In vitro analysis of mushroom proteases that may tenderize beef

Jing-Wei Lee[1*], Michelle N. LeMaster[1], Eric M. England[1]

[1] Deparment of Animal Sciences, The Ohio State University, 2029 Fyffe Rd, Columbus, OH 43210

*lee.7181@osu.edu

Abstract

Meat tenderness is an important characteristic that influences consumer purchasing decisions. Protease extracts from natural plant sources such as pineapple have exhibited broad proteolytic activity that can over-tenderize the meat and negatively affect texture and quality. Therefore, identification and evaluation of other proteases capable of tenderizing beef is necessary. Previously, mushrooms have been shown to enhance flavor and nutritional composition of meat dishes, as well as having beneficial antioxidant and health effects. Mushrooms also contain a variety of proteases that were analyzed in this study for their ability to proteolyze beef proteins using an *in vitro* model system. Eight mushroom varieties were tested including white button (white immature *Agaricus bisporus*), crimini (brown immature *Agaricus bisporus*), portobello (mature *Agaricus bisporus*), shitakke (*Lentinula edodes*), enoki (*Flammulina velutipes*), oyster (*Pleurotus ostreatus*), king trumpet (*Pleurotus eryngii*), and brown beech (*Hypsizygus tessellatus*). Mushrooms were homogenized in a 20 mM Tris buffer (pH 8.0), filtered, centrifuged, and combined with purified bovine myofibrils for incubation at 25°C. Samples were collected at 0, 30, 60, 240, and 1440 min. Myofibrillar proteins of each sample were solubilized and separated using SDS-PAGE. Densities of protein bands were compared between the time-points to determine which of the eight mushroom varieties proteolyzed myofibrillar proteins including actin and myosin. Mushroom proteolytic activity was also quantified with a standard casein assay. *Pleurotus ostreatus*, mature *Agaricus bisporus*, and *Pleurotus eryngii* had the greatest caseinolytic activity at 1.06 U/mL, 0.70 U/mL, and 0.68 U/mL respectively, while *Flammulina velutipes* had the least at 0.03 U/mL. These results support the possibility that mushroom proteases may be able to tenderize beef, forming the basis for future research trials.

Introduction

Meat tenderness is an important characteristic that influence palatability and consumer purchasing decisions. Aging and postmortem proteolysis are factors that can contribute to tenderness (Voges et al., 2007). Consumer demand for clean-label products has spurred research into natural protease sources that can impact meat tenderness. Protease extracts of bromelain from pineapples, zingibain from ginger, papain from papaya and actinidin from kiwi have exhibited broad proteolytic activity that can quickly over-tenderize the meat and negatively affect texture and quality (Ha, Bekhit, Carne and Hopkins, 2012). Therefore, identification and evaluation of other proteases capable of tenderizing beef is necessary.

Previously, *Agaricus bisporus* powder was shown to enhance flavor and nutritional composition of ground meat, as well as having beneficial health effects and antioxidant effects that could extend shelf life (Alnoumani, Ataman, and Were, 2017). Mushrooms also contain a variety of proteases that can impact texture. *Kamaboko* is a Japanese cooked fish meat gel whose texture and gel strength was negatively affected with the addition of sliced Judas' ear mushroom (*Auricularia auricula-judae*). SDS-PAGE analysis of mushroom extracts depicted the fungi's proteolytic activity on fish myofibrils at an pH optima of 7.0 (Makinodan, and Hujita, 1990).

Given the sensory and antioxidant benefits of mushrooms, and previously studied protease activity on other meats, it is possible for mushrooms to have proteolytic capabilities in beef. The purpose of this study was to focus on the ability of eight commercially available mushrooms to denature beef proteins using an *in vitro* model system.

Objectives

The objective of this study was to analyze commercial mushrooms' proteolytic capabilities on beef proteins in an *in vitro* system for their possible ability to impact tenderness. The objective was achieved though SDS-PAGE analysis and casein assay quantification with 8 available mushroom varieties.

