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Prepared by: Geert Geesink and Xuemei Han

University of New England

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Enzyme Tenderizers for Fresh Meat

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Abstract

Three experiments were conducted within this project to explore the suitability of commercially available enzyme tenderizer to improve the tenderness of beef. In the first experiment, beef topsides were injected to 10% of their weight with 7 different enzyme preparations according to the manufacturers recommendations, aged for 19 days and evaluated for objective quality traits (shear force, compression and colour) and sensory quality (tenderness, juiciness, flavour and overall liking). Based on the objective tenderness measurements, only three of the enzyme preparations (papain, zingibain and actinidin) showed some potential to improve the tenderness after grilling or roasting of the meat. None of the treatments led to a statistically significant increase in the sensory quality score (MQ4) of grilled steaks. However, two of the treatments led to a significant decrease in the MQ4 score (bromelain and a bacterial protease). Colour and colour stability where not affected by any of the treatments.

Given that the manufacturers' recommended concentrations did not lead to a desired impact on tenderness, it was decided to investigate which dose of these enzymes resulted in effective tenderization and what their effects were on myofibrillar proteins and connective tissue (collagen). To this aim, an experiment was conducted at Otago University (New Zealand) in which beef topside samples were injected with up to 5, or 10 times the recommended concentrations, and in-house prepared proteinase extracts from kiwifruit and asparagus. Shear force and compression values of these samples were determined at Otago University at 1 and 21 days after injection, and analysis of degradation of myofibrillar proteins and collagen was performed at UNE. When injected at the highest concentrations tested, only four of the tested enzyme preparations (papain, zingibain, protease G, and the kiwifruit extract [actinidin]) had an appreciable effect on objective measures of tenderness. Results from the collagen analysis indicated that only papain, actinidin, and the kiwifruit extract (actinidin) had a noticeable effect on collagen. Results regarding degradation of myofibrillar proteins using SDS-PAGE and Western blotting of the myofibrillar proteins desmin and troponin-T showed that the different treatments resulted in only minor degradative changes in either the raw or cooked samples.

Although the kiwifruit extract prepared at Otago University was the most effective in causing protein degradation and improving tenderness, the final experiment was conducted using a commercially available kiwifruit (actinidin) extract. In the final experiment, beef short loin sections (about 25 cm in length; 7 days p.m) were injected (10% w/v) with a 10, 25, or 50 g/L solution of the actinidin extract, vacuum packed and aged for 6 days at 2°C. Samples were evaluated for yield, objective quality traits (shear force and colour) and sensory quality (tenderness, juiciness, flavour and overall liking). Yield determination revealed that little of the initially injected volume remained in the muscles during refrigerated vacuum storage. Results from the sensory evaluation and shear force measurements suggested a tenderizing effect, but a dose response effect was not apparent. Results regarding proteolysis of collagen and myofibrillar proteins indicated that actinidin treatment mainly affected collagen and exhibited only marginal effects on myofibrillar proteins.

Based on the results of the experiments within this project, and work published by others, it appears that actinidin can be used successfully as a meat tenderizer. However, the challenge is to design methods to control the uptake of the injected/infused enzyme preparations, without compromising the characteristics/image of fresh meat. If the addition of salts or other additives is considered acceptable for the meat product under consideration, adding actinidin to currently used "enhancement" formulations could be a successful strategy to improve the tenderness of cuts that need improvement regarding this trait.

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1 Background

MLA's Red Meat Innovation program aims at growing demand for red meat by building the industry's capability to develop value added red meat products. The program focuses on developing technology platforms, developing new products, and building the product development capability of red meat processors and value-adders.

The advent of the MSA grading system in Australia has produced a culture where quality and palatability can receive a premium in the market place. This in turn has created an opportunity to add value to meat cuts by enhancing the palatability of less desirable cuts (those that do not reach 3 star or "Good Every Day" status) and offers a real chance to add value throughout the red meat industry supply chain. Exogenous enzymes have been extensively used in food processing for a wide range of applications. Their role in tenderizing meats is well known, but difficulties in controlling the extent of breakdown and also the target tissues which are broken down has meant that they are rarely used in the Australian domestic beef industry. This contrasts with the US where mechanical or enzymatic treatment of meat is more common for food service products.

The use of exogenous enzymes is most suitable for large whole muscle cuts that can be sold in either roast or steak form such as: striploin, cube roll, rump, top side, outside flat, eye round, bolar blade, chuck tender, chuck roll, brisket and tri-tip. As discussed later, the limiting component for quality will vary with specific cuts. For high connective cuts, the factor that would limit palatability is insolubility of the connective tissue (i.e. it contains insoluble cross-links which are not gelatinized with relatively short cooking periods), and therefore optimal palatability is attained with these cuts being sold in roast form and being subject to a long slow cooking process. On the other hand, the limiting factor for striploins (which is a low connective tissue cut) with a MQ4 of less than 46 is generally myofiber toughness which is not corrected by extended application of heat during cooking. Collectively, the muscles that would benefit by application of tenderizing agents comprise approximately 40% of the lean meat yield of a carcass, and the greater portion of the valuable cuts at retail or food service market channels. Thus, enzymes could be used to improve tenderness of higher risk cuts, allow a greater percentage of the carcass to be marketed in a steak form with a desirable eating experience accompanied with a reduced preparation time (grill vs. roast); all of which leads to increased overall satisfaction via greater flexibility in marketing, more convenience for the consumer, and improved palatability.

However, more research is required to develop a management system that optimizes the use of enzymes in enhancing palatability and adding value to products which otherwise would provide consumers with a marginal or unsatisfactory eating experience. As the enzymes have differences in their mode of action and the extent to which they can change the texture of a product, it is important to match the correct enzyme and its concentration with the appropriate muscle cuts and the time and temperature that optimizes its activity along with the appropriate cooking methods to ensure a positive eating experience by consumers and a successful uptake and implementation of the technology by industry.

The aim of the experiments described in this report was to assess the effects of different commercial and experimental enzyme preparations on beef tenderness and other meat quality traits.

2 Project objectives

To characterise enzyme tenderizers in terms of their temperature and kinetics *in situ* (i.e. in the muscle) in order to identify the best for a range of red meat applications.

To quantify the impact of the tenderizers on meat texture (initially shear force and compression, and ultimately sensory characteristics) and to ensure that their use will not produce detrimental impacts on appearance for fresh red meat retail products.

3 Material and Methods

3.1 Experiment 1

3.1.1 Samples and treatment

Topsides were collected at 1 day after slaughter from both sides of 18 carcasses (36 topsides) at John Dee (Warwick). Vacuum packaged topsides were transported to UNE and sample treatment was performed at 2 days p.m. Topsides were halved and cut into blocks of about 9x9x20 cm. Sample treatment consisted of needle injecting the sample blocks to 10% of the original weight with a series of enzyme tenderizers according to the manufacturers' recommendations (Table 1). Each sample was vacuum packaged, stored at 1°C for 19 days and sampled for shear force and compression analysis, colour stability during retail display, and sensory analysis. Sample position for the different analyses was randomized within each sample block.

Table 1. Products and treatments.

Product/Treatment	Treatment Code	Concentration	Manufacturer
None (control)	A	-	-
Water	B	-	-
Papain 25,000 MG	C	0.01g/L	Enzyme Solutions
Bromelain	D	0.05g/L	Enzyme Solutions
Kiwifruit PE (Actinidin)	E	10g/L	Ingredient Resources
DigestEasy (Zingibain)	F	100 mL/L	Biohawk
Fungal protease 60,000	G	0.01g/L	Enzyme Solutions
Fungal protease 31,000	H	0.02g/L	Enzyme Solutions
Protease G	I	0.08g/L	Enzyme Solutions

3.1.2 Shear force and compression

For grilling, 2.5 cm thick steaks were grilled during 5 min. on a Silex grill set at 220°C. Steaks were stored overnight at 1°C and subsequently sampled for determination of shear force.
For roasting, cooking blocks (about 9x9x12.5 cm) were placed in aluminum baking trays and placed in a fan forced oven set at 160°C. Samples were grilled for 1.5 hours which led to a temperature of 65-70°C in the centre of the meat. Roasted samples were stored overnight at 1°C and subsequently sampled for determination of shear force and compression.
Shear force and compression values were determined as described by Perry et al. (2001).

3.1.3 Colour and colour stability

Steaks (2.5 cm thick) were placed in polystyrene trays and covered with oxygen permeable plastic foil. L*-, a*-, and b*-values were recorded using a Minolta CR-300 Chromameter after 1, 3, and 5 days of storage at 1°C. The light source of the color meter was D65 and the instrument was calibrated on a white tile according to the manufacturer's specifications.

3.1.4 Sensory analysis

Steaks (2.5 cm thick) were grilled on a grill plate (200°C) for about 7 min. to a medium degree of doneness. Thereafter, samples were cut into 4 pieces and served warm to the panellists. Panellists were asked to judge the quality of the meat as described by Watson et al., 2008 with regard to tenderness, juiciness, flavour and overall liking.

3.1.5 Statistical analysis

Significance of differences was determined by analysis of variance with the factors animal, carcass side, cut (within the topsides) and treatment. The analysis for both experiments was carried out using R v 2.7.

3.2 Experiment 2

3.2.1 Samples and treatment

Cold-boned topsides from prime cattle (2-3 years old) were obtained from Alliance Group Ltd (Mataura Plant, South Island, NZ). The topsides from both sides of 18 carcasses (36 topsides in total) were collected at 1 day post-mortem and processed immediately. Topsides were halved and cut into steaks (average weight ± SD was 268.6 ± 90.2 g) that were assigned to one of the treatment (control untreated, injected with water, or injected with one of the test enzymes).
The enzyme preparations that did not show a significant effect in the previous trials (Geesink, 2010; Bekhit, 2010) were used at concentrations up to 10 times the recommended level of use by the supplier whereas those demonstrated some effect on meat tenderization in the previous trials (commercial actinidin and zingibain), up to 5 fold of the recommended concentration were used (Appendix 1). Furthermore, two in-house extracts prepared as described earlier (Bekhit, 2010) were investigated at 5 concentrations as outlined in Appendix 1. All of the samples were subjected to needle injection (to 10% of the original weight), vacuum packed and half the samples were assigned to 1 day post-mortem (1 d PM) and the other half was stored at 4°C for further 20 days (21 d PM). Subsequently, the samples were analyzed for shear force, compression force, analysis of degradation of myofibrillar protein and collagen solubilisation. The samples for protein analysis were frozen in liquid nitrogen, vacuum packed and stored at -30°C until analysis. Sample position for the different analyses was randomized among different animals. Only results for the highest concentration (Conc 5) are reported in this report.

