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ARTICLE

Effect of Proteolytic Enzymes and Ginger Extract on Tenderization of M. *pectoralis profundus* from Holstein Steer

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Abstract The effects of proteolytic enzymes (bromelain and bromelain+papain) and a ginger extract were assessed on collagen content and solubility, thermal shrinkage temperature of connective tissue, pH, cooking loss, drip loss, and Warner-Bratzler shear force (WBSF) of M. *pectoralis profundus* isolated from the beef brisket cut. Both proteolytic enzymes and ginger extract led to a significant increase in cooking loss and collagen solubility compared with untreated controls. On the other hand, the peak (T_p) thermal shrinkage temperature markedly decreased in all treatments compared with those in controls. Samples treated with bromelain, bromelain + papain, and ginger extract showed a significant decrease in WBSF by 36%, 40%, and 37%, respectively, compared with untreated controls. Our findings suggest that ginger extract are useful for post-mortem tenderization of meat containing high levels of collagen, compared to control even though, bromelain and bromelain + papain treatments have higher collagen solubility than ginger extract.

Keywords collagen, ginger, proteolytic enzyme, tenderization

Introduction

The meat industry is under increasing pressure to meet consumer demands, including safety and palatability, at low cost. Beef brisket has traditionally been considered a low quality cut due to its high content of connective tissue (Bailey 1972). Meat palatability is determined by the interactions of a number of traits, with meat tenderness representing one of the most important traits contributing to the palatability of beef (Savell and Shackelford, 1992; Boleman et al., 1997). For this reason, the meat industry has frequently focused its efforts on improving the tenderness of beef from aged cows and tough cuts, such as the chuck and round muscle. Collagen content influences meat tenderness (Thu, 2006), and consequently, the high levels of collagen solubility is increased in meat tenderness resulting from conversion of collagen to gelatin on cooking (Vasanthi et al., 2007).

To improve the tenderness of older and tougher cuts, previous studies have tested different post-mortem treatments. Those include electrical stimulation (Dransfield et al., 1992; Hertzman et al., 1993; Olsson et al., 1994; Hwang et al., 2003), proteolytic enzymes (Bernholdt, 1975; Syed Ziauddin et al., 1995; Naveena and Mendiratta, 2001; Naveena et al., 2004), carcass suspension (Herring et al., 1965), the tender cut method (Ludwig et al., 1997), and blade tenderization (Benito-Delgado et al., 1994). The injection of various artificial and natural metabolites and/or tenderizers (Morgan et al., 1991b) has been particularly interesting.

Collagen is one of the main protein components of the connective tissue. To this end, total, soluble, and insoluble collagen content affect meat tenderness (Light et al., 1985). Proteolytic enzymes, typically of plant origin, including papain, bromelain, and ficin, have been used as meat tenderizers (Allen Foegeding and Larick, 1986; Asif-Ullah et al., 2006). Proteolytic enzymes can also play an important role during meat tenderization resulting from the breakdown of myofibrillar proteins (Wang et al., 1958; Kang and Rice, 1970; Rattrie and Regenstein, 1977). These studies also showed that bromelain is more effective than papain for collagen breakdown, but less effective for the hydrolysis of actomyosin compared with papain and ficin. Ginger is widely used as a flavoring ingredient in bakery, sausage seasonings, and Asian cooking. In addition, several studies have assessed ginger as a potential source of plant proteolytic enzymes for use as a meat tenderizer (Thompson et al., 1973; Syed Ziauddin et al., 1995; Naveena and Mendiratta, 2001; Naveena et al., 2004). Ginger contains thiol proteinase with an optimal activity at 60°C, which rapidly denatures at 70°C. In addition, its proteolytic activity is higher against collagen than against actomyosin (Thompson et al., 1973).

Previously, the plant proteases, including cucumis, ginger, and papain on buffalo meat chunks marinated for 48 h at 4°C were indicated the tenderizing effect, due to increased collagen solubility and reduced shear force (Naveena et al., 2004). In this study, we investigate the effects of injecting proteolytic enzymes and ginger extract on beef cuts in tenderization. We assess the influence of proteolytic enzymes in collagen breakdown and Warner-Bratzler shear force (WBSF) to develop more effective industrial meat tenderization methods.

