DEGRADATION PRODUCTS OF BOVINE MYOFIBIRL PROTEINS UNDER OXIDATION AND REDUCTION CONDITIONS BY m-CALPAIN AND CATHEPSIN B

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Abstract - The active site cyteine of calpain is susceptible to oxidative inactivation. Reducing conditions are needed for calpain to remain active and oxidative conditions are decreased to calpain activity. Postmortem changes in muscle are accompanied by weakening of the anti-oxidation defense system. Therefore, oxidative processes in postmortem muscles affect the rate of tenderization by negatively influencing calpain activity. The present study was designed to identify myofibril proteins degraded by μ-calpain and cathepsin B under oxidation and reduction conditions. Purified bovine myofibrils were incubated with one of following mixtures at 25°C for 24 hr: 1) 100 μM H_2O_2, 2) 1% MCE, 3) μ-calpain with 100 μM H_2O_2, 4) μ-calpain with 1% MCE, 5) cathepsin B with 100 μM H_2O_2, and 6) cathepsin B with 1% MCE on bovine myofibrils. Degradation products of myofibrils were determined by a 2DE SDS-PAGE, accordingly identified by LC/MS-MS. Data from 1DE-SDS-PAGE and western blotting for Troponin-T and desmin revealed that both proteases showed greater activity in reduction condition than control and oxidation conditions in that both proteases resulted in a similar proteolysis. A total of 33 protein spots (including creatin kinase M, myosin, actin, myosin heavy chain, myosin light chain, troponin-T, titin, HSP 27, and crystalline) were apparently changed during incubation period with μ-calpain and cathepsin B under oxidation or reduction conditions. Based on the number of protein sported affected by the treatment, myofibril proteolysis by μ-calpain and cathepsin B was greatly affected by biological status of protein (i.e., oxidation or reduction). It was particularly notable that myosin and actin are greatly changed by both proteases under reduction condition, but not under oxidation condition. Furthermore B cathepsin B was more greatly affected by incubation environment compared to these for μ-calpain.

***Index Terms*— μ-calpain, cathepsin B, reduction, oxidation**

I. INTRODUCTION

Variation in beef tenderness is a major problem in quality control program for beef industry and texture is rated as most important by the consumer. It has been known for a long time that meat tenderness improves during cooler storage, and it was suggested almost a century age that this is enzymatic activity (Hoagland et al., 1971). Cytoskeletal protein proteolysis is considered the major contributor to meat tenderization during ageing (Sentandreu, Coulis, & Quali, 2002) and three main proteolytic systems, which are cathepsins (the lysosomal protease), calpain (calcium-dependent proteases), and proteasomes participate.

Calpains are a family of cystein proteases that depend on calcium and a decreased calpain activity modulated by oxidation state *in vitro* (Guttmann et al., 1997). The reason for this is likely due to the mechanism by which μ-calpain hydrolyzes a peptide bond. The effect of an oxidizing environment on μ-calpain activity are important to understand because oxidative stress has been linked to pathological states in which μ-calpain has been suggested to play a role. Although the role of calpains in meat tenderization and in cytoskeletal proteins degradation post-mortem is well recognized, however, they cannot univocally explain all the changes and cytoskeletal protein breakdown observed post-mortem. More to the point, the active site cysteine of calpain is susceptible to oxidative inactivation. Reducing conditions are needed for calpain to remain active. Postmortem changes in muscle are accompanied by weakening of the antioxidant defense system. Consequently, oxidative processes in postmortem muscles affect the rate of tenderization by negatively influencing calpain activity. Likewise, cathepsin B is the most abundant lysosomal protein, but its ability to degrade cytoskeletal proteins is dependent on its release post-mortem in the cytosol. As a result, although cathepsins appear to play considerable role *in vivo* in muscle protein turnover during animal growth and development and in muscle fiber destruction in inflammatory myopathies, their role in muscle tenderization and their ability to degrade cytoskeletal proteins is still controversial, even though release of cathepsins from lysosomes during post-mortem storage has been clearly demonstrated.