3.2.2 Shear force and compression

Shear force and compression analysis was performed at the University of Otago as described by Bekhit (2010). Briefly, samples were cooked individually in plastic bags immersed in a water bath at 80°C until they reached an internal temperature of 75°C. The cooked meat was chilled on ice and the shear force and compression values were determined according to Chystall and Devine (1991) and Perry et al. (2001), respectively.

3.2.3 Determination of total and soluble collagen

The protocol for collagen content was adapted from ISO (1978). About 25g of muscle tissue was freeze dried and subsequently homogenized into a fine powder.
For determination of total collagen content, freeze dried muscle powder (0.10 g) was hydrolysed with 3.5 M H_2SO_4 (3 mL) overnight for 16 h at 105°C. The hot hydrolysate was diluted to 50 ml using MQ water and filtered using filter paper. One ml of filtrate was diluted with 3.75ml water and neutralized with 0.25ml NaOH (2M). An aliquot of the filtrate (0.5 mL) was mixed with 0.25 mL chloramine-T solution and incubated at room temperature for 20 min. Subsequently, 0.25 ml

of the colour reagent was added, and the samples were incubated at 60°C for 15 min. After cooling under running tap water, the absorbance was measured at 558 nm. The amount of hydroxyproline was then calculated using a standard curve. The amount of collagen was calculated in mg/g of freeze dried muscle by multiplying the amount of hydroxyproline by 7.25. For determination of heat soluble collagen, 1.5 g of freeze dried muscle powder was suspended in 10ml of MQ water and heated for 2 hours in a water bath at 80°C with mixing every half hour. Insoluble material was pelleted and the supernatant was filtered using filter paper. An aliquot of the supernatant (0.5 mL) was hydrolysed with 3.5 M H_2SO_4 (3 mL) overnight for 16h at 105°C. The hot hydrolysate was diluted to 10ml using MQ water. One ml of the diluted hydolysate was neutralized with 1ml NaOH (2M), and the hydroxyproline content was determined as described above.

3.2.4 SDS-PAGE and Western blotting

A portion of the freeze dried muscle powder (approximately 0.2 g) was extracted in 5 mL of ice-cold extraction buffer (50 mM Tris/HCl, 10 mM EDTA, pH 8.3). Tissue was homogenized for 15 s using a polytron on high speed. Insoluble material was pelleted using a centrifuge at 2,000 x g_{max} and the supernatant was discarded. Insoluble material was washed twice by suspension in 5 mL of ice-cold buffer (50 mM Tris/HCl, 10 mM EDTA, pH 7.5) and centrifugation. The final suspension was mixed with an equal volume of 2x SDS-PAGE sample buffer (0.125 M Tris/HCl, 4% SDS, 20% glycerol, pH 6.8) and heated at 50°C for 20 min. After centrifuging the solution at 16,000 x g_{max} for 5 min at room temperature, the supernatant was collected and the protein concentration was determined using a Pierce BCA protein assay kit (Pierce Laboratories, Rockford, IL). Samples were diluted to 2.5 mg/mL of total protein using SDS-PAGE sample buffer containing 0.5% (v/v) 2-mercapthoethanol and bromophenol blue (0.04% v/v). SDS-PAGE was performed as described by Laemmli (1979) using 8 x 10 x 0.075-cm 12% or 7.5 acrylamide minigels using a 37.5:1 acrylamide to bisacrylamide stock solution. After electrophoresis, at 200 V, gels were either stained using Coomassie Brilliant Blue, or proteins were transferred onto Hybond-P polyvinylidine fluoride membranes (Amersham Biosciences, Uppsala, Sweden) at 200 mA for 1 h using a wet transfer apparatus (BioRad Laboratories, Hercules, CA). All the following steps were performed at room temperature. Membranes were blocked with 3% (wt/vol) nonfat dry milk in Tris-buffered saline containing Tween (TTBS: 20 mM Tris/HCl, 137 mM NaCl, 5 mM KCl, 0.05% Tween, pH 7.5). After blocking for 1 h, the membranes were exposed to the following primary antibodies diluted in 3% nonfat dry milk in TTBS: mouse monoclonal antibody anti-desmin (DE-U-10; dilution 1:2,500; Sigma-Aldrich, St. Louis, MO) and mouse monoclonal antibody anti-troponin T (JTL-12; dilution 1:5,000; Sigma-Aldrich, St. Louis, MO). Blots were incubated for 1 h before being washed with TTBS. The secondary antibody used was alkaline phosphatase conjugated antibody against total mouse IgG (A3562; dilution 1:10,000; Sigma-Aldrich, St. Louis, MO). Blots were exposed to the secondary antibody for 1 h before being extensively washed with TTBS. Antibody binding was visualised using an alkaline phosphatase conjugate substrate kit (BioRad Laboratories, Hercules, CA).

3.3 Experiment 3

3.3.1 Samples and treatment

Forty shortloin sections (about 25 cm long) were randomly selected from a batch of 88 samples which originated from trial 2 of the MSA “long distance trial”. The animals used in this trial were killed on 18/6/2012 at Dinmore.
A 7 days post mortem the shortloin sections were randomly assigned to 1 of 4 treatments:

- Control (no treatment)

- Injection (10% w/v) with Kiwifruit PE (Actinidin) at 10 g/L
- Injection (10% w/v) with Kiwifruit PE (Actinidin) at 25 g/L
- Injection (10% w/v) with Kiwifruit PE (Actinidin) at 50 g/L

Injection was performed manually with a single needle on both sides of the muscle at intervals of about 1 cm. The initial weight of the samples and the injection volumes were recorded. After injection, samples plus excess fluid were vacuum packed and stored at about 2°C for 6 days.

After the storage period, samples were removed from their vacuum bags and weighed to determine yield. Subsequently, the samples were subdivided for analyses according to the following sequence from cranial to caudal:

- Slice 1 (about 1 cm): Waste
- Slice 2 (2.5 cm): Colour stability
- Slice 3 (2.5 cm): Grill samples (sensory analysis)
- Slice 4 (2.5 cm): Grill samples (shear force, collagen, SDS-PAGE)
- Slice 5 (13 cm): Roast sample (sensory analysis, shear force, collagen, SDS-PAGE)
- Slice 6 (2.5 cm): Collagen and SDS-PAGE
- Slice 7 (1 cm): Waste

3.3.2 Colour and colour stability

Steaks (2.5 cm thick) were placed in polystyrene trays and covered with oxygen permeable plastic foil. L*-, a*-, and b*-values were recorded using a Minolta CR-300 Chromameter over a 1 week storage period at 1°C. The light source of the color meter was D65 and the instrument was calibrated on a white tile according to the manufacturer's specifications.

3.3.3 Sensory analysis

Grill samples

Steaks were divided in two part and grilled on a Silex at 220°C for 4.5 min. Each grill session contained 1 sample (2 pieces) of each treatment. The sensory panel consisted of eight groups of four people. The groups were asked to cut the steaks into four bite size portions and evaluate the quality of the meat as described by Watson et al. (2008) with regard to tenderness, juiciness, flavour and overall liking.

Roast samples

Samples were roasted in aluminium trays at 160°X to a core temperature of 65°C, after which they were kept at 50°C until serving. Roast were carved into three 1.5 cm thick slices and one 3 cm thick slice. The 1^{st} slice was discarded. The 2^{nd} and 3^{rd} slice were used for sensory analysis and the 4^{th} slice was bagged and stored in the cold room until sampling for shear force, collagen analysis and SDS-PAGE the following day. Sensory analysis was conducted in a similar manner as described for the grilled samples.

3.3.4 Shear force

Shear force of the grilled and roasted samples was determined according to Perry et al. (2001).

3.3.5 Determination of total and heat-soluble collagen

Total and heat-soluble collagen were determined as described under 3.2.3 with the following modification. To assess the possibility that the apparent collagen solubility is an artefact of

residual enzyme activity during the solubilisation step in the determination (2 hours at 80°C), this step was performed without and with the addition of a proteinase inhibitor cocktail (Complete, Roche Diagnostics GmbH).

3.3.6 SDS-PAGE and Western blotting

SDS-PAGE and Western blotting against desmin were performed as described under 3.2.4

3.3.7 Statistical analysis

Statistical analysis was performed using a general linear model in Minitab v. 14.

4 Results and discussion

4.1 Experiment 1

4.1.1 Shear force and compression

The injection treatment *per se* did not result in a tenderizing effect given the similar results for shear force and compression in non-treated (A) and water injected (B) samples (Table 1).
Treatment with papain (C) resulted in a significant decrease in shear force in the roasted samples, but not the grilled samples. Given that the temperature for maximal activity of this enzyme is relatively high, it suggests that most of the tenderizing action occurred during the roasting process, but not, or only to a very limited extent, during the aging period or during grilling.
Treatment with bromelain (D) did not result in a tenderizing effect. Given the well-known tenderizing effects of this enzyme, it is likely that the concentration used was too low for effective tenderization. An alternative explanation is that the enzyme was inactivated during the aging period, and therefore, was not active during the temperature conditions for maximal activity (cooking phase).
Treatment with actinidin (E) resulted in a significant decrease in shear force after roasting and a trend towards a decrease in shear force after grilling. Analysis of muscle protein degradation patterns is needed to determine whether the tenderizing effect occurred during the aging period and/or cooking.
Zingibain (F) treatment resulted in a tenderizing effect as assessed by shear force and compression measurement. Given that compression values reflect the strength of the connective tissue after cooking, these results suggest that the tenderizing effect of Zingibain is at least partly due to breakdown of collagen.
Treatment with the fungal proteases (G and H), or the bacterial protease (I) did not result in a significant tenderizing effect. A likely explanation for this is that the concentrations used were too low to exert a significant tenderizing effect.

