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Protein Changes in Longissimus Dorsi Muscle of Chinese Yellow Crossbred Bulls as a Result of Electrical Stimulation

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Abstract: Protein changes induced by low-voltage Electrical Stimulation (ES; 42V for 40 sec) in the insoluble protein fraction of bovine Longissimus Dorsi (LD) muscle at 3 days post-ES were investigated by proteomics. Protein abundance patterns from ten Chinese yellow crossbred bulls were compared and significant changes due to ES were found. Seven protein spots showed lower expression abundance in 3 days post-ES samples including myofibrillar and cytoskeletal protein (myosin binding protein H, histone H3.3-like isoform 2) two metabolic enzymes (creatine kinase, triosephosphate isomerase) and an unnamed protein due to ES. Reduced abundance of these proteins in ES samples indicated an accelerated proteolysis due to ES which finally improves tenderness. The most important finding was that an unnamed protein product was found to change in abundance due to ES at 3 days post-ES and this protein could provide a new target as a potential meat quality biomarker. These findings provided a better understanding of the biochemical processes taking place as a result of ES during postmortem storage of meat.

Key words: Chinese yellow crossbred bulls, muscle protein changes, beef quality, electrical stimulation, proteolysis, two-dimensional gel electrophoresis

INTRODUCTION

Meat quality is determined by various factors such as meat color, fat color, tenderness and intramuscular fat content (Geay *et al*., 2001; Maltin *et al*., 2003; Hocquette *et al*., 2005). Among these factors, meat tenderness is the most important which determines the commercial value of meat products (Kim *et al*., 2008). Numerous studies have shown that variation in postmortem glycolytic rates in muscles from different carcasses yields aged meat of varied tenderness (O'Halloran *et al*., 1997). It is generally accepted that degradation and denaturation of proteins during postmortem aging is responsible for the tenderization of meat (Koohmaraie, 1996; Kwasiborski *et al*., 2008) e.g., activities of calpains and their inhibitors, calpastatins (Huang and Forsberg, 1998; Doumit and Koohmaraie, 1999) have been associated with tenderization processes. Several structural proteins such as actin, myosin, troponin-T and metabolic proteins such as glycogen phosphorylase, creatine kinase and dihydrolipoamide succinyl transferase have been described to undergo postmortem changes in Longissimus dorsi.

As slowly glycolysing muscles yield tough meat, Electrical Stimulation (ES) is widely used to accelerate postmortem glycolysis resulting in a rapid pH decline and earlier development of rigor mortis and finally improve tenderness (Olsson *et al*., 1994; Polidori *et al*., 1999; Luo *et al*., 2008). However, the mechanisms that are unleashed by ES stimulation are not well understood. Previous studies have also reported that ES enhances the proteolysis of myofibrillar and cytoskeletal proteins postmortem (Ferguson *et al*., 2000; Rhee *et al*., 2000; Lonergan *et al*., 2010) including proteins such as desmin, troponin-T, titin and nebulin (Ho *et al*., 1996, 1997; Bjarnadottir *et al*., 2011). Furthermore, ES has been reported to be related to the activation of Calpains system and metabolic enzymes (Hwang and Thompson, 2001; Lametsch *et al*., 2002). The techniques generally applied in these studies are sensitive and specific for looking at target proteins or protease activity. However, proteome analysis allows for a more global investigation of the dynamic process unleashed by ES and postmortem storage. As with electrical stimulation, biomarkers for muscle growth and meat quality traits will allow scientists to build and test better hypotheses and meat producing industry and consumers will benefit from better indicators for meat quality (Li *et al*., 2006). The aim of this study was to identify proteins in the insoluble protein fraction of bovine Longissimus Dorsi (LD) muscle that changed in abundance as a result of ES and to investigate their potential as biomarkers for tenderness.

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MATERIALS AND METHODS

Animals and sampling: Ten Chinese yellow crossbred bulls (Yan-bian×Simmentals) aged 18 months were selected, randomly divided into two groups and slaughtered. The mean slaughter weight was 629.0±50.3 kg. One group was subjected to Electrical Stimulation (ES: 42V, 50 Hz, 0.7 A for 40 sec) with a commercial ES unit (EST-608, Freund, Germany) immediately after bleeding. The stimulation was applied via the neck region of the carcass using the rail as the earth. The other group that was not subjected to ES was referred to as the Non-Stimulated control (NS). The experiment was undertaken following the guidelines of the Animal Ethics Committee in Shandong Agricultural University and all experimental procedures were approved by the State Scientific and Technological Commission (China, 19881114).

