# DETECTION OF MULTIPLE PROTEASE ACTIVITIES IN BOVINE SKELETAL MUSCLES BY TWO-DIMENSIONAL ZYMOGRAPHY

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## Introduction

It is widely recognized that proteases in skeletal muscles play principal roles in the degradation of myofibrillar proteins in *post mortem* muscles. Two groups of proteases are known to be involved in this process, namely calpains and cathepsins (Ouali *et al.*, 1987), although several proteases other than these two groups may be involved in the meat ageing process. It is important to examine the quantity and activity of these proteases for the investigation of their effects on *post mortem* muscles and numerous studies have been performed with beef samples. However, most studies deal with only one or a small number of proteases and require large beef samples (e.g. from 10 to over 50 g). It is necessary to detect multiple proteases simultaneously in beef samples to comprehensively understand the cooperation of these proteases in the degradation of myofibrillar proteins in *post mortem* muscles. In addition, when the sample size is limited, it is desirable to apply a method to accurately detect protease activities in these muscle samples (Kent *et al.*, 2005). Zymographic analysis is widely used for the detection of multiple protease activities in relatively small volumes of animal tissues and body fluids. One-dimensional casein zymography has been applied for the detection of neutral μ- or m-calpain activities in meat samples (Veiseth *et al.*, 2001). However, as far as we know, there are no reports using this technique for the detection of acidic protease activities in beef samples.

It is well known that two-dimensional electrophoresis (2-DE) is more effective in separating large number of proteins than one-dimensional electrophoresis, and the two-dimensional (2-D) zymography method, a combination of casein zymography and 2-DE was reported to detect multiple proteolytic enzymes (Kaino *et al.*, 1998). The purpose of this study is to clarify whether 2-D zymography gives better results for detecting multiple protease activities in beef samples, eliminating the need for large muscle samples.

## Materials and Methods

One Japanese Shorthorn steer was slaughtered at 24 months of age and 640 kg bodyweight in our experimental slaughterhouse. All of the slaughter procedures were performed following The Guidelines for Experimental Animal Handling, National Agricultural Research Center for Tohoku Region.

*Longissimus lumborum* (LL), *Biceps femoris* (BF), *Psoas major* (PM) and *Semitendinosus* (ST) muscles were collected within two hours of slaughter and snap frozen in liquid nitrogen. The samples were then stored at -80°C until further analysis.

One gram of frozen sample was homogenized with 3 ml ice-cooled homogenization buffer (50 mM Tris-HCl, 10mM EDTA-2Na and 1/100 concentration of protease inhibitor cocktail (SIGMA), pH 7.5). The homogenate was centrifuged at 4°C and the aliquot was dispensed into two microtubes. The microtubes were centrifuged at full speed for 10 min at 4°C to purify the aliquot which was vacuum condensed and desalted by passing through a Sephadex® G25 gel column, followed by quantification of the protein content. An immobilized pH gel strip (BioRad, 11 cm length, for pI 5-8) was rehydrated with 185 μl of rehydration solution containing 2mg of desalted sarcoplasmic protein. 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.

A polyacrylamide slab gel 12.5% (w/v) containing 0.25% (w/v) casein without SDS was prepared for two- dimensional electrophoresis, with buffer containing 30 mM Tris base, 200 mM glycine and 10 mM EDTA. 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 120 mA current for 16 h, maintaining the buffer temperature below 10°C throughout electrophoresis.

Fifty mM sodium-acetate buffer was prepared for gel incubation to restore the protease activities. The pH was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. Calcium chloride was added at a concentration of 10 mM or 100 mM to investigate whether the proteases were Ca-dependently activated. Upon completion of electrophoresis, the gel was washed twice by gentle shaking in incubation buffer for 15 min at room temperature to wash off EDTA and protease inhibitors. The gel was then transferred into fresh incubation buffer and incubated for 48 h at 37°C with gentle shaking, changing the buffer after 24 hrs of incubation. The gel was fixed and stained with Coomassie Brilliant Blue R-250 (Waco Chemicals) dissolved in fixation buffer (10% acetic acid, 30% methanol and 70% ion-exchanged water) and destained with fixation buffer.

## Results and Discussion

Figure 1 shows the typical two-dimensional zymographic pattern using bovine LL and ST muscles. More than ten protease activity spots could be detected under the conditions mentioned in the Legend. Western blot analysis suggested that two of the spots corresponded to m-calpain and cathepsin-B. PM exhibited stronger activities for most of the spots than BF, LL and ST muscles. However, several spots exhibited higher intensity in muscles other than PM, suggesting that the composition of the proteases differed by muscle.

At pH 6.5 and 6.0, no activity spots were detected. In contrast, most spots showed strong caseinolytic activities at pH 5.5 and 5.0. At pH 4.5 and 4.0, proteolytic activities were generally weaker than at pH 5.0 and 5.5, however, several spots retained relatively strong activities. These results indicate that the pH of the incubation buffer should be adjusted corectly when particular protease activities are to be investigated with this method. The concentration of calcium ions apparently affected caseinolytic activity. Although proteolytic activities were seen at 10 mM of $Ca^{2+}$, most activity spots were brighter at $Ca^{2+}$ concentrations of 100 mM than at 10 mM. It is unlikely that all activated spots are calpains and one possible explanation for the phenomenon is that the substrate casein in the gel was affected by the increase in $Ca^{2+}$ concentration and became prone to degradation by the proteases in muscles.

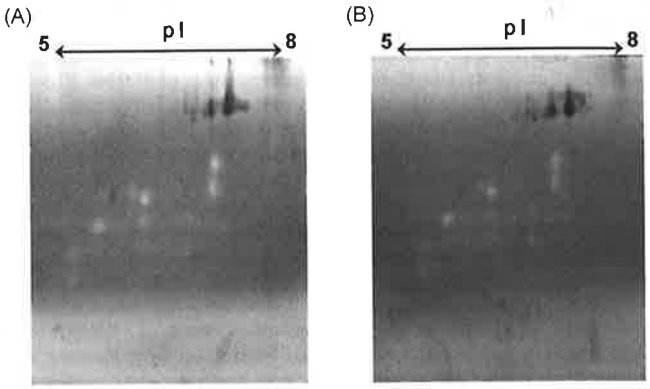


**Figure 1:** Two-dimensional zymography of the water-soluble proeins of bovine LL (A) and ST (B). Caseinolytic activity spots are seen as CBB-unstained bright spots. Protein content: 2.0 mg, incubation buffer: 50 mM sodium acetate, 100 mM calcium chloride, pH=5.5, incubation: 48 hrs at 37 °C.

## Conclusions

Although the two-dimensional casein zymography used in this study needs to be significantly improved (e.g., identification of the proteases, longer incubation time and difficulty in quantifying protease activities), it seems to be a good method of detecting multiple protease activities with a small amount of skeletal muscle, enabling us to investigate proteases in muscle not only *post mortem* but also in living animals, facilitating knowledge on the differences in muscular protease activities influenced by factors such as cattle breed, sex, age, feed and exercise.

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