Table 2. Effect of treatment of beef topsides with enzyme tenderizers on the shear force and compression values after grilling and roasting.

	Treatment								
Item	A	B	C	D	E	F	G	H	I
SF Grill (kg)	5.0^{a}	4.8^{a}	4.9^{a}	4.7^{a}	4.3^{ab}	3.8^{b}	4.8^{a}	4.4^{ab}	4.5^{ab}
SF Roast (kg)	6.2^{a}	6.2^{a}	4.9^{b}	6.2^{a}	4.9^{b}	5.2^{ab}	5.6^{ab}	6.0^{a}	5.6^{ab}
Comp. Roast (kg)	1.9^{ab}	2.1^{a}	1.8^{ab}	2.1^{a}	1.9^{ab}	1.6^{b}	2.1^{a}	1.9^{ab}	1.9^{ab}

^{ab}means, within rows, not containing a common superscript, differ significantly ($p < 0.05$). A = control, B = water injected, C = papain, D = bromelain, E = actinidin, F = Zingibain, G = fungal protease 60,000, H = fungal protease 31,000, I = protease G.

4.1.2. Colour and colour stability

The results of the colour measurements are graphically depicted in figure 1. Injection with the different enzyme solutions did not result in adverse effects on colour or colour stability. At the end of the storage period, steaks were visually inspected for browning. None of the steaks showed signs of browning at this stage.

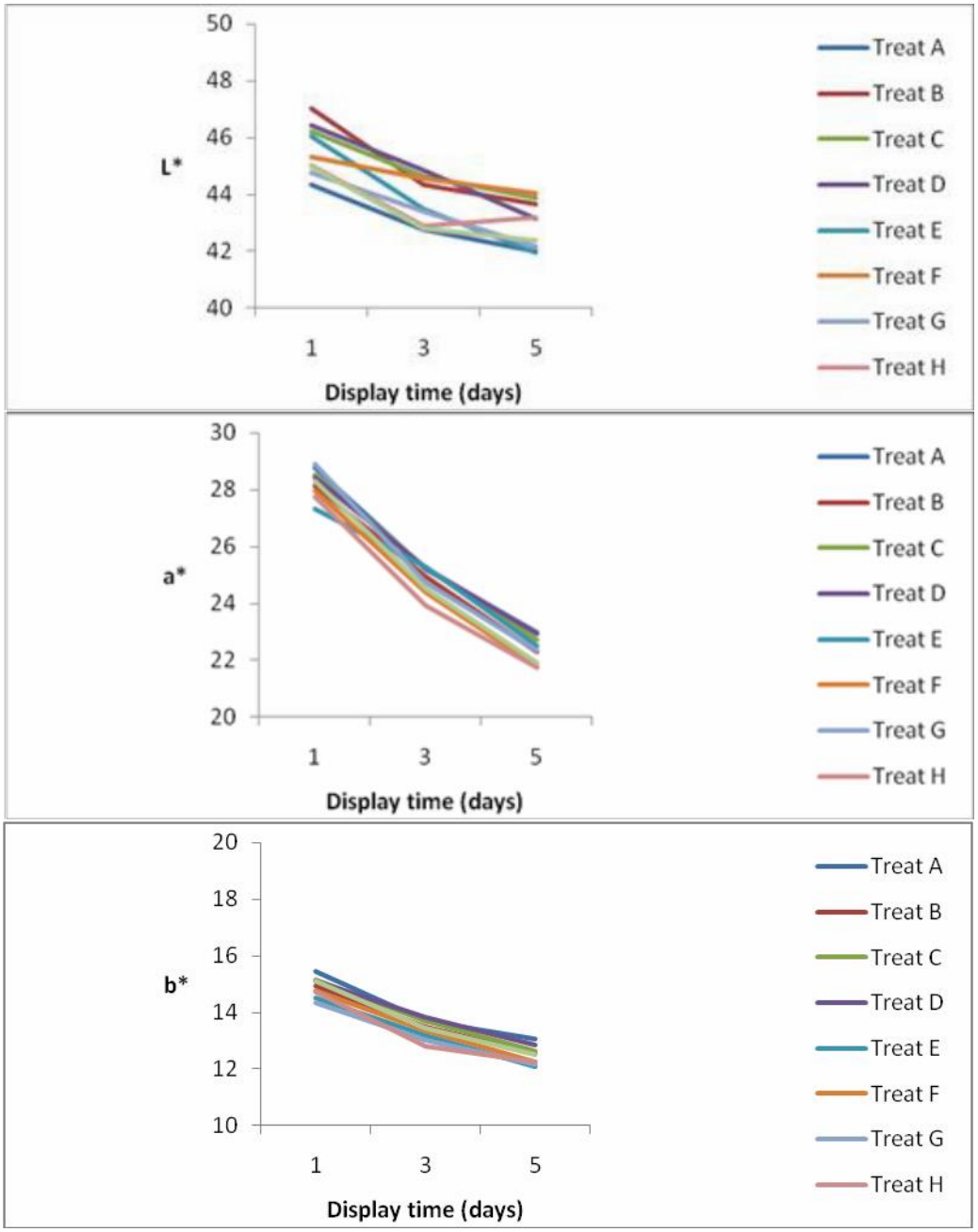

Figure 1. Colour and colour stability (L*-, a* and b*-values) during display storage as affected by the enzyme treatments. A = control, B = papain, C = bromelain, D = actinidin, E = Zingibain, F = fungal protease 60,000, G = fungal protease 31,000, H = protease G.

4.1.3 Sensory analysis

Enzyme treatment did not result in a significant improvement in MQ4 score for grilled samples (Figure 2), but a significant decrease in palatability when bromelain or the bacterial protease where used.

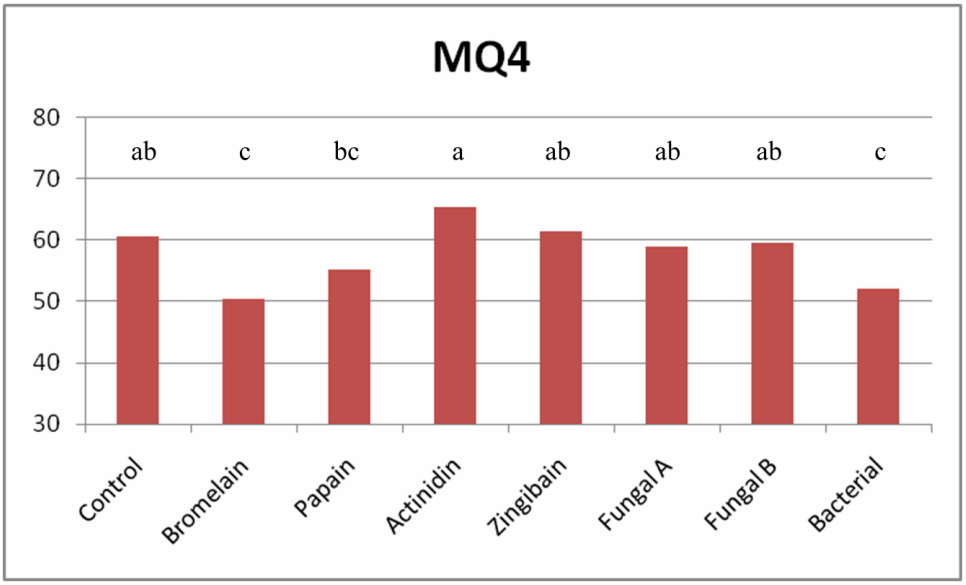

Figure 2. Palatability score (MQ4) of grilled topside samples after treatment with different enzyme preparations. ^{a,b,c}Means, not containing a common letter in the superscript, differ significantly ($p<0.05$).

General discussion

The results of the first experiment within this project showed that some of the enzyme tenderizers show potential to improve the tenderness of fresh meat. However, it is also evident that the manufacturer's recommendations for use of the enzymes did not always result in an improvement in objective tenderness measures, or even in a decrease in palatability score.

For reasons of efficiency, a tenderizing intervention that can be implemented after boning and before vacuum storage is preferable. The results of the present experiment show that this is a possibility with at least some of the enzymes tested. In addition, it was shown that this treatment has no adverse effects on color or color stability. Since the selection of fresh meat at retail level is largely based on appearance, this is an important observation.

Before further evaluation of the sensory characteristics, a number of issues need to be addressed. Analysis of myofibrillar and connective tissue proteins should clarify which structural proteins are the primary targets of the different enzymes, and therefore, which enzymes are most suitable for treatment of muscles with specific tenderness problems (myofibrillar, connective tissue, or a combination thereof). This analysis will also yield information about the stage at which the tenderizing effect occurs (aging, cooking or both). This information will aid in developing protocols for effective use of tenderizing enzymes under different scenarios regarding aging time and cooking options. These issues will be addressed in the following experiments.

4.2 Experiment 2

4.2.1 Shear force and compression

Shear force and compression were both determined because the first is assumed to mainly reflect the contribution of myofibrillar proteins to toughness whereas the latter should be a better measure for the contribution of connective tissue to toughness.
For ease of interpretation, the shear force and compression values are expressed as a percentage of mean of the control and water injected samples (C/W) 1 day after injection (Figures 3 and 4).