Direct evidence of the abilities of calpains and cathepsins to degrade cytoskeletal proteins at the molecular level would give a better insight of the proteolytic processes taking place during meat ageing and their implications for muscle food quality. Reactive oxygen species or free radicals produced either as a natural consequence of cellular metabolism or as a result of pathological events are known to attack lipids and protein, resulting in cellular injury. Oxidative processes are known to be the major causes of meat quality deterioration such as flavor, color, and nutritional

composition (Asghar et al., 1988). The present study was designed to identify myofibril proteins degraded by µ-calpain and cathepsin B under oxidation and reduction conditions.

II. MATERIALS AND METHODS

A. Samples preparation for SDS-PAGE and isolated myofibrils

Samples were from three beef carcasses. The muscles were taken from the *longissimus* muscles immediately after halving. Approximately 10 g of sample were frozen in liquid nitrogen as a 0 day sample, whereas the other sample was aged for an additional 7 days at 4°C and then frozen at -80°C. Samples were initially powdered in liquid nitrogen using a homogenizer, for SDS-PAGE according to the method of Laemmli (1970). Myofibrils were isolated from bovine skeletal muscle by the procedure of Goll et al. (1974), suitably modified. *Longissums* muscle at 300 mg was homogenized in 10 times the volume (enough to cover the tissue) of standard salt salution (SSS buffer: 20 mM potassium phosphate, 2 mM $MaCl_2$, 2 mM ethylendiamintetraacetic acid (EDTA), 1 mM Sodium NaN_3, and 100 mM KCl, pH 6.8) in a Waring blender. The homogenized samples were centrifuged for 10 min at 1,000 × g at 4°C in cold room. The pellet was washed with SSS buffer for three times followed by washing with SSS buffer plus 1% Triton X-100 (three times), 100 mM KCl (six times) and 100 mM NaCl (three times). Lastly, the myofibrils were washed with 100 mM Tris (pH 7.0) for at least three times.

B. Incubation

The purifed myofibrils were washed three times in an incubation buffer (12 mM NaCl, 12.6 mM MgSO4- $7H_2O$, 70 mM KH_2PO_4, 3.4 mM NaOH, 64.2 mM KOH, 11.1 H_2SO_4, 132 mM lactic acid, 100 mM Tris, and 1 M Tris added to pH 5.8, 4°C). This buffer was formulated on the basis of the postrigor sternomandibularis muscle composition as described by Winger and Pope (1980-81). Aliquots of 400 µl containing 500 µg purified myofibrils with the following six treatments: 1) 100 µM H_2O_2, 2) 1% MEC, 3) 10 U µ-calpain (1367U/mg, Porcine Erythrocytes, calbiochem) with 100mM H_2O_2, 4) 10 U µ-calpain (1367U/mg, Porcine Erythrocytes, calbiochem) with 1% MEC, 5) 155 mU cathepsin B(26.19 U/mg, bovine spleen, sigma) with 100 µM H_2O_2, and 6) 155 mU cathepsin B (26.19 U/mg, bovine spleen, sigma) with 1% MCE, were incubated in a temperature-controlled water bath maintained at 25°C for 24 hrs. Moreover, $CaCl_2$ was added to µ-calpain only. Control, which is isolated myofibrils in the same buffer but without proteases (µ-calpain or cathepsin B) were also kept under the same conditions without oxidation or reduction treatments.

C. Two-D gel electrophoresis and analysis

After incubation, the protein was extracted from samples using lysis buffer (8 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, and 0.8% E-lyte pH 4-7). Protein expression was assessed according to the procedure described by Hwang et al. (2005) with suitable modifications. Briefly, duplicate gels for each treatment, which the samples were run in triplicate for each given treatment analyzed using 2DE image analysis software (PDQuest, Bio-Rad, USA) according to the manufacturer's instruction. Spots appearing in more than two gels within 50% of variation in optical density were accepted as analytical spots, and an average of normal volume was used for the final analysis. MS/MS spectra were generated by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The data were processed using a Mass Lynx Windows NT PC system. Peptide masses from MALDI-TOF MS were matched with the theoretical peptides of proteins in the NCBI database using MASCOT and/or Profound software. Also, all MS/MS spectra recorded on tryptic peptides were searched against protein sequences from NCBInr and EST databases using the MASCOT search program.

