PE2.02 Differences in protease expression patterns in individual bovine skeletal muscles detected by two-dimensional zymography 94.00

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***Abstract*—Two-dimensional (2D) zymography method developed by our laboratory was applied to detect the difference in multiple protease activities in bovine individual muscles. *Biceps femoris* (BF), *Longissimus lumborum* (LL), *Psoas major* (PM), and *Semitendinosus* (ST) muscles of Japanese Shorthorn steers were subjected to the analysis. BF and PM were classified as red muscles, while LL and ST were classified as white muscles. 2-D zympgraphy revealed that most protease activities including cathepsin-D and μ-calpain were higher in red muscles (BF and PM) than in white muscles (LL and ST). 2-D zymography is capable of detecting the difference in protease activities in individual muscles. However, problems still remain for practical use and the study to improve the method is in progress.**

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***Index Terms* — cattle, skeletal muscle, proteases, zymography.**

I. INTRODUCTION

PROTEASES in skeletal muscles play principal roles in meat aging process including the degradation of myofibrillar proteins and the release of flavour substances such as peptides and amino acids in *post mortem* muscles. Two groups of proteases are known to be involved in this process, namely calpains, a group of calcium-dependent neutral proeases and cathepsins, a group of lysosomal acidic proteases (Ouali et al., 1987), although several other groups of proteases may be involved in the process.

The protease activities and their inhibitor expressions vary by muscle contractile types, resulting in the difference in myofibrillar protein degradation speed by individual muscles. Generally, red muscles that contain higher proportion of slow-twitch oxidative myofibers exhibit higher protease activity than white muscles with higher ratio of fast-twitch glycolytic fibers (Ouali and Talmant, 1990; Whipple and Koohmaraie, 1992; Therkildsen et al., 2002).

It is advantageous to detect multiple proteases simultaneously in muscles to understand the cooperation of the proteases in the aging process comprehensively. Zymographic analysis is widely used for the detection of multiple protease activities in various animal tissues and body fluids (e.g. Kaino et al., 1998). One-dimensional (1D) casein zymography has already been applied to detect the activities of neutral μ- and m-calpains with meat samples (Veiseth et al., 2001). It is also known that two-dimensional electrophoresis (2-DE) is more effective to separate a large number of proteins than 1D electrophoresis.

We developed a two-dimensional (2D) zymographic method for detecting of multiple protease activities in bovine muscles by combining casein zymography and 2-DE. We applied this method to investigate the differences in the patterns of proteolytic activities in bovine individual muscles.

II. MATERIALS AND METHODS

A. Muscle samples

Japanese Shorthorn steers were slaughtered at 24 - 25 months of age and 715 kg average bodyweight in the experimental slaughterhouse in National Agricultural Research Center (Tohoku Region) (NARCT). All of the slaughter procedures were performed following The Guidelines for Experimental Animal Handling, NARCT. *Biceps femoris* (BF), *Longissimus lumborum* (LL), *Psoas major* (PM) and *Semitendinosus* (ST) muscles were collected within two hours of slaughter. A portion of the sample was diced and frozen in dry ice-cold acetone for preparing frozen sections. The rest was snap frozen in liquefied nitrogen. Both samples were vacuum-packed and stored at – 80 °C until use.

B. Muscle fiber typing

Frozen sections, 8 μm in thickness were made from the acetone-fixed sample by a cryostat. The sections were pasted on slide glass and subjected to double staining (NADH-DH activity and alkali - preincubated myosin ATPase activity). Five hundred muscle fibers were counted under optical microscopy and each myofiber was classified into Type-I (slow-twitch oxidative), Type-IIA (intermediate oxidative-glycolytic) and Type-IIB (fast-twitch glycolytic). The proportions of the three fiber types were calculated.

C. Preparation of muscle proteins

The frozen samples were homogenized with three volumes of ice-cooled homogenization buffer (50 mM Tris-HCl and 10mM EDTA-2Na, pH 7.5). The homogenate was centrifuged at 4 °C and the aliquot was isolated. Ammonium sulfate (AS) powder was added to the aliquot until the saturation reached to 55% to precipitate the water-soluble protein fraction. The precipitate was re-dissolved in deionized water and dialyzed against deionized water overnight at 4°C to remove AS and other ions. This solution was condensed in a vacuum condenser until the protein concentration reached to 40 mg/ml.

D. 2-D electrophoresis

An immobilized pH gel strip (BioRad, 17 cm length, for pI 5-8) was rehydrated with 300 µl of rehydration solution containing 9 mg of the processed protein fraction. First dimension isoelectric focusing was performed, applying 6,000 V voltages to the rehydrated strip for 5 hours. Rehydration and isoelectric focusing were performed at 10 °C.