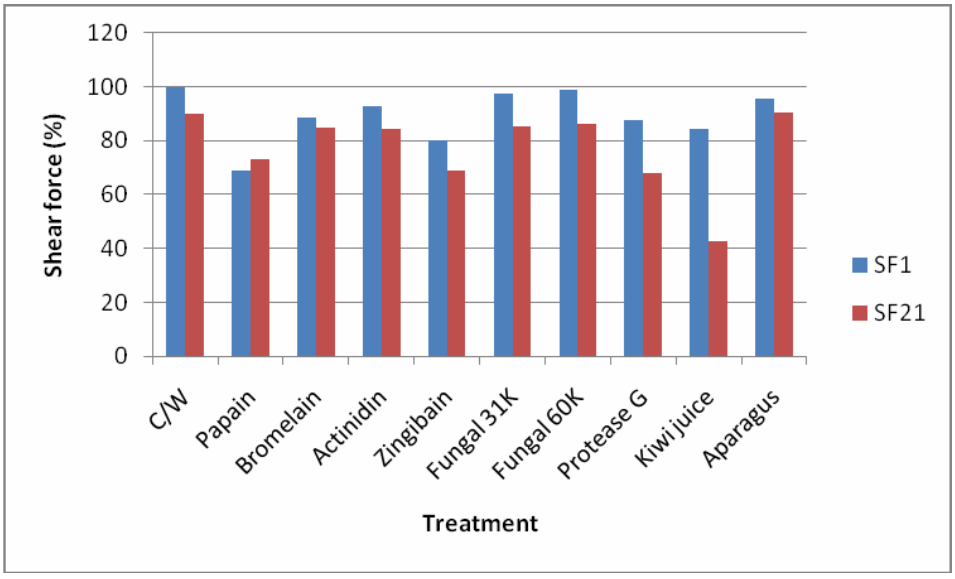

Figure 3. Shear force on 1 and 21 days after injection with the different enzyme preparations (shear force is expressed a percentage of the shear force of the control/water-injected samples on day 1)

From the presented results it is clear that there was little improvement in tenderness between d1 (3 days p.m.) and day 21 (23 days p.m.) in the samples that were not treated with any enzymes.
Regarding the enzymes there are a number of enzyme preparations that did not have appreciable effect on the shear force. These include bromelain, actinidin, the fungal proteases, and the asparagus extract.
The impact of the papain, zingibain and protease G treatments appear similar regardless whether the meat is cooked 1 day or 21 days after the treatment. This suggests that the enzymes only exert their effect during the cooking process, and that they don't lose their activity during chilled storage of the meat. In contrast, aging of the meat after injection with kiwi juice extract resulted in an appreciable drop in shear force. This suggests that the proteases contained in this extract are active at refrigeration temperatures

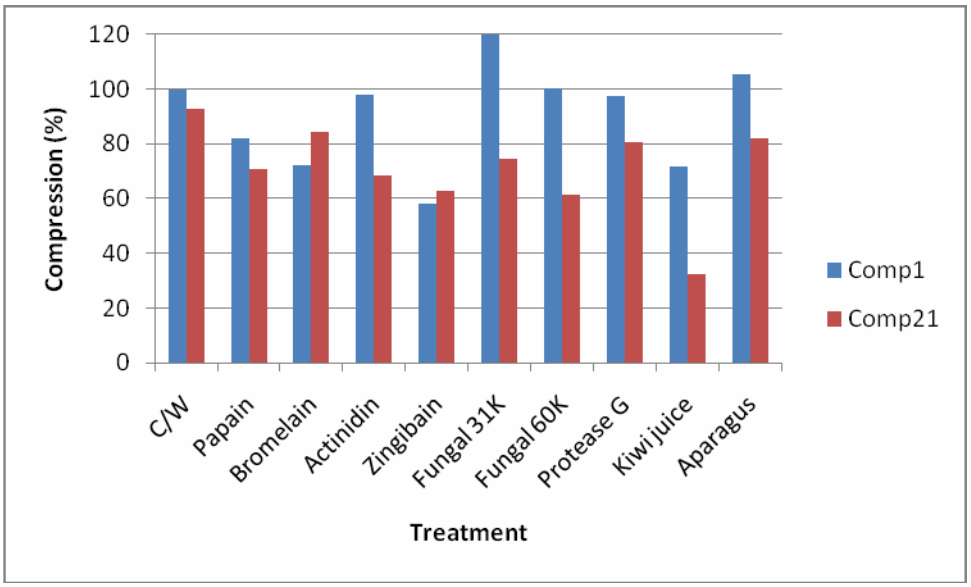

Figure 4. Compression values on 1 and 21 days after injection with the different enzyme preparations (Compression values are expressed a percentage of the compression value of the control/water-injected samples on day 1)

Results from the compression analysis can be interpreted in a similar fashion. The impact of papain and zingibain appears independent of the aging period, suggesting that these enzymes are stable during aging, but mainly exert their effect during cooking. Bromelain appears most effective when the meat is cooked at day 1. This suggests that the enzyme loses its activity during aging, and is only active during cooking. The effects of actinidin, the fungal proteases and the kiwi juice extract appear most pronounced after aging for 21 days. This suggests that these enzyme extracts exert some of their effects during aging. Finally, protease G and the asparagus extract do not appear to have a major effect.

4.2.2 Total and soluble collagen

Total collagen amounts were quite variable, which is probably a reflection of an inhomogeneous distribution of connective tissue over the muscle (Table 1). However, the total amount of collagen does not appear to be affected by the treatments other than that aging and cooking resulted in an apparent increase in connective tissue content. The latter can be explained by a loss of some protein in drip and cooking loss, and thus, a relative increase in connective tissue as a proportion of dry matter.

Table 1. Total collagen (mg/g freeze dried tissue; mean + standard error).

	Day 1 raw		Day 21 raw		Day 1 cooked		Day 21 cooked	
Treat	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	35.8	3.7	45.3	2.5	30.8	0.8	28.0	1.2
Water	**33.9**	3.0	27.3	3.9	36.5	2.3	45.2	4.5
Papain	37.6	2.9	36.2	2.9	46.9	10.2	40.0	2.3
Bromelain	32.0	7.0	36.9	3.7	36.0	3.4	46.6	7.5
Actinidin	30.3	5.3	40.2	1.5	28.9	5.5	41.2	2.3
Zingibain	29.2	4.5	42.0	7.8	38.9	4.9	53.0	15.9
Fungal 31K	30.0	2.0	39.5	3.7	27.6	1.7	46.0	1.8

Fungal 60K	42.1	12.5	36.1	6.3	50.0	13.9	40.4	0.4
Protease G	35.0	4.1	34.6	0.7	32.8	3.1	38.5	0.2
Kiwi juice	31.4	1.0	36.5	3.0	37.1	9.5	41.2	2.1
Asparagus	32.0	9.2	25.7	7.7	37.3	8.9	47.7	3.0
Mean	33.6		36.4		36.6		42.5	
Min	29.2		25.7		27.6		28.0	
Max	42.1		45.3		50.0		53.0	

Results for the determination of soluble collagen in the raw and cooked samples are given in Figure 5. From these results it appears that a number of the enzyme preparations did affect collagen solubility, but mostly in the raw meat. The latter is probably an artifact of the method to determine soluble collagen. One step in this assay is heating a suspension of the freeze dried muscle powder at 80°C for a period of 2 hours. Most likely, most of the observed degradation/solubilisation of connective tissue occurred during this step, and not during chilled storage. However, comparing the results for the raw muscle samples after 1 and 21 days of storage, the following tentative conclusions can be drawn. Papain, zingibain and the asparagus extract retain their activity during three weeks of chilled storage, whereas actinidin and the kiwi juice extract lose some of their activity during chilled storage.

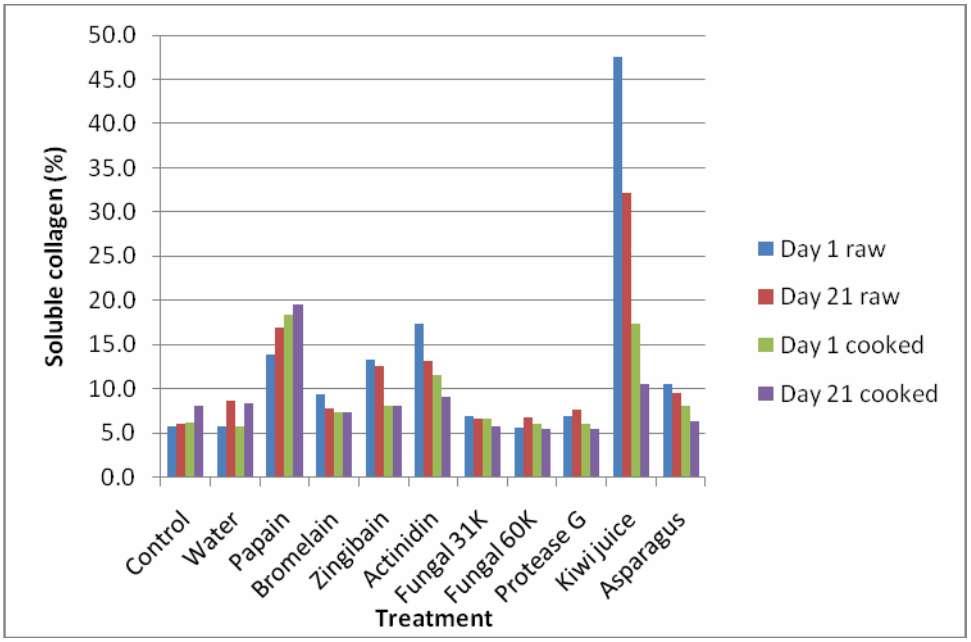

Figure 5. Soluble collagen (%) in raw and cooked samples at 1 and 21 days after injection with different enzyme preparations.

Although it cannot be excluded that the results for the cooked samples are also partially an artifact of the methodology, it is expected that these results to a certain extend reflect the combined activity during chilled storage and the cooking process. With the exception of papain, most of the enzymes may be expected to have lost most of their activity during the cooking process (Bekhit, 2010).

The results of the analysis for soluble collagen in cooked samples alone are given in Figure 6.

