EFFECT OF PROTEASES FROM *BACILLUS SUBTILIS* AND *ASPERGILLUS ORYZAE* ON THE TENDERNESS OF BEEF

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Introduction

Tenderness, the single most important palatability factor affecting consumer satisfaction of beef, is mainly associated with the structural integrity of myofibrillar and connective tissue proteins. One approach to increasing beef tenderness is to significantly reduce the amount of detectable connective tissues without causing extensive degradation of muscle fibers. This purpose may be achieved by controlled proteolysis of targeted proteins by addition of exogenous enzymes. Although enzyme tenderisation of meat has been used for many years, most commonly using enzymes of plant origin such as papain, bromelain, and ficin, previous reports on the effects of enzymatic tenderisation on tenderness of cooked meat have been unsatisfactory. Due to their broad substrate specificity, these enzymes tend to indiscriminately break down the major muscle proteins which often results in an extensive degradation of the meat structure and undesirable flavour and texture (McKeith *et al.*, 1994; Stefanek *et al.,* 2002).

Current technology in the area of enzyme engineering has revealed other novel sources of proteolytic enzymes, mainly of fungal origin. There has been, however, little research on using novel proteases obtained from alternative sources (Ashie *et al.,* 2002; Stefanek *et al.,* 2002). The application of enzyme technology may provide a useful means of meeting consumer expectations for product quality and consistency.

The current study was conducted to determine the range of acceptable aqueous concentrations of proteinases from *Aspergillus oryzae* and *Bacillus subtilis* injected at 105% of raw weight and to determine if the enzyme preparations were active at refrigeration temperature following injection. In the second study we examined effectiveness of refined levels of the proteases during moist and dry cooking.

Materials and methods

Beef inside rounds from young Canada Grade A carcasses were obtained from the Agriculture and Agri-Food Canada Lacombe Research Centre (Lacombe, AB, Canada) abattoir. The *gracilus* and *adductor* muscles were removed, and *semimembranosus* muscles (SM) were trimmed of all visible fat and connective tissue and then were divided into four (experiment 1) or two (experiment 2) sections.

The major variables investigated in experiment 1 were level of two proteinases from *Aspergillus oryzae* (Amano Enzyme USA Co., Ltd., Elgin, IL) or *Bacillus subtilis* (Enzeco®, Enzyme Development Corporation**,** New York, NY, USA) (to deliver 0.001%, 0.0025%, 0.005% in injected product) and post-injection storage time (1, 7, 14 days). Each SM muscle was cut into four roasts (700 g). The roasts designated for enzyme treatment were injected to 105% over the raw meat weight with enzyme solution and then each section was cut into three 2.5 cm steaks which were individually vacuum packaged and refrigerated (4ºC) for 1, 7 and 14 days. Steaks were cooked in a conventional oven (Model CRSL3400VM-1, Camco Inc., Moncton, NB, Canada) at 163ºC to a final temperature of 71ºC.

In experiment 2, paired SM muscles were divided into two equal portions to yield four sections. One section in each pair was kept as a non-injected control, while the other three were injected to 105% over the raw meat weight with enzyme solution to give 0.0005%, 0.001% and 0.0015% of proteinase from *Aspergillus oryzae* or 0.0005%, 0.0015% and 0.0025% of proteinase from *Bacillus subtilis* in the final product. The sections designated for enzyme treatment were injected to a target weight gain of 5% of the original mass using a multi-needle injector (Model No. FGM20/40, Fomaco Reiser, Burlington, ON, Canada). After injection, the muscle samples were vacuum packaged and stored overnight at 4°C.