Polyacrylamide slab gel 12.5 % (w/v) containing 0.25 % (w/v) casein and no SDS was developed for two- dimensional electrophoresis, using buffer containing 30 mM Tris base and 200 mM glycine. The focused gel strip was put on the top of the slab gel with 1 % (w/v) agarose as an adhesive. The gel was run at 80 mA current for 20 h, keeping the buffer temperature below 10 °C throughout the electrophoresis.

E. In - gel protease reaction

Gel incubation buffer (100 mM sodium acetate, pH=5.5) was prepared to activate the proteases in the gel. Upon completion of electrophoresis, the gel was washed twice by gentle shaking in the incubation buffer for 15 min at room temperature. The gel was then transferred into new incubation buffer and incubated for 24 h at 37 °C with gentle shaking. Then the gel was fixed and stained with Coomassie Brilliant Blue R-250 dissolved in fixation buffer (10 % acetic acid, 30 % methanol and 70 % ion-exchanged water) and destained with the fixation buffer.

III. RESULTS AND DISCUSSION

Table 1 shows the proportions of myofiber types for the five muscles of Japanese Shorthorn steers. BF and PM had higher percentage for I myofibers, while LL and ST contained higher proportions of IIB fibers.

Figure 1 exhibits the typical 2-D zymographic patterns for bovine PM and LL muscle proteins. More than twenty protease activity spots could be detected under the conditions mentioned above. These spots are able to be grouped by isoelectric point (pI) since when two spots with the same pI and different mobility are detected in the zymography one can be regarded as the native form and the other as an activated form by autolysis or the function of other proteases. In this case of Figure 1(a), fifteen groups were observed. Protein phosphorylation affects the pI in the first - dimension isoelectric focusing. We observed the spots that differ in pI but show similar mobility (e.g. groups 11 and 12; 14 and 15 in Fig. 2). These spots may be identical proteases with different modification status by phosphoryl groups. The identification of the spots is presently in progress. So far, µ-calpain (group 5 in Fig.2) and cathepsin-D (groups 13, 14 and 15 in Fig.2) spots could be identified. Overall, PM and BF that have high proportion of Type-I fibers exhibited stronger activities for most of the spots including µ-calpain and cathepsin-D than LL and ST, containing high proportion of Type-IIB fibers. The result is in agreement with the other studies (Ouali and Talmant, 1990; Whipple and Koohmaraie, 1992; Therkildsen et al., 2002).

It is impossible to detect the inhibitory activity directly by the 2-D zymography used in this study. Red muscles exhibit slower myofibrillar protein degradation than white muscles in spite of the higher activities of muscular proteases. It is mainly because of the higher protease inhibitor activity e.g. calpastatin in red muscles than in white muscles (Whipple and Koohmaraie, 1992; Van den Maagdenberg et al, 2007). Therefore, when considering the effect of proteolytic systems on beef tenderization, not only the protease activities, but also the effect of their inhibitors should be taken into account.

2-D zymography still requires improvements. At first, the identification of the protease spots has to be completed. In addition, a difficulty exists in quantifying protease activities in the gel because several activity spots correspond to only one protease. Study is in progress to quantify the activities by image analysis incorporating multiple spots with high accuracy.

IV. CONCLUSION

The study revealed that the 2-D zymography is capable of detecting the difference in protease expression patterns by individual muscles. Although

the method requires further improvement, the method will be a good tool to screen multiple proteases in large number of bovine muscles from different breed, age, sex or aging status in a short period.

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Table1. Muscle fiber composition (%) of Japanese Shorthorn steers at slaughter (n=8) .

Muscles	Type-I (Oxidative)			Type-IIA (Intermediate)		Type-IIB (Glycolytic)		
	Mean	Std		Mean	Std	Mean	Std	
Biceps femoris	40,2	6,4	a	24,5	4,8	35,3	7,3	d
Longissimus lumborum	22,4	3,1	c	21,9	4,4	55,8	4,2	b
Psoas major	30,9	2,3	b	22,2	3,8	47,0	3,5	c
Semitendinosus	9,4	2,0	d	23,4	2,8	67,3	3,1	a

Values with different letters for each muscle type differ at 1% level.