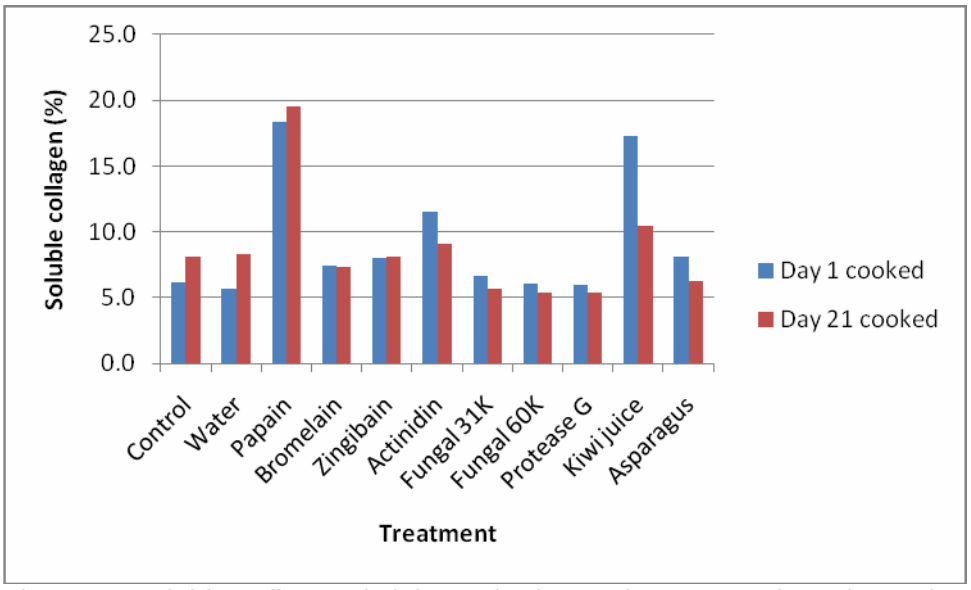

Figure 6. Soluble collagen (%) in cooked samples at 1 and 21 days after injection with different enzyme preparations.

These results show only three of the enzyme preparations; papain, actinidin and the kiwi juice had an appreciable effect on soluble collagen in the cooked meat this experiment.

4.2.3 Degradation of myofibrillar proteins

As shown by Bekhit (2010) all enzyme preparations are able to degrade myofibrillar proteins quite extensively when incubated at their optimal pH and temperature conditions. In the present study we attempted to determine to what extent this occurs during chilled storage and cooking of the samples. Initially, myofibrillar proteins of all raw and cooked samples were evaluated individually (data not shown). The results showed that neither during aging, nor during cooking, clearly noticeable degradation of myofibrillar proteins occurred.

To obtain better separation of relatively large proteins, such as myosin heavy chain, pooled samples were run on 7.5% acrylamide gels (Figures 7 and 8). The separation patterns revealed only subtle changes indicative of protein breakdown. The upper arrow and lower arrows indicate some putative breakdown product, whereas the middle arrow points to a protein band that disappears as a result of some of the enzyme treatments.

The only treatments for which a marked increase in protein degradation appears to occur during the aging period are treatment 5 (actinidin) and treatment 9 (kiwi juice). This observation corroborates the observed effects on measures of tenderness suggesting that actinidin and the kiwi juice extract exert some of their effect during the aging period.

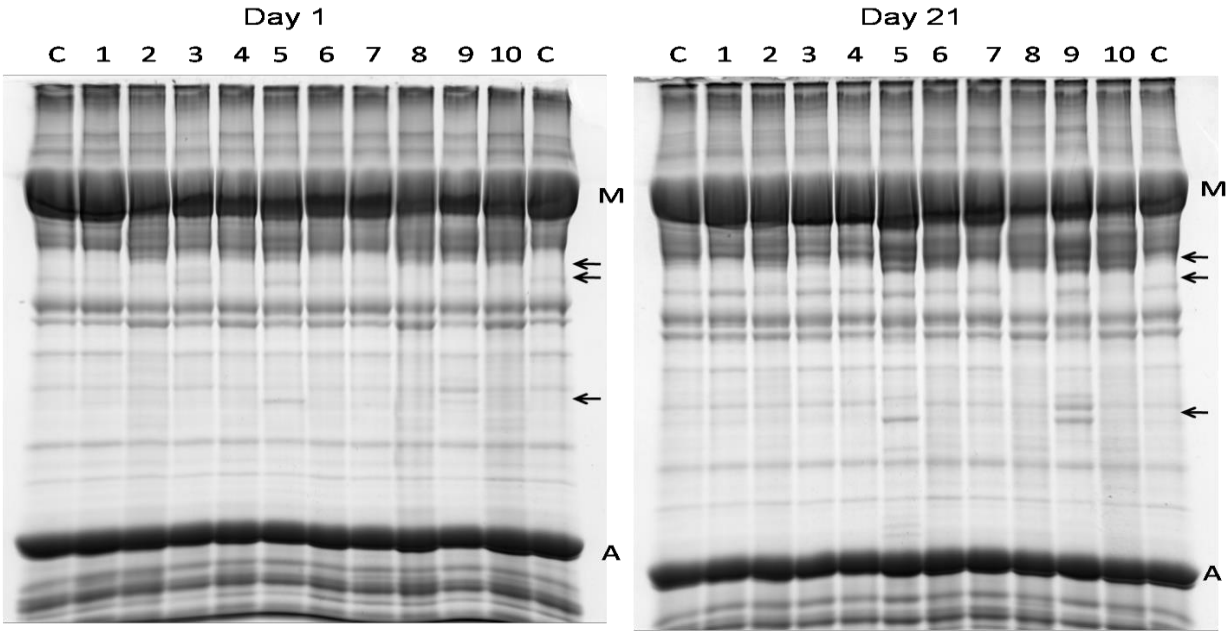

Figure 7. Separation pattern of myofibrillar proteins of raw muscle at 1 and 21 days after injection with different enzyme preparations. (C = control, 1 = water, 2 = papain, 3 = bromelain, 4 = zingibain, 5 = actinidin, 6 = fungal 31K, 7 = fungal 60K, 8 = protease G, 9 = kiwi juice, 10 = asparagus)

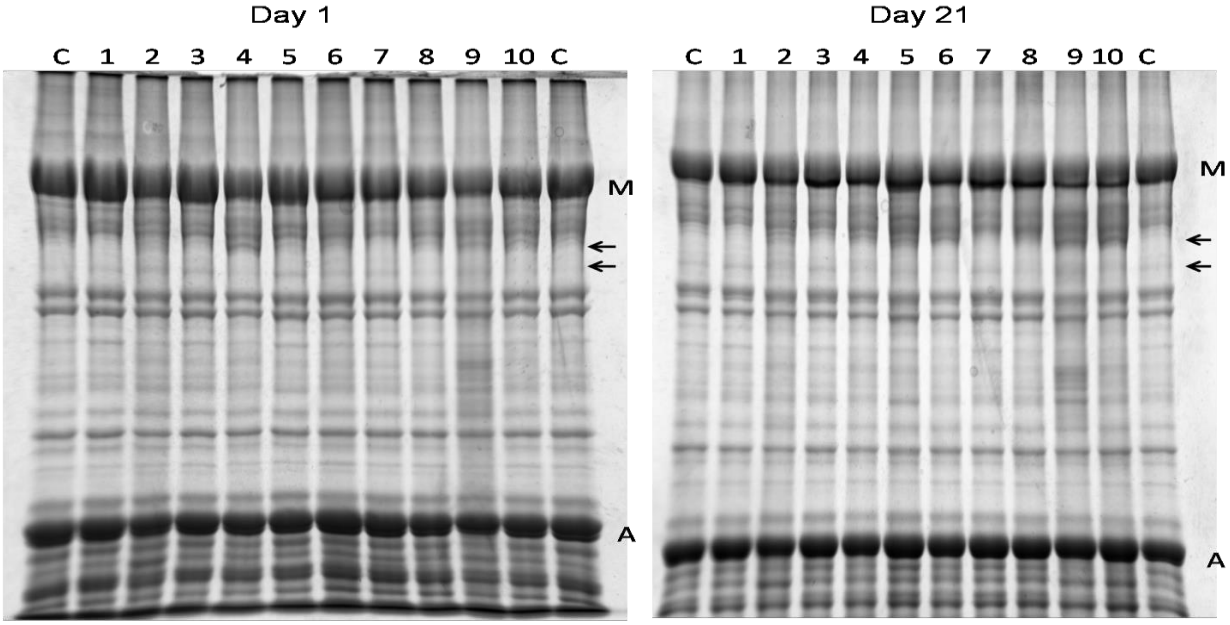

Figure 8. Separation pattern of water-insoluble proteins of cooked muscle at 1 and 21 days after injection with different enzyme preparations. (C = control, 1 = water, 2 = papain, 3 = bromelain, 4 = zingibain, 5 = actinidin, 6 = fungal 31K, 7 = fungal 60K, 8 = protease G, 9 = kiwi juice, 10 = asparagus)

The separation patterns of the cooked muscle samples are somewhat more difficult to interpret. As a result of cooking, many of the soluble muscle and enzyme extract proteins denature, and thus end up in the water-insoluble protein fraction. Nevertheless, the separation patterns do not indicate that any of the enzyme treatments had a major effect on breakdown of myofibrillar proteins during the cooking process (Figure 8).

The observation that rather subtle protein degradation effects result in noticeable changes in measures of tenderness is similar to what is observed during aging of meat. Aging of meat results in little or no observable breakdown of connective tissue and as minor changes in myofibrillar proteins (noticeable using SDS-PAGE). Nevertheless, it has been shown that the degradation of a limited number of structural muscle proteins by µ-calpain results in a marked increase in tenderness. One of these proteins is desmin and another protein whose degradation is indicative of µ-calpain activity and tenderization is troponin-T. To determine to what extent the enzyme treatments affected the degradation of these proteins, Western blots against these proteins were performed (Figures 9 and 10).

