Application of Gel-based Proteome Analysis Techniques to Studying Post-mortem Proteolysis in Meat

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ABSTRACT : This study was conducted to evaluate the possible application of 2 D-SDS-PAGE (2 DE)-based proteome analysis techniques to the assessment of extreme proteolysis in postmortem skeletal muscle. Eight Hanwoo longissimus muscles were incubated immediately after slaughter for 24 h at 5°C, 15°C or 36°C. Warner Bratzler (WB)-shear force and ultrastructural configuration were determined at 24 h, and rate of proteolysis to 24 h was determined by 1 D-SDS-PAGE (1 DE) and 2 DE. In addition, tentative protein identification was performed from peptide mass fingerprints of MALDI-ToF analysis of major protein groups on 2 DE profiles. The result showed that although ultrastructural configuration was similar between the 5°C and 36°C treatments, meat at 5°C had higher WB-shear force (approximately 5 kg greater). A higher rate of protein degradation at 36°C was observed based on Troponin-T degradation, 1 DE, and 2 DE analysis. This indicates that proteolysis during the early postmortem period was a significant determinant of shear force at 24 h. Little difference in proteolysis between 5°C and 15°C treatments was found based on classic 1 DE profile assessment. Meanwhile, considerable differences in the 2 DE profiles between the two treatments were revealed, with substantially higher rate of proteolysis at 15°C compared to 5°C. Nuclease treatment improved 2 DE profile resolution. 400 μg and 600 μg of sample loading appeared to be appropriate for 24 cm pH 3-10 and pH 5-7 IPG strips, respectively. Protein detection and quantification of the 5°C, 15°C and 36°C 2 DE profiles revealed 78, 163 and 232 protein spots respectively that were differentially modified in terms of their electrophoretic properties between approximately p*I* 5.3-7.7 with the molecular weight range of approximately 71-12 kDa. The current results demonstrated that 2 DE was a superior tool to 1 DE for characterising proteolysis in postmortem skeletal muscle. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 9 : 1296-1302)***

Key Words : Proteolysis, Proteome Analysis, Skeletal Muscle

INTRODUCTION

It has been estimated that for the human genome, the predicted 30,000-50,000 genes could encode as many as 250,000-500,000 protein moieties. The gene products go through more than 400 possible chemical modifications prior to their function, but the underlying mechanisms have not been clearly defined (Pennington and Dunn, 2001). For this reason, the rapidly developing field of proteome analysis has established itself as a crucial link to understanding how gene products communicate and work together (Campbell and Heyer, 2003). The prime goal of protein separation in most studies is to reduce the complexity of the mixture, and to characterize electrophoretic properties of proteins and their degradation products. In general, 1D-SDS-PAGE (1DE) separates approximately 50 bands on 15-cm gels, while 2 D-SDS-PAGE (2 DE) is capable of separating approximately 1,000-2,000 proteins depending on sample and detection method (Liebler and Yates, 2002). Although there are a number of limitations to using the 2DE technique, such as lack of repeatability and symmetrical overlapping in spots (Parker et al., 1998), it has been the main-stay for protein chemistry and physiology studies due to its high resolving power since its introduction (Pollard, 1994). In particular, the recent development of immobilized pH gradient (IPG) strips and high throughput downstream technologies such as image analysis, spot picking, digestion, and mass spectrometry analysis have allowed these techniques to become readily accessible for various applications including meat quality investigation (Lametsch and Bendixen, 2001).

Apart from inherent toughness, meat tenderisation is a function of myofibril-related proteolysis and the configuration of muscle fibers including the connective tissue matrix (Koohmaraie, 1996). Postmortem proteolysis is a significant factor determining myofibril-related meat tenderness (Hwang et al., 2003a). Degradation of myofibrillar proteins during the postmortem period has been studied by applying a number of non-gel based chemical measurements, including fragmentation of myofibrils (Hopkins et al., 2000), protein solubility and free amino acids (Claeys et al., 1994), and non-protein nitrogen (Davey and Gilbert, 1966). But the most frequently applied technique is gel based electrophoresis, with various detecting systems such as silver staining and enhanced chemiluminance (Olson et al., 1977; Koohmaraie, 1992; Ho et al., 1997; Wheeler and Koomaraie, 1999). However, one of the challenging problems in gel-based assessment includes limited resolution and repeatability (Hwang et al., 2003a). Application of proteomics in striated muscle was recently reviewed by Isfort (2002), who identified the 2 DE

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technique as a useful tool for determining qualitative and quantitative changes in proteins of striated muscle. In addition, this technique has been adapted in meat science for the assessment of postmortem proteolysis (Morzel et al., 2000; Lametsch and Bendixen, 2001). These previous studies have demonstrated that 2DE is a powerful tool in assessing postmortem proteolysis. This current study was conducted to evaluate the possible application of 2 DE to the assessment of extreme proteolysis in postmortem skeletal muscle.