Materials and Methods

Animals and muscle preparation

The National Institute of Animal Science Institutional Animal Use and Care Committee (NIASIAUCC) gave its approval for all the experimental procedures described here. All the experiments were conducted in accordance with the Animal Experimental Guidelines issued by the NIASIAUCC of the Republic of Korea. Holstein steer (HS) meat present high marbling scores (MS), covering a range between 5 and 8 on a scale of 1-9, with 1 being the lowest and 9 being the highest score, according to the Korean Beef Marbling Standard. The marbling score measures intramuscular fat content, and it is one of the most important traits used to define meat quality in Korean cattle. In this study, we used four Holstein steer carcasses (weight, 368.5 ± 6.4 kg; approximately 28 mon old) with high MS (7-8) slaughtered at the NIAS Meat Laboratory. Holstein steers were fed for 28 mon under the NIAS feeding program. Animals were weaned at a mean age of 3 mon and fed *ad libitum* with 30% concentrate and 70% roughage until 6 mon of age. They were castrated at 6 mon of age, and subsequently, fed concentrates consisting of 15% crude protein (CP) and 70% total digestible nutrients (TDN) until 12 mon, 14% CP and 71% TDN until 21 mon, and 12% CP and 73% TDN to the time of slaughter. After slaughter, the carcasses were stored for 24 h in a cold room (2 ± 1°C). *M. pectoralis profundus* presenting a high proportion of reticular fibers in a brisket cut (average weight = 3.47 kg) were seamed out from chilled carcasses and stored at -20°C for

4 days. We took 4 samples (rectangular-shaped; 1.72-2.15 kg per a sides) from the central portion of the whole brisket of each carcass. We also trimmed any visible external fat and connective tissue from the samples.

Enzyme treatments

We divided the samples from four carcasses into four treatments. Samples were more softened and inflated by injection, but retained its shape.: (1) control (without proteolytic enzyme), (2) 50 ppm bromelain (bromelain), (3) a mixture of 50 ppm bromelain and 20 ppm papain (bromelain + papain), and (4) 5% ginger extract (ginger extract). Bromelain (Activity, 400 casein protease u/g; biological source, *Ananas comosus*; off-white powder form; pH, 5.0-7.0; optimal temperature, 45-55°C) and papain (Activity = 154 casein protease units/g; biological source, microbial; yellow liquid form; pH, 5.0-7.0; optimal temperature, 50-60°C) were purchased from Biocatalysts (Cardiff, UK). The bromelain treatment was prepared in water to a final concentration of 50 ppm of bromelain. The bromelain + papain treatment was prepared in water to a final concentration of 50 ppm of bromelain and 20 ppm of papain. The ginger extract was prepared from fresh ginger roots. To prepare the ginger suspension, we purchased fresh ginger roots from a commercial store, and subsequently peeled, sliced, and blended them in cold distilled water (50%, w/w) for 2 min using a Robot Coupe blender (Robot Coupe Ltd., UK). The homogenate was then pressed to produce a crude ginger by heavy duty manual press with four layers of cheesecloth for approximately 1 min. The 5% ginger suspension (w/v) was prepared in water. The treatments were injected into *M. pectoralis profundus* using a Dorit PSM-21 Inject-O-Mat brine injector, 36 multi-needle injector at 1.5 mB pressure, at a volume accounting for 10% of the initial weight of the meat cut by using 500 mL measuring cylinder. After injection, the sample shape barely changed and the weight (986.3 ± 68.6 g) did not differ significantly among treatments. Water was used as a control treatment. Excess brine was allowed to drain off after re-weighing. After injecting the solution, each treatment was vacuum-tumbled dried for 10 min and vacuum-packed. The samples were then incubated in a chiller at 0°C for 48 h. Subsequently, 2.54 cm steaks were cut from each sample, vacuum-packed and frozen at -20°C until further analysis. We also took samples for drip loss measurement immediately after incubation.

pH values

We measured muscle pH values before injection and at 48 h post injection using a portable pH meter (Orion Research Inc., USA) fitted with an Amagruss pH electrode (pH/mV Sensors Ltd., Murrisk-Westport, Co., Ireland), adjusted for muscle temperature. Before analysis, the meter was calibrated using standard phosphate buffers (pH 4.0 and 7.0, Radiometer, Denmark). The electrode was rinsed with distilled water between measurements. The pH values were recorded as the average estimated from different muscle regions.