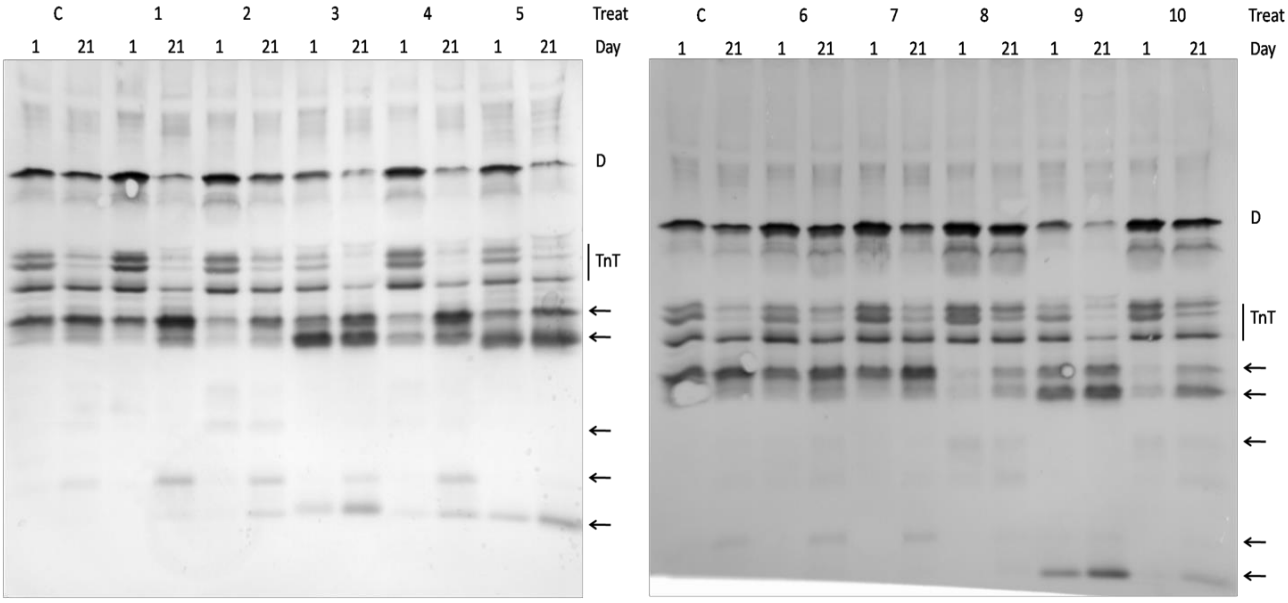

Figure 9. Western blots against desmin (D) and isoforms of troponin-T (TnT) in raw muscle at 1 and 21 days after injection with different enzyme preparations. Arrows indicate degradation products of desmin or troponin-T. (C = control, 1 = water, 2 = papain, 3 = bromelain, 4 = zingibain, 5 = actinidin, 6 = fungal 31K, 7 = fungal 60K, 8 = protease G, 9 = kiwi juice, 10 = asparagus).

Evaluation of the extent of degradation in the raw muscle appears easiest using the intensity of the desmin band (D), the intensity of one of the troponin-T degradation products (second arrow from the top), and a low molecular weight degradation product (bottom arrow). Using these indicators it appears that treatment with bromelain (3), actinidin (5), and kiwi juice extract (9) exert an additive effect on degradation of desmin and troponin-T to the action of µ-calpain during aging. Conversely, treatment with protease G (8) appears to inhibit the action of µ-calpain during aging.

Cooking of the muscles did not result in a noticable increase in degradation of desmin and troponin-T for any of the treatments (Figure 10).

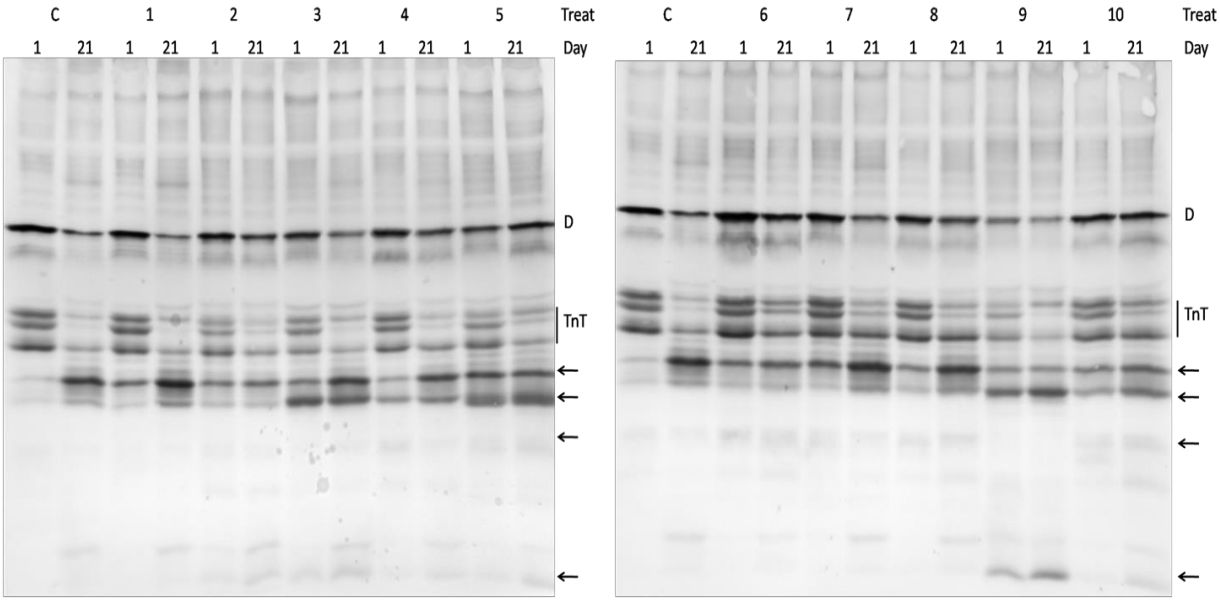

Figure 10. Western blots against desmin (D) and isoforms of troponin-T (TnT) in cooked muscle at 1 and 21 days after injection with different enzyme preparations. Arrows indicate degradation products of desmin or troponin-T. (C = control, 1 = water, 2 = papain, 3 = bromelain, 4 = zingibain, 5 = actinidin, 6 = fungal 31K, 7 = fungal 60K, 8 = protease G, 9 = kiwi juice, 10 = asparagus).

4.2.4 eneral discussion

An ideal enzyme tenderizer for fresh meat can be described as having the following characteristics:

1. Application should fit with normal processing operations. Therefore, treatment between boning and vacuum packaging would be most suitable.
2. The tenderizer should be effective in degrading both myofibrillar and connective tissue proteins.
3. Ideally, some level of activity should be expressed during chilled storage (aging), thereby making the tenderizing action independent of cooking conditions.
4. The tenderizing action should be self-limiting to prevent over-tenderizing, i.e. the enzyme used should lose its activity after expression of a certain amount of activity. Examples of this type of enzymes are calpain (naturally present in muscle) and actinidin (from kiwi).
5. The tenderizers should not have a negative effect on other meat quality traits such as colour and flavour.

For the interpretation of the present results it should be realised that the treated samples were relatively small (steaks) and that the concentrations used were 5 to 10 times higher than the manufacturers' recommendation. Based on the results of the sensory analysis in experiment 1, negative effects on the flavour can be expected when using the bromelain, bacterial protease and zingibain preparations. Based on published studies and the results of the experiments performed within this project, it appears that the kiwi juice extract produced by the University of Otago is the most suitable candidate for further study. However, this does not present a commercially available product. Based on these evaluations it was decided to perform a final experiment with relatively large cuts of meat injected with different concentrations of the actinidin preparation.

4.3 Experiment 3

4.3.1 Yield

During the injection of the samples (1.64 ± 0.14 kg), roughly half of the liquid exuded from the muscles directly after injection and was poured into the vacuum bags with the muscles before sealing the bags. After the 6 days aging period, the injected samples weighed 100.3% of the original weight versus 98.4% for the controls. Thus, only a small amount of the volume injected remained in the muscles.

4.3.2 Colour and colour stability

Similar to the results of experiment 1, no effects on colour or colour stability were observed (data not shown), indicating that the treatments were compatible with the aim of maintaining the appearance of fresh meat.

4.3.3 Shear force

Although numerically lower, injection with the actinidin preparation did not result in a significantly lower shear force of either grilled (p = 0.12), or roasted samples (p = 0.20) (Figure 11).

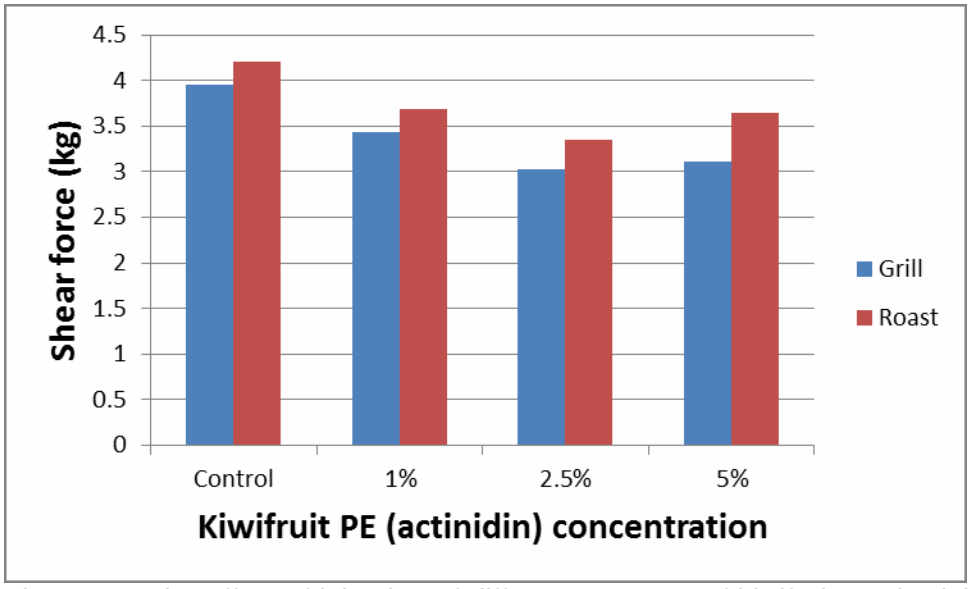

Figure 11. The effect of injection of different amounts of kiwifruit PE (actinidin) on the shear force of grilled and roasted striploins.