MATERIALS AND METHODS

Sample preparation

M. longissimus thoracis et lumborum (from the 6th thoracic vertebrae to the last lumbar vertebrae) was obtained from eight Hanwoo steers immediately after slaughter, and incubated at 5°C, 15°C, or 36°C for 24 h. At the completion of incubation, muscle temperatures were equalized in running water and muscles immediately prepared for WB-shear force and electron microscope observation. For proteolysis analysis, approximately 3 g of tissue was taken at the beginning and completion of incubation, frozen in liquid nitrogen, and stored at -70°C until analysis.

WB-shear force and electron microscope

Steaks of 2.54 cm thickness were cooked in a 70°C water bath for 60 min and cooled in running water (ca. 18°C) for 30 min so that core temperature reached below 30°C. Eight cores were made 1.27 cm in diameter and shear force was measured using a V-shaped shear blade at 400 mm/min speed (Wheeler et al., 2000). Ultrastructural observation was made according to the method described by Hwang and Thompson (2002) using an electron microscope (Carl Jeiss, Japan) at an accelerating voltage of 100 K vol.

1D-SDS-PAGE and immunoblotting

Frozen tissue samples were initially powdered in liquid nitrogen using a mortar-based homogenizer (Warning, Dynamics Corp., USA), and 300 mg of the homogenized sample was used for SDS-PAGE according to the method of Laemmli (1970). Samples were separated on resolving gels of 12.5% (8.3×5.5 cm) or 6-15% (16×22.5 cm) acrylamide (37.5:1 ratio of acrylamide to bisacrylamide) with 4% acrylamide (37.5:1) stacking gels. The discontinuous gels were run at 200 V for 45 min for the homogenous gel, and at 20 mA/gel for 2 h, followed by at 40 mA/gel for 6 h for the gradient gel. Gels for western blotting were transferred to Hybond-P polyvinylidine fluoride (Amersham, Arlington Heights, IL) at 200 mA for 60 min in ice-cold transfer buffer (25 mM Tris, 193 mM glycine, and 10% methanol). Membranes were then blocked with 5% non-fat milk in tris-buffered saline, pH 7.4, containing 0.05% Tween-20 (TTBS) for 60 min at room temperature with gentle rocking. Blots were incubated with anti-Troponin-T (Clone JLT-12, Sigma) for 60 min under the same conditions. The bound primary antibodies were then labeled (60 min at room temperature) with goat anti-mouse IgG alkaline phosphatase conjugated secondary antibody (Promega, WI). The bound antibodies were visualized by incubating membranes with BCIP/NBT substrate (Bio-Rad laboratories, CA), according to the method described by Hwang (2004).

2D-SDS-PAGE and visualization

Frozen tissue samples were initially powdered as described above, and 50 mg of the sample was homogenized using a handheld homogenizer for 1 min in 1 mL lysis buffer, containing 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, and 0.8% Pharmalyte pH 3-10 nonlinear. After the homogenization, the lysate was vortexed for 1 min and incubated at room temperature for 30 min with rotational shaking. The lysate was then centrifuged at 40,000×g for 1 h at 20°C, and the second layer of supernatant was collected. Protein concentration was determined in triplicate according to the method described by Westermeier and Naven (2002) which is compatible with the sample preparation reagents such as 1% DTT, 8 M urea, 2 M thiourea, 4% CHAPS, and 2% Pharmalyte (Amersham Pharmacia Biotech, Amersham, UK). Protein concentration was measured spectrophotometrically at 480 nm with the 0-50 μg of BSA for the standard curve. The samples were diluted with rehydration solution, containing 8 M urea, 0.5% CHAPS, 0.2% DTT, and 0.2% Pharmalyte pH 3-10. Isolectric focusing (IEF) was performed using IPG strips of pH 3-10 nonlinear (Amersham Pharmacia Biotech, Amersham, UK). The strips were rehydrated for 12 h with the sample mixed rehydration buffer (450 μg/strip) and focused at 50 μA/strip for 70 kVh at 20°C. At the completion of focusing, the strips were equilibrated in 50 mM Tris-HCl buffer, pH 8.8, containing 6 M Urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 1% DTT for 15 min, followed by an additional incubation for 15 min with the same buffer replacing DTT with 4.8% iodoacetamide. The second dimension SDS-PAGE was performed with 12.5% acrylamide (37.5:1 ratio of acrylamide to bisacrylamide) using the Ettan DALT system (Amersham Pharmacia Biotech, Amersham, UK) at 17 W/gel and 15°C. Gels were stained by using a silver staining kit (Amersham Pharmacia Biotech, Amersham, UK), or by 0.1% colloidial Coomassie Brilliant Blue G-250 in 2% phosphoric acid, 10% ammonium sulphate, and 20% methanol for 48 h. The remaining dye solution was removed with a brief washing with distilled water and