The following day each section was cut into four equal-sized roasts that were used for texture measurements after dry- and moist-heat cooking. All roasts were cooked in a preheated

conventional oven (Model CRSL3400VM-1, Camco Inc., Moncton, NB, Canada) set at 165°C. Roasts assigned to dry-heat cooking were placed individually on mesh racks in aluminum pans and a copper constantan thermocouple was inserted into the geometric center of each in order to monitor the internal temperature. Roasts for moist-heat cooking were wrapped in aluminum foil and cooked as described above. In both methods of thermal processing, roasts were cooked to a final internal temperature of 71°C or 79°C. After roasts had reached the final end-point temperature they were removed from the oven, placed in individual plastic bags and submersed in an ice bath to arrest cooking. Once the roasts were cooled to ~20°C, they were transferred to a cooler and stored at 4°C until further testing. The temperatures of the oven and roasts were monitored using the thermocouples attached to a scanner (Model 692-8000, Barnant Co., Barrington, IL, U.S.A.) and a computer, which recorded the change in temperature at 30 s intervals.

Cooking yield = (cooked weight /raw weight at cooking) x 100, was recorded for each roast. The shear force of 7-9 core samples (1.27 x 1.27 x 2.54 cm), cut parallel to the fibre direction from each cooked roast, was determined. Samples were sheared perpendicular to the fibre direction using a TMS-90 texture system (Food Technology Corp., Rockville, MD, USA) fitted with a Warner-Bratzler shear attachment. Maximum peak force recorded during the test was reported as shear force. The textural characteristics of beef roasts were analyzed according to texture profile analysis (TPA) (Bourne, 1982) using a TMS-90 texture system (Food Technology Corp., Rockville, MD, USA). Six cores were cut (1.27 x 1.27 x 1.27 cm) from the center slice of each cooked roast and compressed twice to 30% of their original height. A cross-head speed of 100 mm/min was applied. The TPA parameters, namely hardness (peak force on first compression [N]), cohesiveness (ratio of the active work done under the second force-displacement curve to that done under the first compression curve [dimensionless]), springiness (distance the sample recovered after the first compression [mm]), and chewiness (hardness x cohesiveness x springiness [N*mm]) were computed.

Expressible moisture (EM) of roast samples was determined 24 h after thermal processing by a modified method as described by Pietrasik & Li-Chan (2002).

Statistical analysis

All data were analyzed using SPSS analysis of variance (SPSS 11.0.1 for Windows, SPSS Inc., Chicago, IL, USA). Enzyme treatments were applied to each round (1st experiment) or muscle pair (2nd experiment) with location within muscle balanced to ensure that all treatments were assigned twice to all locations. In first experiment data were analysed as a 4x3 factorial design with enzyme level and post-injection storage time (1, 7, 14 days) as the main effects. In 2nd experiment data were analysed as a 4x2x2 factorial design with enzyme level, cooking method (dry, moist) and endpoint temperature (71^{o}C, 79^{o}C) as the main effects. In both experiments *Aspergillus* and *Bacillus* injected samples were analysed separately. The least significant difference (LSD) test ($P<0.05$) was used to determine differences between treatment means.

Results & Discussion

Experiment 1:

Roasts had significantly ($P<0.01$) lower cooking yield with higher levels of enzyme addition (Table 1). Enzyme-treated meat showed a gradual reduction in shear force with an increase of enzyme concentration. The lowest concentration (0.001%) of enzyme reduced WBSF by about 25% as compared to the control while maintaining acceptable appearance and texture. However, steaks injected with the highest (0.005%) concentration of proteinase had localized spots with unacceptable mushy and creamy texture. At these levels, the mushy texture caused by the activity of enzymes during cooking made them undesirable and may cause consumer rejection of steaks formulated with these levels of enzymes.

Our results indicated no obvious difference amongst shear values following the different lengths of refrigerated storage (1, 7, 14 days) after injection (Table 1). This suggested that these enzymes were relatively inactive at refrigerated temperatures and the tenderising effect occurs mainly during cooking. It appeared that the protease functioned quite effectively once cooking commenced and substrate temperature increased.

Stefanek *et al*. (2002) also reported a lack of shear force difference following extended post-injection storage times of up to 4 weeks. In contrast, Ashie *et al.* (2002) found that papain retained

maximal activity even after cooking to 75°C thus increasing the risk of both texture and flavour defects.