Materials & Methods

Sample Preparation

The following eight mushroom varieties were obtained from a local grocery store (Lucky's Market, Columbus, OH) for analysis: white button (white immature *Agaricus bisporus*), crimini (brown immature *Agaricus bisporus*), portobello (mature *Agaricus bisporus*), shitakke (*Lentinula edodes*), enoki (*Flammulina velutipes*), oyster (*Pleurotus ostreatus*), king trumpet (*Pleurotus eryngii*), and brown beech (*Hypsizygus tessellatus*). Mushrooms (5g) were homogenized (Polytron; Brinkmann, Riverview, FL) in 10mL of 20mM Tris buffer (pH 8.0) and centrifuged (accuSpin^{TM} 3R, Fisher Scientific, Hampton, NH) at 1300xg for 5 min at 23°C to obtain the crude mushroom supernatant.

Beef (5g) was homogenized and washed in 20mL Rigor Buffer (pH 7.2, stored at 4°C; 75mM KCl, 10mM Imidazole, 2mM $MgCl_2$, 2mM EGTA, 1mM NaN_3) before centrifugation at 1000xg for 10 min at 4°C. This procedure was repeated twice with 20mL of Rigor Buffer to obtain purified bovine myofibrils. Finally, 20mL Rigor Buffer and 0.1mM phenylmethylsufonyl fluoride were added to the pellet with 50% v/v glycerine for -20°C storage.

SDS-PAGE

300μL of myofibrils and 700μL of Rigor Buffer were centrifuged (GeneMate SpinMate24; BioExpress, Kaysville, UT) at 3000xg for 5 min and the supernatant was removed. 1mL of 200mM MES buffer (pH 6.5) and 300μL of crude mushroom extract was added to the pellet, vortexed, and incubated at 25°C, with samples being collected at 0, 30, 60, 240, and 1440 min. 200μL aliquots from each timepoint were centrifuged at 13,000xg for 5 min, and the supernatant was discarded. The pellet was then mixed with 191μL Titin buffer and 10μL Bromophenyl blue dye. Myofibrillar proteins were solubilized and separated in 1.5mm SDS-PAGE 8% gels with 40μL samples against 10μL ladder. Gels were electrophoresed at 70V for 10 min., then 140V for 2 hr, and stained overnight with coomassie blue solution (250mL methanol, 50mL acetic acid, 30-35mg Brilliant Blue G, 200mL distilled water), destained (10% methanol, 7.5% acetic acid, 82.5% distilled water) for 2 hr, then imaged (Azure c600, Azure Biosystems, Dublin, CA) and analyzed using ImageJ. Density of protein bands were visually compared across time.

Caseinolytic Activity Assay

Crude mushroom protease extracts (200 μL) were combined with 1mL of 0.65% w/v casein in 50 mM potasium phosphate buffer (pH 7.5), 500μL 100mM $CaCl_2$, and 300 μL distilled water and incubated at 25°C for 1 hr. To stop the reaction, 1mL of 10% w/v TCA was added. The solution was centrifuged (accuSpin^{TM} 3R, Fisher Scientific, Hampton, NH) at 4100xg for 10 min before NanoDrop quantification (ND-1000; Thermo Fisher Scientific, Waltham, MA) at 280 nm.

Results & Discussion

Note that the common and scientific names of the observed mushrooms are: white button (white immature *Agaricus bisporus*), cremini (brown immature *Agaricus bisporus*), portobello (mature *Agaricus bisporus*), shitakke (*Lentinula edodes*), enoki (*Flammulina velutipes*), oyster (*Pleurotus ostreatus*), king trumpet (*Pleurotus eryngii*), and brown beech (*Hypsizygus tessellatus*).

Proteolysis of bovine myofibrillar proteins can be observed by a decrease in band density with time and the emergence of many thin bands below the myosin band, as the protein is broken down into smaller components. The enoki sample most resembles the control across the timepoints, except at 1440 min, but overall had little observed proteolytic activity as the band widths did not change. Shitake hydrolyzed myosin, was observed to have little to no effect on actin across all timepoints. Oyster, portobello and king trumpet samples had the greatest observable myosin and actin hydrolysis over time, and the myosin band cannot be seen at 1440 min for these samples. The thick actin band of portobello at 240 min could explained by actin's hydrolyzed components being grouped together to look like a large band, as this band is thinner and faded at 1440 min (Figure 1).