4.3.4 Sensory analysis

Although numerically higher, injection with the actinidin preparation did not result in a significant improvement in MQ4 score of either grilled (p = 0.26), or roasted samples (p = 0.14) (Figure 12).

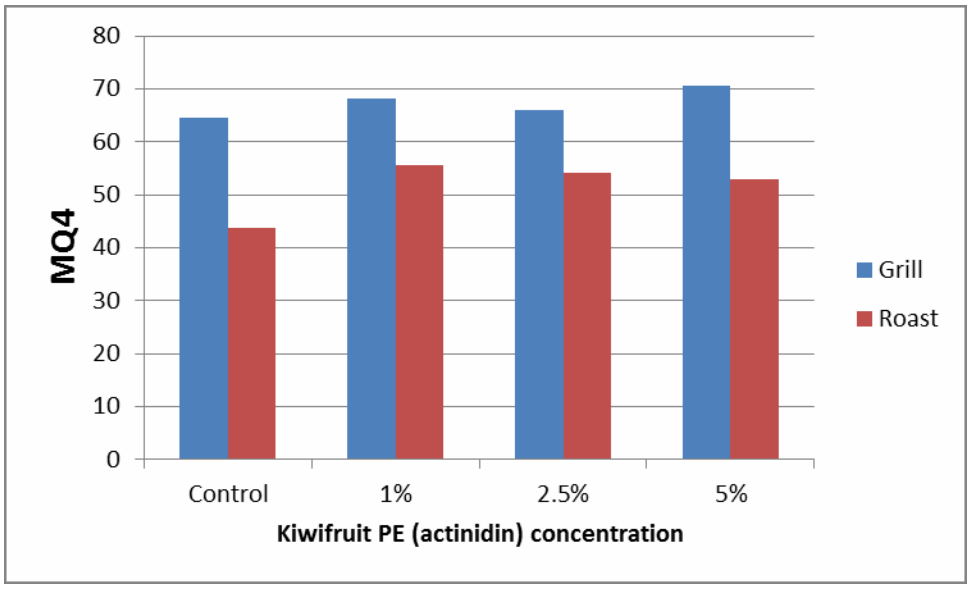

Figure 12. The effect of injection of different amounts of kiwifruit PE (actinidin) on the MQ4 score of grilled and roasted striploins.

4.3.5 Collagen

To assess whether the amount of soluble collagen are partly an artefact of the determination method (see section 4.2.2), soluble collagen extract were prepared in the presence and absence of a proteinase inhibitor cocktail. From the results presented in Figure 13, it appears that part of the apparent solubilisation of collagen is indeed an artefact of the methodology, with the amount of soluble collagen extracted in the absence of proteinase inhibitors generally being higher than those extracted in the presence of the proteinase inhibitor cocktail.

Considering the results generated when inhibiting proteinase activity during extraction, it appears that little degradation of collagen as a result of actinidin treatment occurs during chilled storage of the muscles. In the grilled samples, a dose dependent increase in soluble collagen is observed. However, for the roasted samples an almost inverse dose-response effect was observed. The reason for this is unclear. The most straightforward explanation is that somewhere in the sampling process a mix-up has occurred in the transfer of samples between bags, tubes, etc., and that consequently “Roast 1%” represents “Roast 5%” and vice versa. If this is indeed the case, the results would suggest that there is a dose and heat treatment dependent effect of the actinidin treatment on collagen degradation. In addition, the comparison of the values obtained with and without using the proteinase inhibitor cocktail would suggest that little or no actinidin activity remains after roasting the samples.

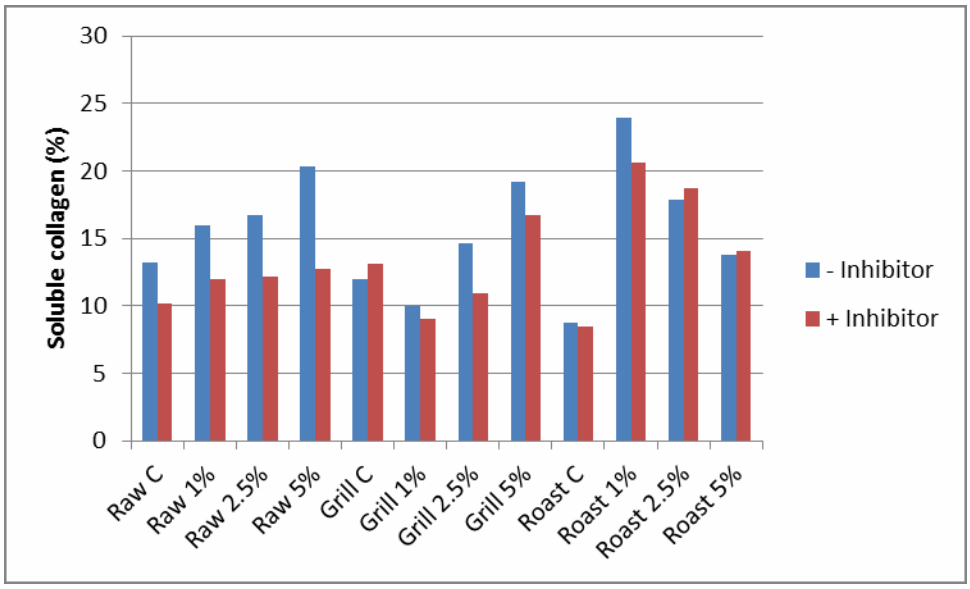

Figure 13. Soluble collagen (%), extracted in the presence or absence of proteinase inhibitors, in raw, grilled and roasted samples injected with different concentrations of actinidin.

4.3.6 Degradation of myofibrillar proteins

To evaluate general degradation of myofibrillar proteins, samples were run on 7.5 and 12% polyacrylamide gels (Figure 14). Similar to the results of experiment 2 (section 4.2.3), only minor indications of protein degradation could be detected (indicated by the arrow). When evaluating degradation of the myofibrillar proteins troponin-T (Figure 15) and desmin (Figure 16) a similar conclusion can be drawn. For troponin-T it appears that treatment with actinidin did result in a dose responsive increase in some degradation products (indicated by the arrows). However, this increase was relatively minor to the degradation that occurred in the control samples. With regard to desmin, no clear indication of an increased degradation due to actinidin treatment was apparent.

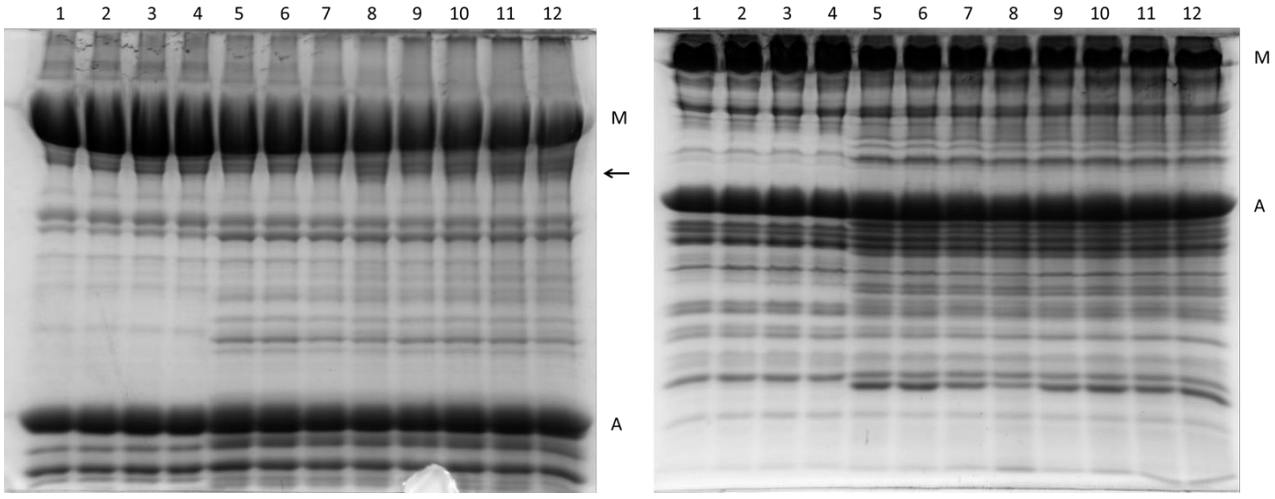

Figure 14. Separation pattern of water insoluble proteins of raw (lanes 1-4), grilled (lanes 5-8) and roasted (lanes 9-12) samples injected with different concentrations of actinidin. (lanes 1, 5 and 9: control, lanes 2, 6, 10: 1% actinidin, lanes 3, 7, 11: 2.5% actinidin, lanes 4, 8, 12: 5% actinidin).

Left panel: 7.5% acrylamide gel. Right panel: 12% acrylamide gel).