Experiment 2

From exp. 1 we determined that the tenderizing effect of enzyme injection was primarily manifested during cooking and meat could therefore be stored without any adverse enzymatic changes in product characteristics.

Overall initial results indicated the potential use of these enzymes in meat tenderization and justified further experimentation at lower levels of these enzymes to try and reduce the mushy, overtenderized textural problems.

The effects of dry and moist cooking in a conventional oven after injection with different levels of the two enzymes were assessed. The cooking method employed had significant ($P<0.01$) effects on the cooking time (min/100 g) through its interaction with endpoint temperature. Significant differences in the cooking time due to method of cooking were observed only for roasts heated to an internal temperature of 79°C. As expected, roasts cooked to 79°C using the dry method required the longest amount of time followed by roasts cooked to 79°C by moist regime, whereas there was no significant difference between cooking methods in cooking time of roasts cooked to an internal temperature of 71°C; typical heating curves from these cooking regimes are depicted in Fig. 1.

As expected, the different endpoint temperatures investigated also affected cooking yield of roasts. Regardless of the cooking method, roasts cooked to a higher end-point internal temperature appeared very dry/grainy and had lower cook yield compared to those cooked to 71°C (Tables 2 and 3). Some studies have indicated that cooking to the higher end-point temperature, therefore the increased cooking time, increases moisture loss, especially for intact muscles (Beilken *et al.*, 1986; Boles and Swan, 2002; Dhanda *et al.*, 2002). This observation was well supported by the values obtained for EM, with roasts cooked to 79°C having significantly ($P<0.001$) lower EM, thereby indicating lower amounts of free moisture due to loss during cooking.

Enzyme-injected muscles had lower cook yield than non-injected control samples. Janz *et al*. (2005) also reported a lower cooking yield, compared to uninjected controls, following 105 and 110% injection of beef *semitendinosus* muscles with a water solution of liquid porcine pancreatin. Enzyme-treated roasts had also significantly lower EM than did roast beef made from non-injected meat indicating that there was less free moisture in the product as a result of larger cook loss.

Textural characteristics of the roasts injected with protease from *Bacillus subtilis* were not significantly affected by cooking method. The significant improvement of shear force values of roasts due to enzyme treatment had been achieved at 15 ppm *B. subtilis* level (Table 2). *Bacillus subtilis* injected SM muscles were also progressively less hard with increasing level of enzyme. Roasts injected with protease from *Aspergillus oryzae* cooked to 71°C exhibited lower TPA hardness, chewiness and springiness, but were more cohesive than those processed to 79°C. *Aspergillus oryzae* injected roasts processed by the moist cooking regime had lower shear force values than those cooked by the dry method (Table 3). However, the moist cook regime appeared to be more effective in the enzyme treated roasts. Enzyme injected roasts cooked by moist method were more tender (lower shear values) in comparison to non-injected control; while with the exception of the highest (0.0015%) enzyme treatment, there were no differences in shear force among samples baked in the dry environment (Table 4). In addition, a significant reduction of WBSF values of moist cooked roasts had already been achieved at 0.0005% enzyme level; whereas dry cooked roasts required 0.0015% enzyme. Regardless of the endpoint temperature, increased level of protease resulted in a gradual decrease in hardness of moist cooked roasts, but its effect was insignificant for roasts cooked by the dry regimen. The significant decrease of hardness and shear force values due to enzyme treatment in moist cooked roasts compared to roasts processed by the dry method might have been due to the initially slower heating rate of the moist method and approx. 15 min longer period of time in the temperature range for increased activity of injected proteinase (20°C to 50°C).

Conclusions

Exploratory testing indicated that a negligible amount of proteolysis occurred at refrigeration temperature, suggesting that enzyme-treated meat could therefore be stored without any adverse enzymatic changes in product characteristics. This result shows the superiority of the proteinase from *Bacillus subtilis* or *Aspergillus oryzae* to currently used enzymes such as papain which retains

its activity at refrigerated temperature even after cooking thus increasing the risk of both texture deterioration and flavour defects.