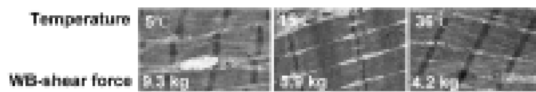

Figure 1. Representative electron microscope and WB-shear force for longissimus muscle incubated at 5°C, 15°C or 36°C for 24 h. The images demonstrating ultrastructural configuration of cold and rigor shortening were similar, but shear force for the 5°C treatment was ca. 5 kg higher than that at 36°C.

scanned with an optical densitometry scanner (UMAX PowerLook 1100 (Amersham Pharmacia Biotech, Amersham, UK).

Image analysis and identification

Triplicate gels for each treatment were analyzed using a 2 DE image analysis software (ImageMaster, Amersham Pharmacia Biotech, Amersham, UK) according the manufacturer's instruction. Intensity calibration was set and manually adjusted where necessary. Spots appearing in more than two gels within 50% of variation in optical density were accepted as analytical spots, and average normal volume was used for the final analysis. For peptide mass fingerprint (PMF)-based protein identification, spots of interest were excised and destained with 100 μL aliquot of 50% acetonitrile in 25 mM ammonium bicarbonate until visual dye was removed. The selected samples were then finally washed with 100 μL of 100% acetonitrile, followed by drying in a centrifugal evaporator. Samples were rehydrated in 10 μL (250 ng) of methylated porcine trypsin (Promega, Madison, USA) in 25 mM ammonium bicarbonate (pH 8.8) for 45 min, the remaining trypsin solution was removed, and then the samples were incubated at 37°C overnight. Digestion was stopped by adding 50 μL aliquot of 50% acetonitrile and 5% trifluoroacetic acid, sonicated at 37°C in ultrasonic water bath for 10 min, and agitated for 60 min at room temperature. The recovered peptide extract was concentrated by reducing the final volume to approximately 10 μL in a vacuum centrifuge, and prepared for spotting for PMF analysis using Voyager Elite MALDI mass spectrometer (PE Biosystems, Framingham, MA, USA). Peptides were bound to a ZipTip (Millipore, USA) and spotted into MALDI target by mixing with 2 μL of α-cyano-4-hydroxycinnamic acid (10 mg/ml). Data were averaged for 200-300 laser shots in the positive ion reflectron mode at 20 kV accelerating voltage, with 66 grid percentage and 120 nsec delay time. Tentative protein identification was performed by matching PMF at SWISS-PROT and NCBI protein database using ExPASy search engine (http://us.expasy.org) with 50 ppm of mass tolerance, maximum missed cleavage of one (1) and a possible modification of carbamidomethylation of cystein.

RESULTS AND DISCUSSION

WB-shear force and 1D-SDS-PAGE

The first report on muscle shortening and its relation to meat toughness was a significant breakthrough for meat tenderness control, and numerous studies have followed in order to understand the underlying mechanism for cold and rigor shortening (Locker, 1960; Devine et al., 1999; Hwang et al., 2003a). As seen in Figure 1, under the current experimental condition, muscles placed at 5°C and 36°C appear to have visually shorter sarcomeres compared with those at 15°C. This is consistent with the previous study by Bendall (1973) where sarcomere shortening occurred either below 10°C with pH higher than 6.2, or higher than 30°C with pH lower than 6.2. It was noticeable that visual objective assessment of ultrastructural configuration for muscles at low and high temperature (i.e., cold and rigor shortening) were similar, indicating that physical configuration of shortened muscle was independent of shortening temperature.

