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Designer enzyme tenderisers

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Abstract

Several plants and microbial enzyme preparations have been researched to determine whether the exogenous application of proteases can reliably tenderise meat. Successful use of these enzymes in fresh meat requires their enzymatic kinetics and characteristics to be determined, together with an understanding of the impact of the surrounding environmental conditions (pH, temperature, release and accumulation of metabolites during meat storage) on enzyme function. This enables the optimal conditions for tenderising fresh meat to be established, and the elimination or reduction of any negative impacts on other quality attributes.

This project characterised the activity of seven commercial proteases (papain, bromelain, zingibain, actinidin, bacterial protease G, fungal 31K and fungal 60k) sourced from Australian suppliers, and two in-house preparations (Kiwifruit and Asparagus extracts) using synthetic substrates (BODIPY-casein, CBZ-Lys-p-nitrophenol and Azocoll) and meat protein fractions (myofibrils, collagen and elastin). Furthermore, the effect of some commonly used additives on the proteolytic activity of the enzymes was investigated using meat myofibrils. The effects of the commercial enzyme preparations (when applied according to the manufacturers' recommendations), and the two in-house preparations on the eating and keeping qualities and the protein degradation in the sarcoplasmic and myofibrillar fractions of hot-boned topside (*M. semimembranosus*) from cull dairy cows was also investigated.

All of the protease enzyme preparations were demonstrated to be impure extracts and displayed variable proteolytic activities dependent on the substrate used and the assay conditions (pH and temperature). The kinetic data obtained from BODIPY-FL casein assay parallels those results obtained using meat proteins (myofibrils and connective tissue). Therefore the kinetic results from the BODIPY-FL casein assay can be used to reliably estimate the 'in meat' binding affinity and the rates of reactions.

Each enzyme preparation targeted different meat proteins. The efficacy of the enzymes in degrading all meat proteins was demonstrated and the structural proteins targeted by each enzyme preparation were identified at the molecular level for the first time. All rates and affinity to different structural proteins were significantly increased or decreased by the use of ascorbic acid and cysteine respectively. Use of the commercial proteases at their recommended level was not sufficient to cause significant proteolysis and tenderness in hot-boned beef from dairy cows. However, the use of the in-house kiwifruit preparation was successful in achieving significant tenderization.

The colour and lipid stabilities of steaks from the beef topsides during display and post-mortem storage benefited from the treatment with the protease preparations (with the exception of commercial actinidin). Therefore, other quality attributes in addition to tenderness may benefit from the application of the enzyme preparations.

The characterisation of the enzymatic target protein substrates; enzyme kinetics; and effective enzyme to substrate ratio required for protein degradation undertaken through this project provides the critical information base upon which a strategy can be developed to optimise and control tenderness through the use of a combination of enzymes and additives. It may also offer the industry new value-adding opportunities to enhance other meat quality attributes such as colour and lipid stability.

Executive Summary

Background

Variation in meat tenderness is dictated by several factors that are spread along the production chain (biological, on farm, processing and consumer factors). Setting standards along the production chain for animal selection, farm management, and processing conditions can improve average meat tenderness, but cannot eliminate the biological variation in meat tenderness due to physiological and anatomical differences between different animals and muscles/meat cuts.

Meat tenderness is largely regulated by post-mortem protein degradation via the actions of endogenous proteases in meat. Increased meat tenderisation can be achieved by stimulating the activity of endogenous proteases through exposure to relatively high post-mortem temperatures and/or addition of Ca^{2+}; however, these treatments often have a negative impact on other meat quality attributes (e.g., colour and flavour). The use of exogenous proteases to improve meat tenderness has attracted much interest recently, with a view to consistent production of tender meat products and added value to lower-grade meat cuts. Several plant enzymes (such as papain, bromelain and ficin from papaya, pineapple and figs, respectively) have been extensively investigated as meat tenderisers. These proteases, however, have not been adopted at an industrial level as their indiscriminate hydrolysation of meat proteins can lead to the generation of 'off' flavours. Additionally, these proteases can result in the over-tenderisation of meat products as high temperatures (>80°C) are required for their complete inactivation. Recent interest in plant proteases has been directed toward proteases from kiwifruit (actinidin) and ginger (zingibain), which exhibit mild tenderising effects and have other potential beneficial effects on lipid oxidation, colour stability, and microbiological status. Bacterial and fungal proteases have also generated interest due to their controlled meat tenderising effects.