Generally, the positive effect of increased enzyme concentration on tenderness of beef was more pronounced in roasts cooked by a moist heat method. Owing to a longer time in the temperature range from 20°C to 50°C, the moist cooking regime yielded products with lower shear force values than those cooked by the dry method.

References

Ashie, I.N.A., Sorensen, T.L. and Nielsen, P.M. (2002). Effects of papain and microbial enzyme on meat proteins and beef tenderness. *Journal of Food Science*, 67:2138-2142.

Beilken, S.L., Bouton, P.E. and Harris, P.V. (1986). Some effects on the mechanical properties of meat produced by cooking at temperatures between 50° and 60°C. *Journal of Food Science*, 51:791-796.

Boles, J.A. and Swan, J.E. (2002). Heating method and final temperature affect processing characteristics of beef *semimembranosus* muscle. *Meat Science*, 62:107-112.

Bourne, M.C. (1982). Food texture and viscosity. Academic Press, New York.

Dhanda, J.S., Pegg,, R.B., Janz, J.A.M., Aalhus, J.L. and Shand, P.J. (2002). Palatability of bison *semimembranosus* and effects of marination. *Meat Science,* 62:19-26.

Janz, J.A.M., Pietrasik, Z., Aalhus, J.L. and Shand, P.J. (2005). The effects of enzyme and phosphate injections on the quality of beef *semitendinosus*. *Canadian Journal of Animal Science,* 85:327-334.

McKeith, F.K., Brewer, M.S. and Bruggen, K.A. (1994). Effects of enzyme applications on sensory, chemical and processing characteristics of beef steaks and roasts. *Journal of Muscle Foods*. 5:149-164.

Pietrasik, Z. and Li-Chan, E.C.Y. (2002) Response surface methodology study on the effects of salt, microbial transglutaminase and heating temperature on pork batter gel properties. *Food Research International*, *35*:387-396.

Stefanek, J.L., Scanga, J.A., Belk, K.E. and Smith, G.C. (2002). Effects of enzymes on beef tenderness and palatability traits. [On-line].
Available: http://ansci.colostate.edu/ran/beef/2002/pdf/jls02 [3 April 2006].

Table 1. Effect of enzyme level and storage time on cook yield and shear force of *semimembranosus* steaks

	Aspergillus oryzae		*Bacillus subtilis*	
Enzyme level (%)	Cook yield (%)	WBSF[x] (N)	Cook yield (%)	WBSF (N)
0	68.1a	80.4a	67.1a	76.3a
0.001	64.6b	63.2b	64.7b	62.5b
0.0025	65.1b	49.3c	65.2b	54.3c
0.005	64.9b	45.9c	64.4b	49.5c
SEM	0.63	2.11	0.59	2.30
Storage time (days)				
1	65.5	62.6	65.1	63.5
7	65.4	57.0	65.7	59.2
14	66.2	59.5	65.2	57.3
SEM	0.48	2.17	0.51	1.99

a-c, Means with different letters in the same column (within each main effect) are significantly different ($P<0.05$).
[x]WBSF, shear force value (N=newton, SI unit of force).

Table 2. Processing and textural characteristics of cooked beef *semimembranosus* muscle roasts injected with protease from *Bacillus subtilis*