III. RESULTS AND DISCUSSION

The meat tenderizing process is unanimously recognized to be enzymatic in nature and these are significantly affected by biological status of muscle proteins including proteases (Sentandreu, Coulis, & Ouali, 2002; Lonergan et al., 2009). The current experiment was designed to examine a hypothesis that proteolytic activity of µ-calpain and cathepsin B are greatly affected under reduction and oxidation conditions. In a preliminary study, purified bovine myofibrils were incubated under oxidation or reduction condition with µ-calpain and cathepsin B, and degradation proteins were assessed by using one-dimension PAGE and western blotting. The results showed that degradation products were prominent for alpha-actin, myosin light chain, heat shock 27kDa protein 1 and mutant beta-actin. Oxidation condition for both proteases resulted in a similar protein profile while cathepsin B under reduction condition showed prominent productions of mutant beta-actin and myosin light chain. Western blotting for Troponin-T and desmin also confirmed the observation in that both proteases showed greater activity in reduction condition than control and oxidation conditions (data not shown). The results mirror that status of muscle proteins including proteases significantly affects postmortem proteolysis including cathepsin B.

A total of 33 protein spots were apparently changed during incubation period with µ-calpain and cathepsin B under oxidation or reduction conditions (Table 1, Figure 1). The results reveal that myofibril proteolysis by µ-calpain and cathepsin B was greatly affected by biological status of protein (i.e., oxidation or reduction; These present results were in adherence to the previous studies conducted for meat tenderization experiments (Baron et al., 2004; Kristensen et al.,

1997; Maddock et al., 2006) including creatin kinase M, myosin, actin, myosin heavy chain, myosin light chain, troponin-T, titin, HSP 27, and crystalline. It was particularly notable that myosin and actin are greatly changed by both proteases under reduction condition, but not under oxidation condition. Baron et al. (2004) reported a similar result for μ-calpain. Based on the number of protein spots changed, cathepsin B was more greatly affected by incubation environment (e.g., oxidation and reduction) compared to these for μ-calpain. When protein spots altered under reduction condition was identified by LC/MS-MS, majority of sports were fragments of actin and myosin.