Beef muscles that enter rigor mortis at approximately 10-18°C result in the most tender meat (Tornberg, 1996; Devine et al., 2002), which is attributed to minimum sarcomere shortening and maximum ageing rate (Devine et al., 1999; Hwang and Thompson, 2001a). As muscle shortening takes place under abnormal pH-temperature conditions during rigor development, the toughening effect of cold and rigor shortening *per se* could mirror changes in the biological environment of muscle tissue that influence endogenous proteolytic activity (Koohmaraie, 1996; Moon et al., 2003). Both muscle shortening and proteolysis require free calcium ions in the cytoplasm, which is a function of the pH-temperature interaction during rigor development in spite of the complexity in biological regulation by sarcoplasmic reticulum and mitochondria (Jaecocke, 1993; Westerblad and Allen, 1993). This implies that muscle shortening and proteolysis is a concomitant process, which could have been the case in this current experimental condition. In other words, higher rate of proteolysis occurred at higher temperatures as a consequence of a faster decline in pH under which μ-calpain-mediated proteolysis was accelerated (Hwang and Thompson, 2001b, 2003b; Min et al., 2002; Koohmaraie, 1996).

The extreme range of incubation temperatures applied in this study rarely occurs in the beef carcass, but was used in this experiment to induce a large difference in proteolysis and meat tenderness. The experimental design appears to have achieved extremes in sarcomere length and WB-shear force and proteolysis. These results show that the impact of extreme conditions at rigor are reflected in shortened sarcomeres at both the 5°C and 36°C treatments. In spite of the similarity in sarcomere length, the 5°C treatment showed a substantially higher WB-shear force than that for

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메모 [m1]: A general comment on this paper is that you are drawing very strong conclusions from your data. I don't think that your analysis can support some of your assertions. I would be more reserved.

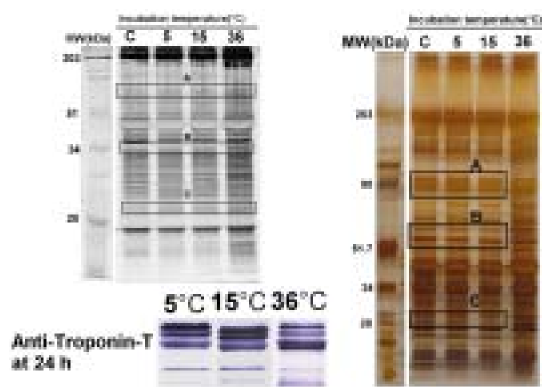

Figure 2. Effect of incubation temperature on proteolysis for 24 h, assessed by homogenous 12.5% min-gel (left upper), large gradient gel (6-15%) and western blotting for Troponin-T. C: immediately after slaughter. Muscle incubated at 15°C had a tendency to have faster rate of proteolysis than that at 5°C, but resolution was very limited.

the 36°C treatment. This result was rather anticipated, considering that an extensive proteolysis was expected at 36°C as compared to that at 5°C. This was supported by 1 DE and 2 DE assessments including Troponin-T degradation (Figures 2 and 3). The importance of proteolysis for meat tenderness was further supported by shear force at 15°C with longer sarcomere length being higher than that of the shortened muscle at 36°C (Dransfield et al., 1994). Some aspects of the results were reported by Hwang et al. (2004). This suggests that ultrastructural conformation had a limited effect on meat toughness compared to postmortem proteolysis. In this regard, these results raised a question about the relative effect of changes in connective tissue matrix in shortened muscle on meat toughness. As previous studies have shown, meat toughness is also related to structural rearrangement of the connective tissue matrix (Bailey and Light, 1989).

At first glance, it appeared that proteolysis between 5°C and 15°C was similar by visual assessment using classic western blotting for Troponin-T, which has been identified as a good indicator for postmortem proteolysis (Ho et al., 1997; Hopkins and Thompson, 2001; Geesink et al., 2001). In addition, the trend was also similar for silver stained mini-gel system (8.3 cm×5.5 cm) where 7 μg of SDS-bound sample was separated. On the other hand, the tendency of a higher rate of proteolysis at 15°C became more evident when samples were separated by a long gel system (16 cm ×22.5 cm) by which 100 μg of sample was resolved, followed by an extensive silver staining (Figure 2). This suggested that proteolysis at 15°C was greater than that at 5°C, and that extra caution should be taken when interpreting the result of electrophoresis using mini-gels.