Drip loss

We analyzed drip loss following the procedure by Honikel et al. (1994). For this analysis, we cut samples (2.54 cm thickness) of similar dimensions, weighing approximately 100 g, from each brisket cut and suspended them in an expanded bag so that the meat did not come in contact with the bag. The samples were then stored at 2°C for 96 h, and subsequently, the surfaces were blotted lightly with tissue and re-weighed. Drip loss was expressed as a percentage of the original weight of the meat.

Cooking loss and WBSF determination

We measured cooking loss from samples of similar size and weight (2.54 cm thickness, 100 ± 5 g) after thawing them for 24 h at 4°C in a chiller before cooking. After thawing, we patted all samples dry using paper towels and cooked them in a water bath at 72°C (Model Y38, Grant Instruments Ltd., UK) until a core temperature of 70°C was reached. The steaks were then patted dry again and re-weighed to determine the cooking loss. Cooking loss was calculated as the percentage of the raw weight lost, based on the weights of all steaks before and after cooking. The mass changes were expressed as a percentage of the initial mass (w/w, wet basis).

The WBSF from the different steak samples (2.54 cm thickness) were measured using the method described by Shackelford et al. (1991) with modifications. The steaks were cooked to a core temperature of 70°C (Minitherm HI8751 temperature meter and probe, Hanna Instruments Ltd., Eden Way, Pages Industrial Park, Leighton Buzzard, Bedfordshire, LU7 8TZ, UK) in a water bath (model Y38, Grant Instruments Ltd., Barrington, Cambridge CB2 IBR, UK), left to cool to room temperature, and subsequently left to cool overnight at 4°C. We then collected four random cores per steak shortly after removing them from the refrigerator. The cores (1.25 cm diameter; n=4) were immediately tested for shear force very soon after cut using an Instron model 5543 and Merlin series IX software (Instron Ltd., UK).

Total collagen content and collagen solubility

Prior to collagen analysis, all cooked and uncooked samples were thawed at 4°C for 8 h and then allowed to reach ambient temperature for 30 min. Reproducibility of the results was ensured by following the validation method described by Kolar (1990). To estimate the total and soluble collagen content, we used a method previously described in the literature (Fang et al., 1999; Hill, 1966; Woessner, 1961). Homogenized meat (5 g) was placed in a 50 ml centrifuge tube before adding Ringer's solution (12 ml) and mixing. The mixture was then incubated in a water bath at 77°C for 65 min with stirring at 15 min intervals and centrifuged for 10 min at 3,990 g (Sorvall RC5C, rotor SS-34, Du Pont Company, USA). The supernatant was collected and 8 ml of Ringer's solution was mixed with the precipitate and centrifuged for a further 10 min. After rinsing the precipitate, the supernatants from the two centrifugations were combined. The supernatants and precipitates of all samples were hydrolyzed separately in 30 ml of 6 N H_2SO_4 in a Tecator Digestion System 20 (1015 Digester) at 110°C for 16 h. The hydrolysates were diluted to 250 ml and the hydroxyproline content was determined using a colorimetric reaction after neutralizing the solutions with 4.37 ml of 1 M NaOH; the absorbance at 560 nm was then recorded (Perkin-Elmer Lambda 2 spectrometer, Germany). A standard calibration curve for hydroxyproline content was established. The percentage of soluble collagen was calculated from the hydroxyproline concentration in the supernatant. Total collagen content was calculated from the sum of the hydroxyproline concentrations in the precipitate and in the supernatant using a conversion factor of 7.25 (Goll et al., 1963) and expressed as mg/g of wet weight. Collagen solubility, which reflects the degree of collagen crosslinking, was expressed as a percentage of the soluble collagen over the total collagen content (w/w).