Research Objectives

Enzyme proteolytic activity varies with concentration and the surrounding environmental conditions (pH, temperature, release and accumulation of metabolites during meat storage). Understanding the impact of these factors on enzyme kinetics is fundamental to establishing the optimal conditions for fresh meat tenderisation and the elimination/reduction of negative impact on other quality attributes. To date, much of the available research on protease characteristics has been obtained using highly purified preparations which are unlikely to reflect the characteristics of crude extracts normally available commercially. Therefore, this report describes research undertaken to:

1. Characterise the proteolytic activity and enzyme kinetics of commercial proteases (papain, bromelain, zingibain, actinidin, bacterial protease G, fungal 31K and fungal 60k) sourced from Australian suppliers, and two in-house preparations (Kiwifruit and Asparagus extracts). Synthetic substrates (BODIPY-casein, CBZ-Lys-p-nitrophenol, and Azocoll) and meat protein fractions (myofibrils, collagen and elastin) were used for these analyses.
2. Examine the effects of some commonly used additives (e.g., ascorbic acid and cysteine) on the proteolytic activity of the enzymes using meat myofibrils.
3. Investigate the effects of commercial proteases (applied according to the manufacturer's recommendations) and Kiwifruit and Asparagus extracts (in-house preparations) on the eating and keeping qualities and protein degradation in the sarcoplasmic and myofibrillar fractions of hot-boned topside from cull dairy cows.

Key Findings

1. Proteolytic Activity & Enzyme Kinetics

All of the protease enzyme preparations used were impure extracts. Analysis of each enzyme extract indicated the presence of several protein species, and some extracts contained more complex protein mixtures than others. All of the investigated enzymes demonstrated variable proteolytic activities, which were dependent on the substrate used and the assay conditions (pH and temperature). Proteolytic activity of the studied enzymes was best reflected through caseinolytic activity (BODIPY-casein) or meat myofibril degradation. The kinetic data obtained from the BODIPY-FL casein assay was largely in agreement with results obtained using meat proteins (myofibrils and connective tissue). As such, the kinetic results from the BODIPY-FL casein assay are likely to be of value in estimating binding affinity and rates of reactions. Key findings are summarised in the below table

Summary of findings from various assays used to characterise commercial and in house proteases preparations.

Enzyme preparation	pH and temp	K_o (1/s) *[2]	E_A kJ*[2]	V_{max}*	K_m *	MM[1] E/S	Col/elas E/S	Remarks
Papain	7.5 and >55°C	$1.84x10^8$	32.8	↑	↓	0.0004	$1.43\ x10^{-3}$	High binding affinity and rapid hydrolysis
Bromelain	5.5 and >55°C	$2.4x10^8$	33.5	↓	↓	0.05	$>1.43\ x10^{-2}$ But <1.43	Very high binding affinity but slow hydrolysis
Fungal 60K	6.5-7.0 and 35-45°C	$1.68x10^{11}$	56.18	↓	↑	0.4	>2.85	Modest binding affinity and slow hydrolysis
In-house kiwifruit	5.0- 6.5 and 55°C 7.5- 8.0 and 55°C	$6.92x10^4$	19.57	↑	≈	20 µl/mg	<20µl/ 0.07mg (20 folds dilution)	Low binding affinity and slow hydrolysis
In-house Asparagus	>7.5 and > 45°C	$1.9x10^3$	15.13	↓	↓	>40 µl/mg	>20µl/ 0.07mg	High binding affinity and slow hydrolysis
Protease G	7.5- 9.0 and 45°C	$2.17x10^{10}$	50.45	↑	↑	<0.2 X >0.02	5.7	Low binding affinity and good hydrolysis
Fungal 31K	5.5 and 45°C	$2.84x10^{11}$	52.5	↑	↑↑	<1 X >0.2	0.57	Low binding affinity but very fast hydrolysis
Zingibain	6.5- 7.0 and 55°C Other pH, > 55°C	$2.6x10^{10}$	46.61	↑	↓	>40 µl/mg	<20µl/ 0.07mg (100 folds dilution)	Modest binding affinity and relatively good hydrolysis rate
Commercial actinidin	4.5-5.5 and 45°C	$3.6x10^5$	21.43	↓	≈	0.4	5.7	modest affinity and slow hydrolysis