Samples (10 g) were removed from the LD muscles (between the 12th and 13th rib interface) by an expert butcher. After 3 days in the conditioning room, the carcasses were transferred to the cutting room and the LD muscles were excised from the carcasses. The boned LD muscles were vacuum-packed and kept at 4°C during the postmortem storage period. A piece of muscle tissue was taken at 3 days post-ES, snap frozen in liquid nitrogen and stored at -80°C until further analysis. Thus, the experiment included two treatments (ES and NES) on ten animals (biological replicates).

Extraction of muscle proteins: 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 (pH3-10, 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.

Two-dimensional gel electrophoresis (2-DE): Samples of approximately 400 μg for analytical gels were applied to 24 cm Immobilized Ph Gradient (IPG) strips (pH 3-10, nonlinear, Bio-Rad). IPG strips were rehydrated containing 8 M urea, 0.5% CHAPS, 0.28% DTT, 10% glycerol, 0.5% bio-lyte ampholyte0s (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, 200, 500 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 coomassie brilliant blue staining.

Image analysis and data analysis: 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 CBB-stained protein spots that demonstrated 2.0 fold up differences in staining densities between 3 days post-Electrical Stimulated (ES) and Non-Stimulated control (NES) samples were selected and the proteins were identified.

Mass Spectrometry (MS) analysis: The differentially expressed spots were excised from the CBB-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^{-1} 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% Trifiouroacetic Acid (TFA), desalted using a C 18 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.

RESULTS AND DISCUSSION

Researchers obtained the protein from the Longissimus dorsi muscle of a total of ten Chinese Yellow crossbred bulls in the present study. Samples were collected from both 3 days post ES and NES samples. Approximately, 669 spots were detected in the coomassie brilliant blue-stained two-dimensional gels by computer-assisted image analysis (Fig. 1).

About 7 spots displayed 2.0 fold up differences in density of coomassie brilliant blue staining between 3 days post-Electrical Stimulated (ES) and Non-Stimulated control (NES) samples (Fig. 2). Figure 3 shows a representative image of the 2-DE pattern where 7 spots are marked. Seven points were identified based on their molecular weights isoelectric points, molecular characterization and function using the known protein databases (Table 1).

All of the seven identified proteins that were found to change significantly in abundance at 3 days post-ES had reduced abundance in the ES samples. Among them myofibrillar and cytoskeletal protein (myosin binding protein H and histone H3.3-like isoform 2) and two metabolic enzymes (creatine kinase, triosephosphate isomerase) were found to change due to ES. In addition, an unnamed protein product was also found to change in abundance due to ES at 3 days post-ES. In this study, the goal was to obtain more knowledge about the biochemical processes caused by ES treatment. Changes in abundance were observed for myofibrillar and cytoskeletal proteins. The proteins were identified as myosin binding protein H and histone H3.3-like isoform 2 which showed lower abundance at 3 days post-ES. Histone is linked to many different types of proteins involved in DNA reparation, cell cycle, apoptosis, oxidative stress, metabolism and calcium signaling. During stress, histone recruits metabolism enzymes to activate energy generation, enhance protein synthesis and recruit anti-stress proteins to protect cells from degradation. Histone modulates different pathways so is an interesting protein to study for tenderness. As histone recruits stress proteins during oxidative stress this protein could play a central role in stress pathways involved in tenderization processes (Yang *et al*., 2010). Myosin binding protein H which belongs to the contractile apparatus (Bouley *et al*., 2004) may modulate the interaction between myosin and actin.