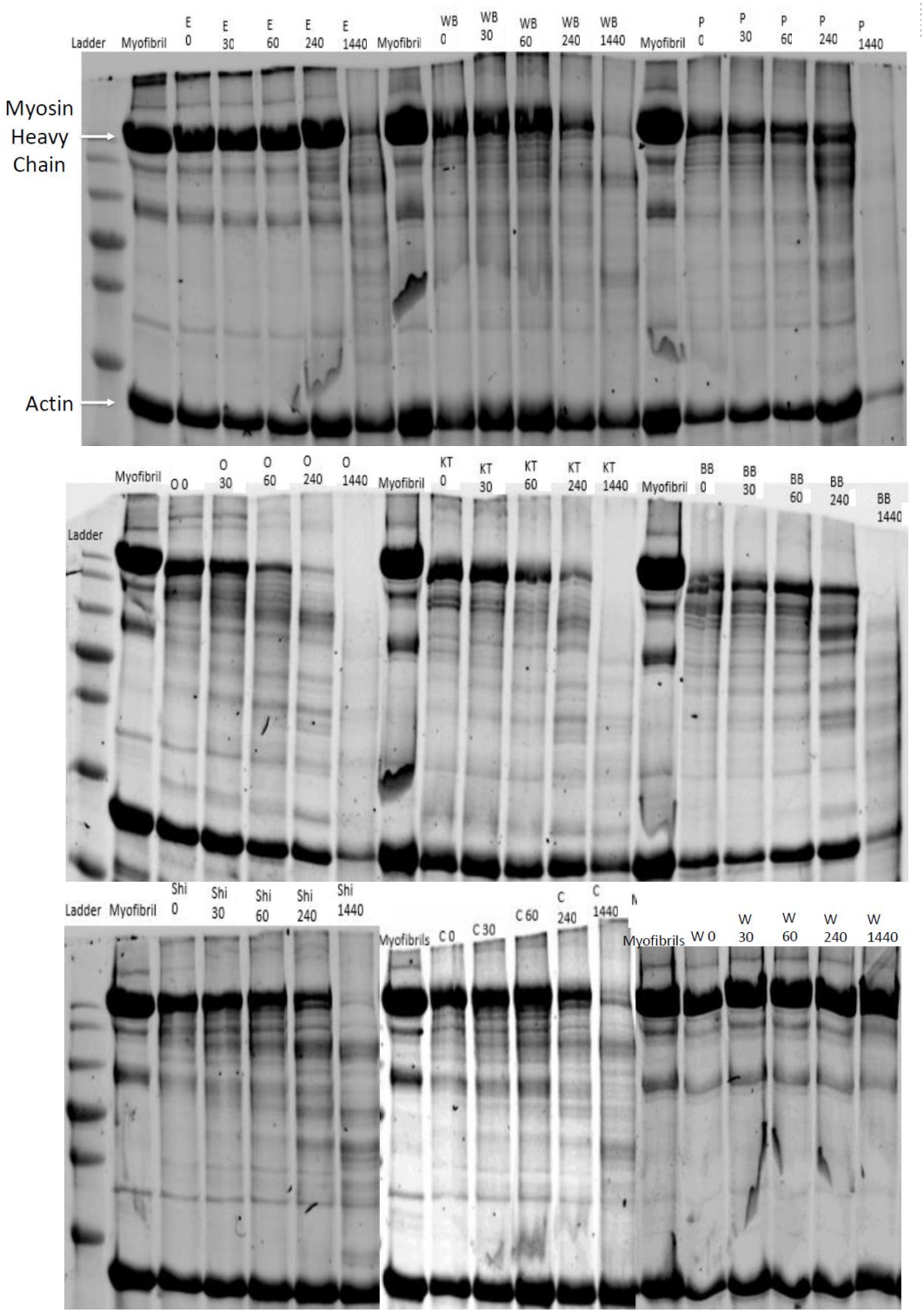

Figure 1. SDS-PAGE gels of enoki (E), white button (WB), portobello (P), oyster (O), king trumpet (KT), brown beech (BB), shiitake (Shi), and cremini (C) mushrooms and water/control (W) on bovine myofibrillar proteins after 0, 30, 60, 240 and 1440 min of incubation.