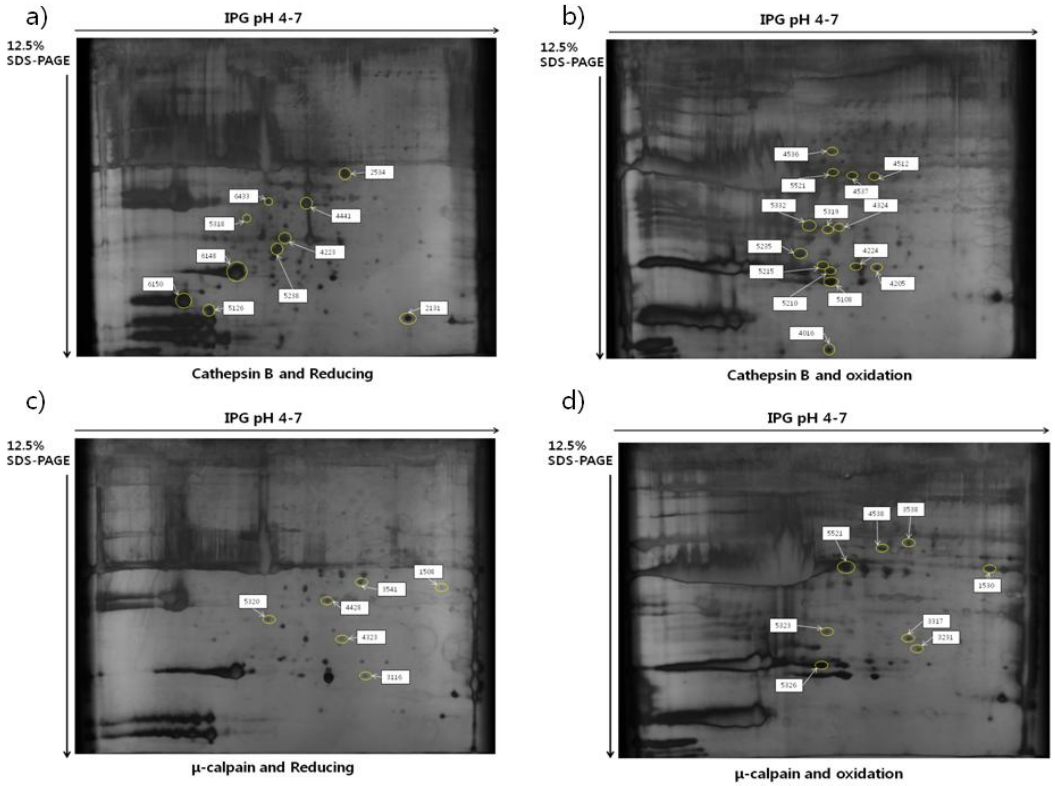

Figure 1 Scanned 2DE image of bovin myofibrils separated using an IPG pH 4-7 strip in the first dimension and 10% SDS-PAGE Representative 2DE-gel on one of the following six treatments: (a) μ-calpain (10 U/ 400 μl) with 100 μM H_2O_2, b) μ-calpain (10 U/ 400 μl) with 1% MEC, c) cathepsin B (155 mU/ 400 μl) with 100 μM H_2O_2, d) cathepsin B (155 mU/ 400 μl) with 1% MCE) with bovine myofibrils.

Table 1 List of spot number of 2DE for the spots having 3-fold higher or lower density from the fair comparison among six treatments: (a) μ-calpain (10 U/ 400 μl) with 100 μM H_2O_2, b) μ-calpain (10 U/ 400 μl) with 1% MEC, c) cathepsin B (155 mU/ 400 μl) with 100 μM H_2O_2, d) cathepsin B (155 mU/ 400 μl) with 1% MCE) with bovine myofibrils.

ID	Identified fragment	NCBI	C/Q	MW/pI	Density*			
					UO	UR	CBO	CBR
3538	Cardiac beta myosin heavy chain [Homo sapiens]	gi|29727	0/1	223647/5.62	189648.5	0	0	0
1530	Creatine kinase M [Homo sapiens]	gi|180576	3/1	43247/6.63	629753.3	0	0	0
5323	Mutant beta-actin (beta'-actin) [Homo sapiens]	gi|28336	4/1	42128/5.22	143016.8	0	0	0
3317	Myosin [Homo sapiens]	gi|558669	0/1	223701/5.63	162392.6	0	0	0
5521	Alpha-actin (aa 40-375) [Mus musculus]	gi|49864	5/1	38016/5.45	613206.4	0	297436.4	0
4535	Alpha enolase [Bos taurus]	gi|4927286	3/1	47589/6.44	171559.8	0	92352.55	0
4536	Alpha enolase [Bos taurus]	gi|4927286	3/1	47589/6.44	56042.95	0	67513	0
4205	Heat shock 27kDa protein 1 [Bos taurus]	gi|61553385	9/1	17602/6.49	169157.8	0	143559.6	0
4324	Mutant beta-actin (beta'-actin) [Homo sapiens]	gi|28336	4/1	42128/5.22	31784.3	0	126770.5	0
5210	Smooth muscle and non-muscle myosin alkali light chain 6B [Bos taurus]	gi|115496556	5/1	23502/5.40	847629.7	0	552554	0
5236	Smooth muscle and non-muscle myosin alkali light chain 6B [Bos taurus]	gi|115496556	7/1	23502/5.40	660061.6	0	999173	0
4537	Alpha-actin (aa 40-375) [Mus musculus]	gi|49864	5/1	38016/5.45	0	541410.3	272941.1	0
4428	Capping protein alpha [Homo sapiens]	gi|433308	3/1	32955/5.58	0	253036.1	61406.85	0
3541	Mutant beta-actin (beta'-actin) [Homo sapiens]	gi|28336	4/1	42128/ 5.22	0	216093.9	0	0
3116	Smooth muscle and non-muscle myosin alkali light chain 6B [Bos taurus]	gi|115496556	12/1	23502/5.40	0	57043.8	0	0
4323	Titin [Oryctolagus cuniculus]	gi|3928489	0/1	223364/8.64	0	80817.85	0	0