[1] MM= Meat myofibrils [2] calculated with the temperature range stated in table7

* determined using BODIPY-casein E/S= Enzyme to substrate ratio for hydrolysis

2. Effect of Additives on Proteolytic Activity

The addition of ascorbic acid or cysteine significantly affected (either by activation or inhibition) both reaction rates and binding affinity to different structural proteins. This demonstrates the potential of these compounds to act as regulators of proteolytic activity. The isoform of ascorbic acid can manipulate protease activity in such a way that it could be used to achieve higher rates of protein degradation (e.g., with fungal and bacterial proteases) or to slow down protein degradation (e.g., with Kiwifruit extracts and D-isoascorbic acid) in order to achieve optimum levels of proteolysis.

3. Proteolytic Activity in Hot Boned Beef Topsides

Commercial proteases (applied according to the manufacturer's recommendations) did not cause significant proteolysis and tenderness in hot-boned beef from dairy cows. While findings from the characterisation studies indicate the potential of several enzymes as tenderisers, the level of these enzymes when used in these circumstances will require some optimisation. In-house kiwifruit preparation was the only effective preparation caused significant tenderization. The colour and lipid stabilities of topsides during display and post-mortem storage appeared to benefit from treatment with the protease preparations, with the exception of commercial actinidin. Therefore, quality attributes in addition to tenderness may benefit from the application of these enzyme preparations.

Conclusions

The efficacy of the enzymes in degrading all meat proteins was demonstrated and the structural proteins targeted by each enzyme preparation were identified at the molecular level for the first time. The characterisation of the enzymatic target protein substrates, enzyme kinetics, and effective enzyme to substrate ratio required for protein degradation undertaken through this project provides the critical information base upon which a strategy can be developed to optimise and control tenderness through the use of a combination of enzymes and additives. It may also offer the industry new value-adding opportunities to enhance other meat quality attributes such as colour and lipid stability. Also, the knowledge generated in this project on the meat substrates and the products generated by different enzyme preparations provide new opportunities to adding value to meat.

Contents

1 Background

Tenderness is arguably the most important quality attribute in red meat. After the meat is cooked or served in cooked form, many of the appearance attributes become irrelevant and flavour is/can be manipulated with other ingredients in the meal or added flavours. Therefore, the consistent production of tender meat is a prime objective for the meat industry in order to retain consumer confidence in red meat and to maximize financial returns due to the higher retail value of tender meat.

Variation in meat tenderness is dictated by several factors that are spread along the production chain (biological, on-farm, processing, and consumer factors). Setting standards along the production chain for animal selection, farm management, processing conditions, can improve average meat tenderness, but cannot eliminate the biological variation in meat tenderness from different animals and muscles due to physiological factors (diet intake, physical activity, level of endogenous proteases,…etc) and anatomical variation in the amount of connective tissue in different muscles/meat cuts.