Differential scanning calorimetry (DSC)

We used DSC analysis to determine the onset (T_o) and peak (T_p) temperatures of thermal shrinkage for the intramuscular connective tissue (IMCT) following a procedure previously described (Aktaş and Kaya, 2001). For this analysis, we prepared a buffer solution for extracting myofibril proteins from IMCT by mixing 0.1 M KCl + 0.02 M KH_2PO_4 or 0.1 M KCl + 0.02 M K_2HPO_4 (pH 5.75). Subsequently, we placed 10 g of meat per sample in 100 ml of buffer

solution (4-6°C) and centrifuged it at 8,000 rpm for 10 s using an Ultra Turrax homogenizer (T25, Janke & Kunkel, GmbH & Co KG, Germany). We then collected any IMCT adhering to the mixer blades and the liquid was discarded. The IMCT was washed twice by agitation for 10 s in 100 ml of distilled water. The sample was kept in the distilled water for 5 min, subsequently dried using filter paper, and approximately 10 mg were placed in a DSC sample pan. After sealing the pans hermetically, the temperature of the sample were left to increase from 10°C to 95°C at a rate of 5°C/min. To determine T_o and T_p values, we used a DSC2010 instrument (TA Instruments, Dublin, Ireland) with a refrigerated cooling system (RCS) calibrated with mercury (mp, -38.8°C; $\triangle H$m, 11.4 J/g), distilled water (mp, 0°C; $\triangle H$m 334.5 J/g), and indium (mp, 156.6°C; $\triangle H$m 28.5 J/g). An empty sample pan was used as a reference. T_o and T_p values were determined according to a procedure previously described by Voutila et al. (2007).

Statistical analysis

All experiments were performed in quadruplicate with replicates in each experiment. The replication analysis was performed with means on a single sample. The significant differences among proteolytic treatments was determined using one way analysis of variance (ANOVA) and multiple comparisons of means were done using Post Hoc (least square difference test (LSD) procedure. A p value of $p<0.05$ was considered as statistically significant.

Results and Discussion

Meat quality and collagen content and solubility

The pH measured before and 48 h after injection did not differ significantly among treatments (Table 1). This follows the results published by Naveena et al. (2004), who found no significant difference in pH after treatments between control and treatments from buffalo *Biceps femoris* muscle.

Table 1. Effects of enzyme treatments on pH, cooking loss, drip loss, and Warner-Bratzler shear force (WBSF) of samples of beef M. *pectoralis profundus* from the brisket cut of Holstein steers

Items	Control	Bromelain (50 ppm)	Bromelain (50 ppm) + Papain (20 ppm)	Ginger extract (5%)	SE[1)]	*p*-values
pH before injection	5.69	5.68	5.66	5.64	0.03	0.189
pH at 48 h after injection	5.70	5.80	5.74	5.73	0.03	0.067
Δ pH increment	0.01	0.12	0.08	0.09	0.06	0.086
Cooking loss (%)	33.9[b]	39.1[a]	37.5[ab]	34.5[ab]	1.66	0.035
96-h drip loss (%)	1.21	1.28	1.13	1.04	0.36	0.268
WBSF(N)	64.0[a]	40.9[b]	38.5[b]	40.6[b]	7.04	0.046

[1)]SE = standard error (n=5).
[a,b]Means in the same row with no superscript letters after them or with a common superscript letter following them are not significantly different ($p<0.05$).

Treating with bromelain caused a significant increase in cooking loss relative to the untreated control, while cooking loss was not significantly different for samples treated with bromelain + papain and those treated with ginger extract. Naveena and Mendiratta (2001) also showed that adding 5% ginger extract did not affect cooking loss relative to a control. Additional, cooking yield is inversely proportional to cooking loss (Ketnawa and Rawdkuen, 2011). Pawar et al., (2000) reported that decreased cooking yield of all treatment when compared to the control may be caused by the degradation of sarcoplasmic and myofibrillar proteins. In this study, the ginger extract was injected into beef muscle and

then vacuum-tumbled. In Mentiratta et al. (2000), on the other hand, the ginger extract was applied to buffalo meat cubes by marination. We found no significant difference in drip loss between any of the treatments and the untreated control.

As expected, no significant differences in the total collagen content were found between any of the treatments and the control; however, all treatments led to a significant increase in collagen solubility relative to the control (Table 3). The samples treated with bromelain showed significantly higher collagen solubility than the control and samples treated with ginger extract and increased collagen solubility compared with those treated with bromelain and papain. These results indicate that the addition of 20 ppm of papain is not affected collagen solubility. Collagen solubility of samples treated with calotropis, papain, papaya, and bromelain has also been reported to increase in different types of meat (Rawdkuen and Benjakul, 2012).