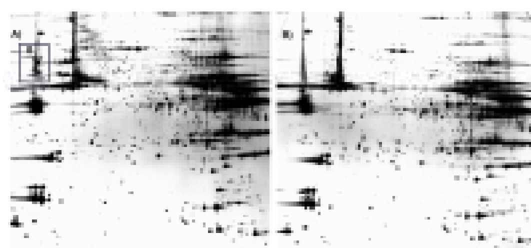

Figure 3. Effect of nuclease treatment (B) compared to non-treated sample (A) on resolution properties of 2DE for pH 3-10. 120 μg was loaded, followed by silver staining. There was a tangled protein complex in near acid area (square S) for non-treated sample.

Proteolysis and 2 DE-SDS PAGE analysis

A typical 1 DE separates approximately 50 bands on 15-cm gel, while 2 DE resolves approximately 1,000-2,000 proteins, depending on the sample and detecting method (Liebler and Yates, 2002). The super resolution presages the powerful potential of the 2 DE technique for studying postmortem proteolysis. This technique has been used largely in protein chemistry and physiology studies since its first introduction in the early 1970s (Pollard, 1994). However, with the recent development of the IPG strip and high throughput downstream technologies, the application of this technique has broadened to a range of scientific disciplines including studying aspects of meat quality. According to Lametsch and Bendixen (2001), changes in 1,000 individual spots can be identified during rigor development in pork longissimus muscle.

For successful proteome analysis, sample preparation for targeted protein groups is the most crucial process. Nucleic acids cause a number of problems during electrophoresis as they behave as polyanions, and are therefore able to bind many proteins through electrostatic interactions. This property prevents electronic forcing, causes background smears, and clogs gel pores (Rabilloud, 1996). This study examined the effect of nucleic acids on electrophoretic property by digesting the acids to mono- or oligonucleotides with a mixture of nucleases. Figure 3 shows that digestion of nucleic acids improved resolution, noticeably at acidic region of approximately 70 kDa.

Amount of sample loading and pre-fractionation is another parameter for successful separation of proteins. As much as 90% of the total protein in a typical cell is made up of only 10% of the 10,000-20,000 different protein species, and consequently, many low-abundance proteins may not be detectable by 2 DE (Lilley et al., 2001). In skeletal muscle, approximately 70% of proteins are associated with the contractile apparatus. When a single protein is applied in very high quantity on an IPG strip, the gel builds a high ridge at the isoelectric point of the protein, and the focusing force exudates a part of the protein through the gel surface,

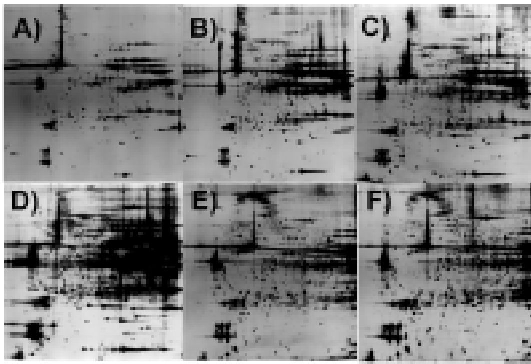

Figure 4. Resolution profiles for sample loading of 100 µg (A), 200 µg (B), 400 µg (C) and 600 µg (D) for pH 3-10 IPG strip, and 300 µg (E) and 600 µg (F) for pH 4-7 IPG strip, followed by silver staining.

resulting in a horizontal streak over the second dimension gel (Westermeier and Naven, 2002). In addition, because a high-resolution 2 DE technique can separate as many as 3,000 spots, some proteins inevitably co-migrate to the same spot position, and many symmetrical spots contain two or more proteins. It has been shown in eukaryotes that approximately 40% of proteins overlapped with two or three proteins (Parker et al., 1998).

This fact indicates that pre-fractionation of sample and amount of sample loaded needs to be optimized for a particular sample, although a one-step procedure for protein extraction would be highly desirable with respect to simplicity and reproducibility. This study initially examined how fractionation affected resolution according to solubility (Scopes, 1994), but the result turned out to have a significant shortage of repeatability, as well as high risk of protein loss and modification during the process (data not shown). Based on this result, a one-step whole extraction method was adapted for protein separation. Figure 4 shows the resolution property for various amounts of sample loading. The gels showed that 400 µg for 24 cm pH 3-10 IPG strip and 600 µg for pH 4-7 IPG strip were appropriate in terms of best resolution of spots. Based on the result, 120 µg for analytical silver staining and 400 µg for preparative Coomassie Blue G-250 staining were used, and approximately 1,500 and 700 spots were detected by silver and Coomassie staining gels, respectively.

