, the ammonium sulfate fractionation increased the relative concentration of the proteins. According to the molecular weight and the pI values, we can choose appropriate gradient ranges of pI and gel percentage to get better resolutions. Based on the experimental conditions and the developed methods, we are going to identify the other designated proteins in Table 1.

For better accuracy and reproducibility, and high loading capacity, it is necessary to employ micro-preparative 2-D gel electrophoresis using immobilized pH gradient (IPG) strip[21,22] (Pharmacia, USA). The high resolution and reproducibility of IPG will provide better pI and MW information on proteins. Accurate protein analysis is necessary to correctly identify proteins. In addition to this rapid and reproducible protein separation ability, the high sample loading capacity of this system makes it unnecessary to run multiple gels and blots.

For cheaper and faster amino acid sequence analysis, recent studies launched an "N-terminal sequence tag" method, which needs only three or four N-terminal residues in identifying proteins rather than 20-30 residues necessitated in conventional Edman degradation approaches.[21,23,24] In theory, 3 or 4 residues can make 8000 (203) or 160,000 (204) possible N-terminal sequence tag of proteins. Due to the immense specificity of sequence data, N-terminal sequence tags should offer a powerful means of screening proteins. This will assist in the identification of novel proteins, and hence genes, from well and poorly molecularly defined organisms studied under a variety of physiological and pathological conditions.

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성장기 소의 등심에 발현되는 단백질들의 분리 및 동정

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초 록 : 각각 다른 성장기의 한우 등심에서 추출한 단백질을 이차원 전기영동법으로 분리하여 젤 상의 단백질 전개 양상을 비교하였다. 성장 0, 6, 12, 24 개월령의 한우 등심 단백질들을 길이 16 cm 튜브젤에서 등전점에 따라 분리하고, 이차원적으로 18×20 cm, 12% SDS-polyacrylamide gel 전기영동 하여 단백질을 분리하였다. 등전점 3.0에서 9.0 그리고 분자량 15,000에서 100,000 Da 사이의 단백질들이 분리되어 Silver 염색법으로 명확히 구분할 수 있었다. 흥미롭게, 성장과정에서 단백질 발현이 증가했거나 감소한 단백질들은 저분자 단백질들 이었다. 성장과정 중 증가된 단백질들을 분리하기 위해 수용성 단백질들을 조직으로부터 1% Triton X-100 으로 추출하였다. 그리고 이를 30%와 50% 황산암모니아로 분획하였다.이 와 같이하여 각 단백질들의 분리조건을 결정하였다. 이들 조건을 이용하여 발현이 증가된 단백질들을 분리하고 PVDF membrane에 옮겨서 아미노산 서열을 결정하여 단백질을 규명하였다.

찾는 말 : 마블링, 한우, 이차원 전기영동, 미세량 서열결정, 분획
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