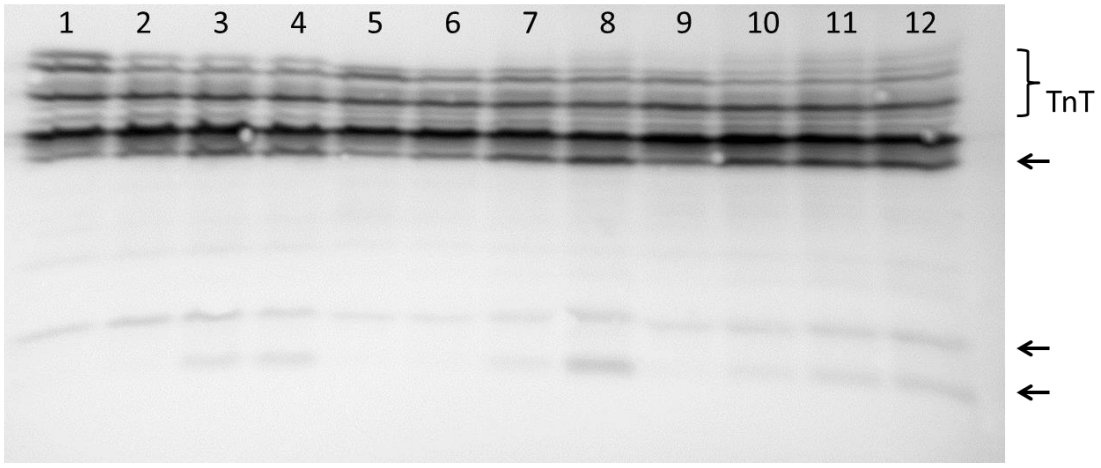

Figure 15. Western blot against troponin-T (TnT) in raw (lanes 1-4), grilled (lanes 5-8) and roasted (lanes 9-12) samples injected with different concentrations of actinidin. (lanes 1, 5 and 9: control, lanes 2, 6, 10: 1% actinidin, lanes 3, 7, 11: 2.5% actinidin, lanes 4, 8, 12: 5% actinidin).

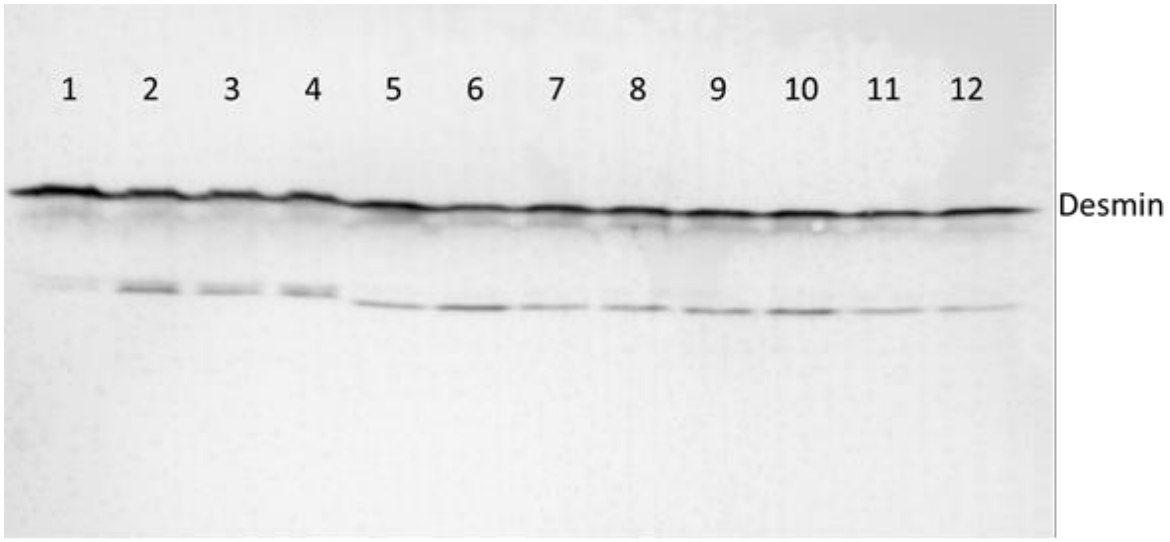

Figure 16. Western blot against desmin in raw (lanes 1-4), grilled (lanes 5-8) and roasted (lanes 9-12) samples injected with different concentrations of actinidin. (lanes 1, 5 and 9: control, lanes 2, 6, 10: 1% actinidin, lanes 3, 7, 11: 2.5% actinidin, lanes 4, 8, 12: 5% actinidin).

4.3.7 General discussion

The results of the present experiment do not present a clear picture regarding the effect of actinidin injection on meat tenderness and degradation of collagen and myofibrillar proteins. Results from the sensory evaluation and shear force measurements suggest a tenderizing effect, but a dose response effect is not apparent. Results regarding degradation of collagen and myofibrillar proteins suggest that actinidin treatment mainly affected collagen and had little effect on myofibrillar proteins. Given the *in vitro* results presented by Bekhit (2010) the actinidin extract

used in the present experiment has the potential to degrade a large number of myofibrillar proteins as well as collagen. The main reason for the limited effects observed in the present experiment may be that injection was conducted without agents that promote the uptake of fluids. Measurement of the sample weights before injection and after injection and aging indicated that only a minor amount of the injected solution remained in the samples.

At present there are a number of published studies regarding the use of kiwifruit extracts to tenderize meat. The results of these studies vary considerably and this is likely due to the different application methods. Han et al. (2009) reported effective tenderization and degradation of myofibrillar proteins after vascular infusion of lamb carcasses directly after slaughter. The presence of actinidin in the muscles was confirmed by assaying muscle extracts for proteolytic activity. Christensen et al. (2009) observed a dose dependent tenderizing effect of porcine *M. biceps femoris* as a result injection with actinidin. In contrast with the present experiment, the actinidin extracts were dissolved in brine instead of water. This treatment evidently enhanced the retention of the solution in the muscles. The initial weight gain after injection was 16% and more than a third of this was retained in the muscle after 2 days of refrigerated storage. The suggestion that a good retention and distribution of the marinade is essential to obtain the desired effect was illustrated by another study from this group (Jorgensen et al., 2008).

In this experiment, injection with kiwifruit extract or ultrasonication of porcine *M. biceps femoris* did not result in increased tenderness. However, a combination of both treatments did. It was suggested that the ultrasound treatment aided the migration of the injected moisture into the tissue thus enhancing the effectiveness of the actinidin treatment.

Thus, based on the result of the present experiment and the work of others discussed above, it appears that actinidin can be used effectively as a meat tenderizer. However, injection *per* se is not a reliable option unless the injected solution includes ingredients that promote the uptake of the injected solution muscle tissue.

5 Conclusions and recommendations

Based on the results of the experiments within this project, and work published by others, it appears that actinidin can be used successfully as a meat tenderizer. However, the challenge is to design methods to control the uptake of the injected/infused enzyme preparations, without compromising the characteristics/image of fresh meat. If the addition of salts or other additives is considered acceptable for the meat product under consideration, adding actinidin to currently used “enhancement” formulations could be a successful strategy to improve the tenderness of cuts that need improvement regarding this trait.

6 Reference list

Bekhit, A.E.D. (2010). Designer enzyme tenderizers (project A.MPT.0021). Final Report.

Christensen, M., Torngren, M.A., Gunvig, A., Rozlosnik, N., Lametsch, R., Karlsson, A.H. and Ertbjerg, P. (2009). Injection of marinade with actinidin increases tenderness of porcine *M. biceps femoris* and affects myofibrils and connective tissue. Journal of the Science of Food and Agriculture 89: 1607-1614.

Chrystall, B.B. and Devine, C.E. (1991). Quality assurance for tenderness. Meat Industries Research Institute of New Zealand Publication No. 872.

Han, J., Morton, J.D., Bekhit, A.E.D and Sedcole (2009). Pre-rigor infusion with kiwifruit juice improves lamb tenderness. Meat Science 82: 324-330.

Jorgensen, A.S., Christensen, M. and Ertbjerg, P. (2008). Marination with kiwifruit powder followed by power ultrasound tenderizes porcine *M. biceps femoris*. Proceedings of the 54th Interanational Congress of Meat Science and Technology, Capetown, South Africa, Session 7B, pp. 1-3.

ISO (1978). Meat and meat products, part II, determination of L(-)hydroxyproline content. International Organisation for Standardisation (ISO) (3946).

Perry, D. Shorthose, W.R., Ferguson, D.M. and Thompson, J.M. (2001). Methods used in the CRC program for the determination of carcass yield and beef quality. Australian Journal of Experimental Agriculture 41: 953-958.

Watson, R., Gee, A., Polkinghorne, R. and Porter, M. 2008. Consumer assessment of eating quality – development of protocols for Meat Standards Australia (MSA) testing. Australian Journal of Experimental Agriculture 48: 1360-1367.

7 Appendices

Appendix 1. Description of proteases used in experiment 2 and their sources.

Code	Product/Treatment	Concentration	Manufacturer	Concentration used
C	None (control)	-	-	-
T1	Water	-	-	-
T2	Papain 25,000 MG	0.01g/L	Supplier A	Conc1 = 1X [1] Conc2 = 2X Conc3 = 4X Conc4 = 6X Conc5 = 10X
T3	Bromelain	0.05g/L	Supplier A	Conc1 = 1X Conc2 = 2X Conc3 = 4X Conc4 = 6X Conc5 = 10X
T4	Kiwifruit PE (Actinidin)	10g/L	Supplier B	Conc1 = 1X Conc2 = 2X Conc3 = 3X Conc4 = 4X Conc5 = 5X
T5	DigestEasy (Zingibain)	100 mL/L	Supplier C	Conc1 = 1X Conc2 = 2X Conc3 = 3X Conc4 = 4X Conc5 = 5X
T6	Fungal protease 60k	0.01g/L	Supplier A	Conc1 = 1X Conc2 = 2X Conc3 = 4X Conc4 = 6X Conc5 = 10X
T7	Fungal protease 31k	0.02g/L	Supplier A	Conc1 = 1X Conc2 = 2X Conc3 = 4X Conc4 = 6X Conc5 = 10X
T8	Bacterial protease G	0.08g/L	Supplier A	Conc1 = 1X Conc2 = 2X Conc3 = 4X Conc4 = 6X Conc5 = 10X
T9	Kiwifruit crude juice	20%	In house prepared	Conc1 = 20% Conc2 = 40% Conc3 = 60% Conc4 = 80% Conc5 = full

				strength
T10	Asparagus crude juice	50%	In house prepared	Conc1 = 20% Conc2 = 40% Conc3 = 60% Conc4 = 80% Conc5 = full strength

1= recommended concentration by the supplier