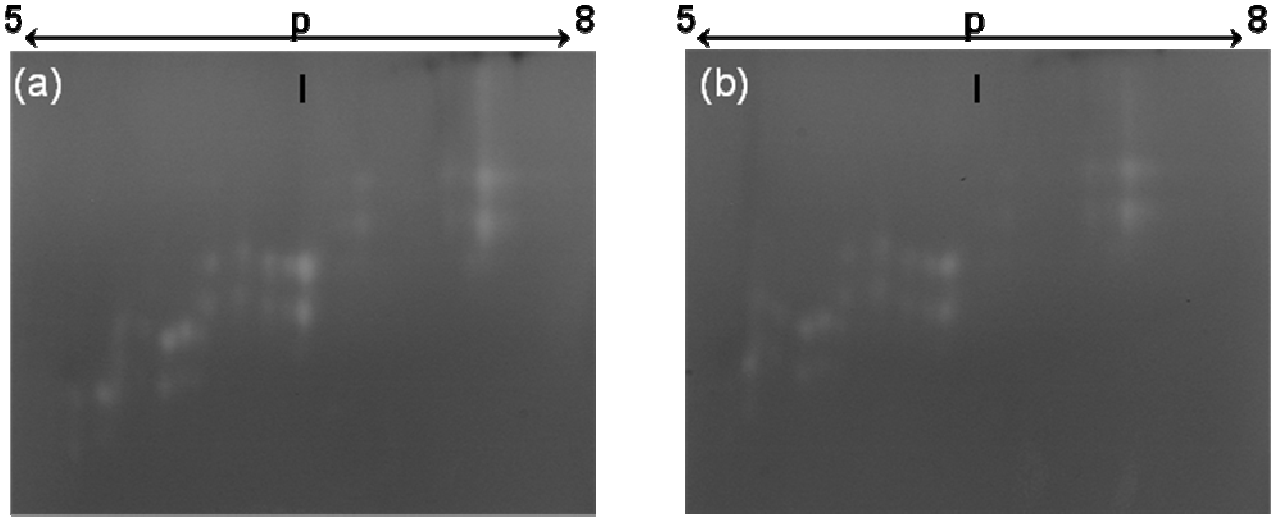

Figure 1: Typical 2-D zymography patterns of the water-soluble proteins from bovine muscles precipitated with 55 % saturation of ammonium sulfate. (a): *Psoas major* and (b): *Longissimus lumborum*. Caseinolytic activity spots are seen as CBB-unstained bright spots.

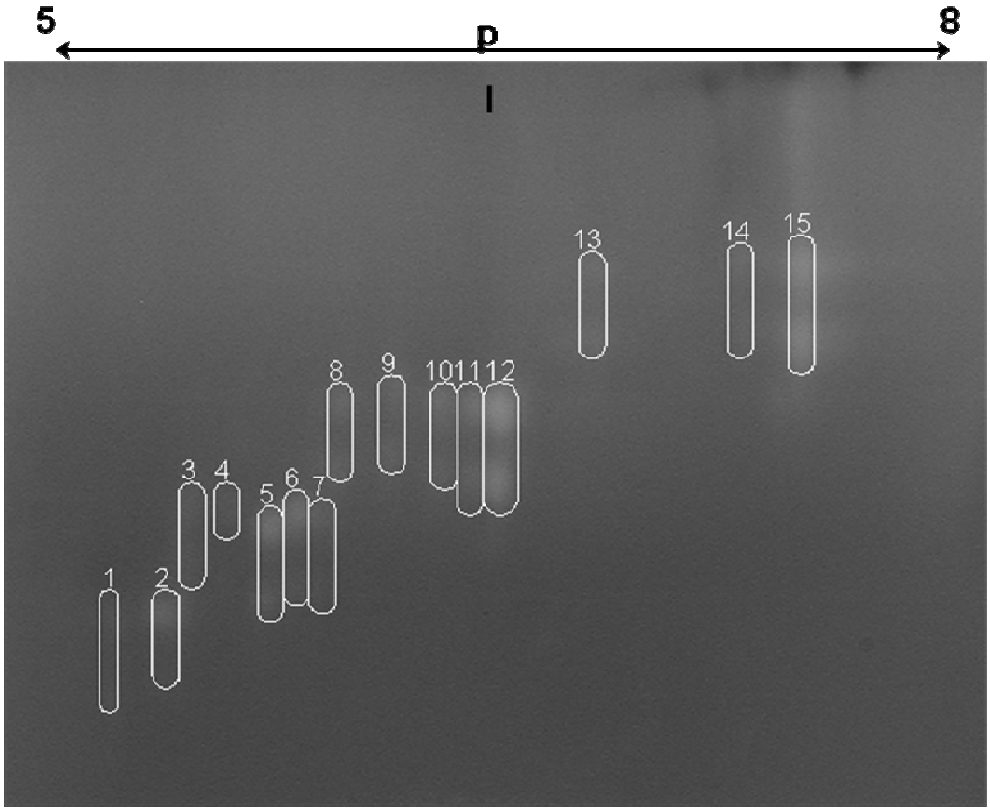

Figure 2. Grouping of the activity spots by pI. The image is the same as Fig.1(a). The spots are classified into fifteen groups in the case.