The gel images correlate with the enzymatic activity quantified by the casein assay. Oyster, portobello, and king trumpet had protease activities of 1.06 U/mL, 0.70 U/mL, and 0.68 U/mL respectively and quickly proteolyzed the myosin band on the gels. On the other hand, enoki displayed comparatively lower proteolytic activity (0.03 U/mL), only proteolyzing actin and myosin bands at 1440 min (Table 1).

It should be noted that while white button, cremini and portobello mushrooms are of the same species, *Agaricus bisporus*, they varied in color, maturity and degrees of proteolytic activity. Portobello (0.70 U/mL) had over double the protease activity of white button (0.31 U/mL) and triple that of cremini (0.19 U/mL) (Table 1). Unlike the other two varieties, which are brown, white button is white in color and is harvested earlier than the others. Portobello is matured *Agaricus bisporus* and is also larger. Cremini is midway between the two in maturity, but has been noted to have a richer, earthier flavor than white button mushrooms. Previous studies have indicated that mushrooms at different maturity stages have varying compositions of sugar, amino acid and flavor concentrations that could explain sensory differences and affect proteolytic activity (Tsai, Wu, Huang, and Mau, 2007).

Table 1. Caseinolytic activity assay of the proteases from eight mushroom varieties

Mushroom Variety	Protease Activity (U/mL)
Oyster	1.06
Portobello	0.70
King Trumpet	0.68
Shitakke	0.45
Brown Beech	0.34
White Button	0.31
Cremini	0.19
Enoki	0.03

Given that this study was conducted *in vitro* using a pH 8.0 buffer, the mushroom proteases may perform differently at the lower meat pH of 5.4-5.7. However, Wang and Ng (2001) previously

isolated a protease from *Pleurotus eryngii*, commonly known as King Trumpet mushroom, that demonstrated optimal activity at pH 5.0 and 50°C, and should be further explored in meat applications.

Conclusions

The data indicated that all eight mushroom varieties proteolyzed myofibrillar proteins, including actin and myosin. Therefore, these results support the possibility that mushroom proteases may be able to tenderize beef. Future research trials focusing on the application of mushroom extracts *in vivo* to beef cuts and processed products are necessary to determine the industrial application of these proteases and their effect on meat quality. Consideration should be given to possible color changes in these products with the addition of mushroom extracts, and how the low beef pH of 5.4-5.7 could affect the proteolytic activity demonstrated in this study at pH 8.0.

Acknowledgements

This research was conducted as part of the OSU Department of Animal Sciences Undergraduate Research Experience (ASURE), and was partially funded by the OSU Second-Year Transformational Experience Program (STEP). Special thanks to the meat science laboratory for their assistance and equipment for the purposes of this study.

References

Alnoumani, H., Ataman, Z.A., Were, L. (2017). Lipid and protein antioxidant capacity of dried *Agaricus bisporus* in salted cooked ground beef. *Meat Science 129*(3): 9-19.

Ha M., Bekhit A.E.D.A., Carne, A., Hopkins, D.L. (2012). Characterisation of commercial papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat proteins. *Food Chemistry, 134*(1): 95-105.

Makinodan, Y. and Hujita M. (1990). Textural degradation of cooked fish meat gel (*Kamaboko*) by the addition of an edible mushroom, Judas' Ear (*Auricularia auriculajudae* (Fr.) Quel). *Journal of Food Science, 55*(4): 979-982.

Tsai, S-Y., Wu, T-P., Huang, S-J., and Mau, J-L. (2007). Nonvolatile taste components of *Agaricus bisporus* harvested at different stages of maturity. *Food Chemistry, 103*(4): 979-982.

Voges, K., Mason, C., Brooks, J., Delmore, R., Griffin, D., Hale, D., . . . Savell, J. (2007). National beef tenderness survey – 2006: Assessment of Warner–Bratzler shear and sensory panel ratings for beef from US retail and foodservice establishments. *Meat Science, 77*(3): 357-364.

Wang, H. and Ng T.B. (2001). Pleureryn, a novel protease from fresh fruiting bodies of the edible mushroom *Pleurotus eryngii. Biochemical and Biophysical Research Communications, 289*(3): 750-755.