	Cooking yield (%)	EM (%)[w]	WBSF (N)[x]	Hardness (N)	Cohesiveness (-)	Springiness (cm)	Chewiness (N*cm)
Cooking method							
Dry	63.8	8.48	63.3	199	0.378	0.25	19.5b
Moist	62.7	8.74	62.9	212	0.388	0.26	22.3a
p-value	*0.10*	*0.82*	*0.94*	*0.21*	*0.31*	*0.05*	*0.01*
Endpoint temperature							
71	65.5a	10.52a	61.4	198	0.392	0.25	20.2
79	61.1b	6.69b	64.8	212	0.373	0.26	21.6
p-value	*0.01*	*0.00*	*0.39*	*0.23*	*0.16*	*0.08*	*0.43*
Enzyme level[z]							
0	65.2a	10.11a	75.2a	244a	0.408a	0.26a	27.1a
5	64.1a	8.95a	68.5a	219b	0.393a	0.26a	23.4b
15	62.0b	7.70b	56.7b	183c	0.369b	0.25ab	17.3c
25	61.8b	7.67b	52.0b	175c	0.360b	0.24b	16.0c
p-value	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*

a-c, Means with different letters in the same column (within each main effect) are significantly different ($P<0.05$).
[w]EM, expressible moisture.
[x]WBSF, shear force value (N=newton, SI unit of force).

Table 3. Processing and textural characteristics of cooked beef *semimembranosus* muscle roasts injected with protease from *Aspergillus oryzae*

	Cooking yield (%)	EM (%)[w]	WBSF (N)[x]	Hardness (N)	Cohesiveness (-)	Springiness (cm)	Chewiness (N*cm)
Cooking method							
Dry	62.89	8.54	76.9a	220	0.392	0.26	22.8
Moist	63.34	8.81	66.3b	208	0.395	0.26	21.9
p-value	*0.75*	*0.52*	*0.03*	*0.08*	*0.80*	*0.30*	*0.72*
Endpoint temperature							
71	66.45a	10.46	67.8	202	0.401	0.24b	20.8b
79	59.77b	6.89	70.7	226	0.386	0.27a	25.9a
p-value	*0.00*	*0.00*	*0.90*	*0.00*	*0.32*	*0.01*	*0.01*
Enzyme level[z]							
0	65.58a	9.65a	84.5a	245a	0.406a	0.27a	27.5a
5	63.06b	9.70a	76.4ab	223ab	0.403a	0.27a	24.3a
10	62.39bc	7.80b	69.5b	204b	0.395a	0.25b	20.3b
15	61.42c	7.56b	56.6c	186b	0.377b	0.24b	17.5b
p-value	*0.00*	*0.00*	*0.00*	*0.00*	*0.02*	*0.00*	*0.00*

a-c, Means with different letters in the same column (within each main effect) are significantly different ($P<0.05$).
[w]EM, expressible moisture.
[x]WBSF, shear force value (N=newton, SI unit of force).

Table 4. Cooking method x enzyme level interactions for processing and textural characteristics of cooked beef *semimembranosus* muscle roasts injected with protease from *Aspergillus oryza*

		Cooking yield (%)	Expressible moisture (%)	WBSF (N)	Hardness (N)	Cohesiveness (-)	Springiness (cm)	Chewiness (N*cm)
Cooking method x Enzyme level								
Dry	0	65.9a	9.15a	86.4a	238.8ab	0.405a	0.27ab	26.1ab
	5	62.6b	9.84a	83.3a	216.4abc	0.407a	0.26abc	22.7bc
	10	62.0b	7.71b	78.8a	217.9abc	0.404a	0.25bc	22.5bc
	15	61.1b	7.46b	59.3c	208.6bc	0.375c	0.25bc	19.9c
Moist	0	65.2a	10.14a	82.6a	250.9a	0.407a	0.28a	28.4a
	5	63.6ab	9.55a	69.5b	229.6ab	0.399ab	0.28a	25.9ab
	10	62.8b	7.88b	59.3c	188.7cd	0.386bc	0.25bc	18.3cd
	15	61.8b	7.65b	53.8c	162.5d	0.379c	0.24c	15.0d
p		*0.51*	*0.61*	*0.02*	*0.03*	*0.12*	*0.11*	*0.02*

a-c, Means with different letters in the same column are significantly different ($P<0.05$).

Figure 1. Temperature of roasts during moist or dry cooking.