1508	Troponin T class Ia alpha-2 [Rattus norvegicus]	gi|207365	6/1	29923/8.39	0	131340.2	0	0
6148	Myosin light chain [Rattus norvegicus]	gi|205474	5/1	20949/4.99	0	523848.4	0	1972488
5235	Alpha-actin (aa 40-375) [Mus musculus]	gi|49864	5/1	38016/ 5.45	0	0	377217.2	0
5320	Capping protein beta 3 subunit [Mus musculus]	gi|1903236	27/1	10266/4.37	0	141273.1	572641.4	0
4224	Heat shock 27kDa protein 1 [Bos taurus]	gi|61553385	9/1	17602/6.49	0	0	309408.7	0
5215	Heat shock protein 27 [Homo sapiens]	gi|662841	5/1	22427/7.83	0	0	535492.2	0
4512	Mutant beta-actin (beta'-actin) [Homo sapiens]	gi|28336	4/1	42128/5.22	0	0	402380.9	0
5322	Mutant beta-actin (beta'-actin) [Homo sapiens]	gi|28336	4/1	42128/5.22	0	0	499914.7	0
5108	Smooth muscle and non-muscle myosin alkali light chain 6B [Bos taurus]	gi|115496556	4/1	23502/5.40	0	0	853525.6	0
2534	Alpha-actin (aa 40-375) [Mus musculus]	gi|49864	6/1	38016/5.45	0	0	0	385911.5
4441	Alpha-actin (aa 40-375) [Mus musculus]	gi|49864	5/1	38016/5.45	0	0	0	468740.8
5238	Alpha-actin (aa 40-375) [Mus musculus]	gi|49864	6/1	38016/5.45	0	0	0	623228
5318	Capping protein beta 3 subunit [Mus musculus]	gi|1903236	27/1	10266/4.37	0	0	0	87955.2
2131	Crystallin, alpha B [Bos taurus]	gi|27805849	14/1	20024/6.76	0	0	0	751816.7
4223	Mutant beta-actin (beta'-actin) [Homo sapiens]	gi|28336	4/1	42128/5.22	0	0	0	614661.5
6433	Mutant beta-actin (beta'-actin) [Homo sapiens]	gi|28336	4/1	42128/5.22	0	0	0	231742.9
6150	Myosin light chain [Rattus norvegicus	gi|205474	5/1	20949/4.99	0	0	0	936578.5

UO: μ-calpain with oxidized for 24 hrs UR: μ-calpain with reduced,
CBO: cathepsin B with oxidized for 24 hrs, CBR: cathepsin B with reduced for 24 hrs

IV. CONCLUSION

On the basis of identification of myofibril proteins affected under oxidation and reduction conditions with μ-calpain and cathepsin B, both proteases showed greater activity in reduction condition than control and oxidation conditions in that both proteases resulted in a similar proteolysis. A total of 33 protein spots (including creatin kinase M, myosin, actin, myosin heavy chain, myosin light chain, troponin-T, titin, HSP 27, and crystalline) were apparently changed during incubation period with μ-calpain and cathepsin B under oxidation or reduction conditions. It was particularly notable that myosin and actin are greatly changed by both proteases under reduction condition, but not under oxidation condition. Furthermore B cathepsin B was more greatly affected by incubation environment compared to these for μ-calpain.

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