Table 2. Effects of enzyme treatments on total collagen content and collagen solubility of samples of beef M. *pectoralis profundus* from the brisket cut of Holstein steers

Items	Control	Bromelain (50 ppm)	Bromelain (50 ppm) + Papain (20 ppm)	Ginger extract (5%)	SE[1)]	*p*-values
Total collagen (mg/g)	12.2	10.8	11.6	11.2	1.07	0.079
Collagen solubility (%)	4.7[c]	38.7[a]	29.6[ab]	10.3[b]	4.98	0.016

[1)]SE = standard error (n=5).
[a-c]Means in the same row with no superscript letters after them or with a common superscript letter following them are not significantly different (p<0.05).

Thermal shrinkage and WBSF

The onset and peak temperature (T_o and T_p, respectively) values for IMCT thermal shrinkage are shown in Table 3. The samples treated with bromelain + papain showed a significantly lower T_o value than the control. And, all treatments showed significantly lower T_p values than the control; however, no significant differences were found among treatments. These results indicate that the addition of 20 ppm papain affected the T_o value.

Table 3. Effects of enzyme treatments on thermal transition temperature of intramuscular connective tissue of samples of beef M. *pectoralis profundus* from the brisket cut of Holstein steers

	Control	Bromelain (50 ppm)	Bromelain (50 ppm) + Papain (20 ppm)	Ginger extract (5%)	SE[1)]	*p*-values
Onset temperature (T_o)	61.1[a]	59.0[ab]	58.3[b]	59.3[ab]	0.89	0.032
Peak temperature (T_p)	65.5[a]	63.4[b]	63.6[b]	63.4[b]	0.03	0.015

[1)]SE = standard error (n=5).
[a,b]Means in the same row with no superscript letters after them or with a common superscript letter following them are not significantly different (p<0.05).

Collagen T_p value depends on the state of hydration of the collagen fiber (Miles et al., 2005). Previous reports have shown that acid marination reduced IMCT T_p (Judge and Aberle, 1982; Horgan, 1990; Aktaş and Kaya, 2001). This suggests that weak acid marination disrupts noncovalent cross-links (hydrogen bonds, dipole or ion-pair interactions, and intermolecular bridges) rather than covalent aldimine cross-links (Aktaş and Kaya, 2001). Aldimine cross-links are sensitive to pH reductions, leading to a decrease in T_o (Horgan et al., 1990).

In contrast, we found no significant differences in pH between the control and the treatment groups. Although we did not measure the content of different collagen cross-link types, we can only speculate that a change in the state of hydration of the collagen fibers and the disruption of noncovalent cross-links by injecting proteolytic enzymes and tumbling

resulted in lower T_o and T_p values. Miller et al. (1983) reported that collagenous tissue with higher cross-linkage would be more resistant to swelling of the muscle tissue, a process necessary for the tenderization of meat.

We observed significant differences between the WBSF values of the treatments and those of the control, but not among treatments (Table 1). WBSF values decreased by 36%, 40%, and 37% relative to the control in the bromelain, bromelain + papain, and ginger extract treatments, respectively. We obtained similar WBSF values in the bromelain and the ginger extract treatments, which are noteworthy given we also found significant differences in collagen solubilities in these two treatments.

These results are in agreement with the findings of Naveena et al. (2004), who reported lower WBSF values in samples treated with ginger extract compared to the control. The proteolytic enzymes and ginger extract used in this study had the potential to cause tenderization due to an increased breakdown of both collagen and myofibrillar protein. Lee et al. (1986), using trans mission electron microscope (TEM) studies of beef steaks treated with ginger extract, reported ginger extract treatment resulted in degradation of thin filaments in the I-bands resulting in extensive fragmentation of myofibrils.

In conclusion, proteolytic enzymes and ginger extract effectively tenderized beef M. *pectoralis profundu*s, increasing collagen solubility, reducing T_0 and T_p temperatures in IMCT, and decreasing WBSF compared to the untreated control. Thus, based on physico-chemical properties, expecially shear force values, treatment with 5% ginger extract can be effectively utilized at the tenderization.

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