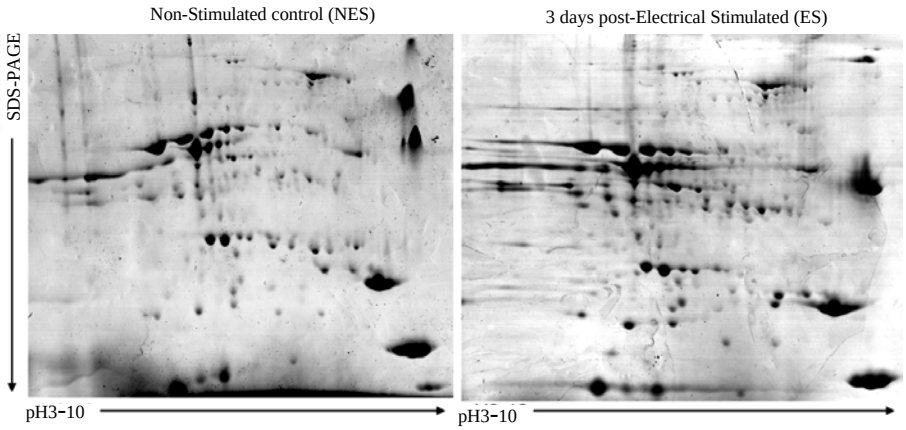

Fig. 1: Comparative analysis of the expressed protein patterns in pre- and post-treated groups. Protein (400 μg) was loaded and separated in the IPG 3-10 NL strip and an SDS gel (12.5% T). This shows only the parts of the 2-DE images that are useful for computer-assisted image analysis

Table 1: Identified proteins from *M. longissimus* muscle of Chinese yellow crossbred bulls changed in abundance between Non-Stimulated control (NES) and 3 days post-electrical Stimulated (ES) samples

Spot No.	Protein name	NCBI Accession No.	PMF (MS) Score	Peptides matched	Peptides obtained	Sequence coverage (%)	Theoretical molecular pI/mass (Da)
Cytoskeletal proteins, 2 of 7 proteins							
6703	Myosin binding protein H	gi|296479376	144	16	92	50	5.82/53686
6705	Histone H3.3-like isoform 2	gi|296202278	54	7	92	38	11.58/13636
Metabolic enzymes, 4 of 7 proteins							
3703	Creatine kinase M-type	gi|60097925	194	24	98	61	6.63/43190
4306	Triosephosphate isomerase	gi|61888856	210	17	95	82	6.45/26901
5310	Triosephosphate isomerase	gi|61888856	127	15	91	75	6.45/26901
3703	Creatine kinase M-type-like isoform 1	gi|60097925	87	12	55	38	6.58/39717
Other							
3302	Unnamed protein product	gi|90075594	81	9	64	50	6.64/15534

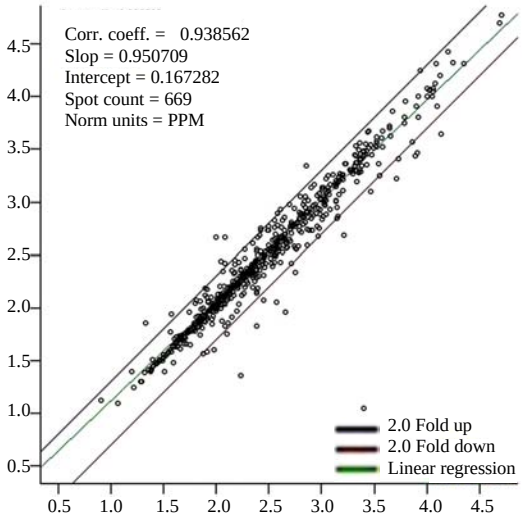

Fig. 2: Scatter plot showing gel relatedness. The spots displayed 2.0 fold up and down differences in density of coomassie brilliant blue staining between 3 days post-Electrical Stimulated (ES) and Non-Stimulated control (NES) samples

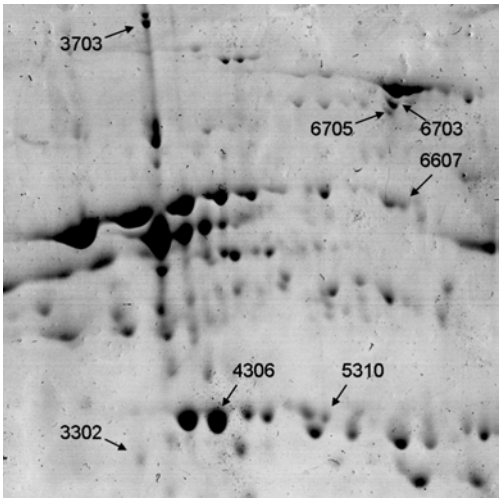

Fig. 3: Representative 2-DE pattern of the protein extracted from *M. Longissimus* muscle of Chinese yellow crossbred bulls. Seven spots are numbered