Figure 5 shows a number of milestone proteins, difference in electrophoretic properties, and number of matched and unmatched spots for samples incubated at 5°C, 15°C and 36°C. The total number of spots were 206, 306, 397 and 432 for samples immediately after slaughter (0 h) and after incubation at 5°C, 15°C and 36°C for 24 h. Respectively, 78, 163 and 232 spots were unmatched and 228, 234 and 200 spots were matched with the 206 spots at

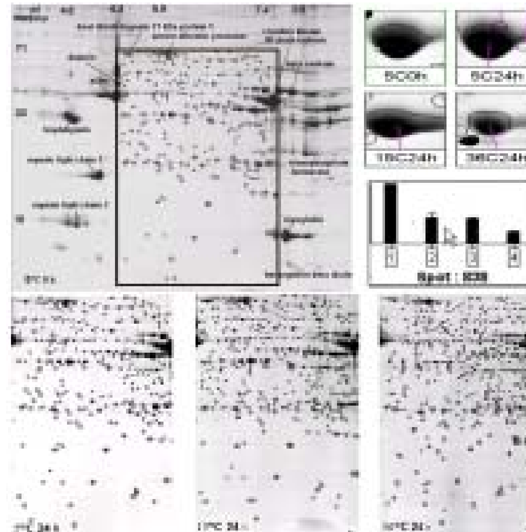

Figure 5. 2DE profiles and tentative identification for longissimus muscle immediately after slaughter (5°C, 0 h), and at the completion of incubation for 24 h at 5°C (5°C, 24 h), 15°C (15°C, 24 h), or 36°C (36°C, 24 h). The total number of spots were 206, 306, 397 and 432 for 5°C, 0 h; 5°C, 24 h; 15°C, 24 h; and 36°C, 24 h, respectively. In comparison with 5°C, 0 h; 78, 163 and 232 spots were unmatched and 228, 234 and 200 spots were matched at 5°C, 15°C and 36°C, respectively. The right upper figures show an example of difference in volume for triosephosphate isomerase (spot 838) at different incubation temperatures, indicating decrease in volume with higher temperature.

0 h. This indicated that approximately 88, 89 and 74% of protein was unchanged, while 26, 41 and 54% of spots were changed at 5°C, 15°C and 36°C at 24 h incubation. Because of the inherent variation in this technique, the average of triplicates was used for the analysis, but changes in volume were not calculated, as there was a large variation between gels. Nevertheless, there was a tendency for the volume to decrease at higher temperatures even for the matched spots. Figure 5 shows an example of a higher rate of degradation in higher temperatures for thiosephosphate isomerase. As discussed earlier, in spite of the use of large gels for 1 DE and extensive silver staining, few bands differed between the 5°C and 15°C treatments. On the contrary, difference in proteolysis between the two groups was clearly demonstrated by using 2 DE analysis, demonstrating that this is a more powerful tool for studying postmortem proteolysis. Figure 5 shows a 2 DE profile and protein names tentatively identified by peptide mass fingerprint in SWISS-PORT and NCBI protein database. Actin isoforms, beta enolase, and creatin kinase M chain isoform groups were dominant protein groups under the current electrophoretic conditions. This profile has a high

homology with rat abdominal and pork longissimus muscles (Lametsch and Bendixen, 2001; Yan et al., 2001). This suggests that these protein groups need to be removed to investigate low abundance proteins in skeletal muscle. The result suggests that increased proteolysis during early postmortem proteolysis could override the detrimental effect of muscle shortening on meat toughness provided that postmortem proteolysis is maximized. In addition, this study proved that 2 DE analysis techniques are a superior tool for accessing subtle difference in proteolysis in skeletal muscle compared to classic 1DE techniques.

IMPLICATIONS

2 DE profiles demonstrated that incubation at 5°C, 15°C, and 36°C for 24 h changed 26, 41 and 54% of spots, respectively, in terms of eletrophoretic property of proteins between approximately p*I* 5.3-7.7 with the molecular weight range of approximately 71-12 kDa. In particular, 2 DE analysis techniques clearly demonstrated a large difference between the 5°C and 15°C treatments, while the difference was not clear for classic 1 DE technique. This result proved that the 2 DE technique is a superior tool to the classic 1 DE technique for characterising proteolysis in postmortem skeletal muscle. However, optimization of analysis conditions is important to ensure the best repeatability and separation for a particular protein group by each laboratory system. The current result also implies that extensive proteolysis could override the adverse effects of shortened sarcomeres on meat tenderness provided that myofibril-related meat tenderization is optimized during the early postmortem period.

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