The postmortem degradation of myosin binding protein H has been reported earlier and is believed to be related to tenderness (Ouali, 1990; Vaughan *et al*., 1993). The observation of decreased abundance of the myofibrillar and cytoskeletal proteins indicates their degradation in the ES treated animals which might be one of the factors giving lower WB shear force and hence improved tenderness in those samples. Furthermore, these findings support the theory that ES affects meat tenderness by accelerating postmortem proteolysis of myofibrillar and cytoskeletal proteins as has been reported by earlier studies (Dransfield *et al*., 1992a, b; Geesink *et al*., 2001; Sun *et al*., 2008).

Previous results indicate that the observed improvements in tenderness by ES were caused by other mechanisms than through accelerated pH decline and prevention of cold shortening (White *et al*., 2006; Hollung *et al*., 2007). After slaughter, the energy metabolism in the muscle is switched from aerobic to anaerobic metabolism and the two most important sources of ATP are degradation of glycogen to lactic acid and the transfer of phosphate from creatine phosphate to ADP (Poso and Puolanne, 2005). Electrical stimulation is known to accelerate postmortem glycolysis (Davey *et al*., 1976; Smulders *et al*., 1986; Eilers *et al*., 1996) and several metabolic enzymes were found to change in abundance due to ES in this study. Creatine kinase catalyses the reversible transfer of a phosphate group between ATP and ADP. This enzyme was in a previous study of bovine muscle found to degrade during the first 24 h postmortem in the soluble protein fraction (Jia *et al*., 2007). Furthermore, fragments of creatine kinase have been found to increase in abundance in the total protein fraction during postmortem storage of porcine (Lametsch *et al*., 2003) and in the insoluble protein fraction of bovine meat (Bjarnadottir *et al*., 2010). In the present study, two creatine kinases were found to decrease in abundance at 3 days post-ES. A previous study reported that approximately, 50% of the creatine phosphate stored in the muscles was consumed during ES and at 6 h postmortem, this protein was depleted in ES samples (Fabiansson and Reutersward, 1986). Moreover, the half-life of ATP was found to be much lower in ES samples compared to NES samples (Fabiansson and Reutersward, 1986). It is therefore not surprising to observe lower abundance of creatine kinase at 3 days post-ES given that the increase in energy consumption is so high during ES.

Triosephosphate Isomerase (TIM) is an enzyme with a role in glycolysis and gluconeogenesis by catalyzing the interconversion between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Bauman *et al*., 2002). This enzyme has been used as a target for drug design (Enriquez-Flores *et al*., 2008). Increased intensity of glycolytic enzymes after slaughter might be a result of enhanced rate of glycolysis to support and maintain the ATP production. A previous study showed that electrical stimulation accelerated the glycolytic processes which resulted in an immediate increase in muscle lactate and about 30% reduction in ATP (Kondos and Taylor, 1987). However in the present study, triosephosphate isomerase was found to decrease

in abundance in the ES samples at 3 days post-ES which indicates that the enzyme was degraded in 3 days post-ES treated animals. In rencent studies of porcine skeleton muscleproteins during postmortem storage, triosephosphate isomerase also degraded after slaughter (Bernevic *et al*., 2011).

Among the decreased proteins visualized on 2-D gels, researchers also identified an unnamed protein. Researchers first analyzed this protein by peptide mass fingerprinting. Nine peptides matched with gi|90075594 giving a sequence coverage of 50%. The unnamed protein may be interesting because it might be muscle specific and play a role in muscle physiology. It may be potential as a biomarker for tenderness. In further research, the structure and function of this unknown protein can be studied in gene level and protein level and can provide a new idea to improve meat tenderness.

CONCLUSION

The results show that these proteins may be useful as biomarkers for meat quality. The unnamed protein was identified as a new muscle protein related to the quality of beef.

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