Meat tenderness is largely regulated by post-mortem protein degradation via the actions of endogenous proteases in meat. Increased meat tenderisation can be achieved by increasing the activity of endogenous proteases through exposure to relatively high post-mortem temperatures and/or addition of Ca^{2+}; however, these treatments often have a negative impact on other quality attributes (e.g. colour and flavour).The use of exogenous proteases to improve the tenderness of meat has attracted much interest recently (Sullivan and Calkins, 2010; Tørngren, 2010), with a view to consistent production of tender meat products and added value to lower-grade meat cuts. Several plant enzymes (such as papain, bromelain and ficin from papaya, pineapple and figs, respectively) have been extensively investigated as meat tenderisers. Plant proteases can hydrolyse meat proteins indiscriminately, with most literature reporting papain to have the highest rate of tenderisation at the same concentration. The primary problem with papain, bromelain and ficin is the generation of off flavours due to indiscriminate hydrolysation of meat proteins, and over-tenderisation as high temperatures (>80°C) are required for their complete inactivation. Enzyme proteolytic activity varies with concentration and the surrounding environmental conditions (e.g., pH, temperature, release and accumulation of metabolites during meat storage). Understanding the impact of these factors on enzyme kinetics is fundamental to establishing the optimal conditions for fresh meat tenderisation and the elimination/reduction of negative impact on other quality attributes. Recent interest in plant proteases has been directed toward proteases from kiwifruit (actinidin) and ginger (zingibain), which exhibit mild tenderising effects and have other potential beneficial effects on lipid oxidation, colour stability and microbiological status. Bacterial and fungal proteases have also generated interest due to their controlled meat tenderising effects (Ashie et al., 2002).

To date, much of the available research on protease characteristics has been obtained using highly purified preparations which are unlikely to reflect the characteristics of crude extracts normally available commercially. Also, the enzyme preparations from different suppliers can vary in composition and activity due to different extraction procedures and different bulking agents used. To date no information has been reported on the effects of additives (ascorbic acid and cysteine as reducing agents and maltodextran as a bulking agent) on the efficacy of proteases in the meat model system. The effective and successful use of any of the

aforementioned proteases lies in understanding the characteristics and strength of the protease to be used and the environmental conditions in which it will be used.

As mentioned above, the efficacy of a protease in tenderising meat can be affected by the environment in which it will function (pH, temperature and level of toughness). Hot- boned meat has different biochemical conditions to cold-boned meat; this may affect the performance of the tenderisation process and lead to the advantage of reduced PM holding and handling of the meat. Also, a different tenderisation profile due to the higher temperature of the injected meat (activation of the protease) and the loss of the skeleton support during rigor (potentially greater sarcomere contraction) could be expected.

2 Project Objectives

1. To characterise the activity of commercial proteases sourced from Australian suppliers and two in-house enzyme preparations from Kiwifruit and asparagus using synthetic and meat myofibrils as substrates.

2. Examine the effects of some commonly used additives on the proteolytic activity of the enzymes using meat myofibrils.

3. To investigate the effects of different commercial enzyme preparations (applied according to the manufacturer's recommendations) and two in-house preparations (Kiwifruit and Asparagus extracts) on hot-boned topside (*M. semimembranosus*) from cull dairy cows.

3 Material and methods

3.1 Characterization of proteases from Kiwifruit, pineapple fruit and stem, asparagus spear and ginger rhizome

Clarified juice extracts were prepared and preliminary enzyme assay data were obtained. Several assays have been used to explore the proteolytic activities as follows:

3.1.1. *An assay for esterase activity*. This assay involves hydrolysis of the substrate, CBZ-Lys-*p*-nitrophenol (#96893 SIGMA), which when hydrolysed generates *p*-nitrophenol. This can then be measured spectrophotometrically and quantitated from a standard curve of *p*-nitrophenol. Preliminary assay data have been obtained over the pH range 4-9 and temperature range 25-45°C for all four enzymes. The method was carried out as described in Han et al (2009), originally described by Boland and Hardman (1973) and modified by Tsuruhami et al. (2006), which is described in detail in section 3.2.1.1 in relation to the pH and time course measurements.

3.1.2. *An assay for protease activity on casein*. This assay involves hydrolysis of the substrate, green fluorescent-labelled (BODIPY-FL)-casein (#E6638, Molecular Probes Company, supplied

through Invitrogen) in which the fluorescence is highly quenched. When the Bodipy-FL-casein is hydrolysed, fluorescent labelled peptides are released and the fluorescence is unquenched, proportional to hydrolysed peptide bonds. Preliminary assay data have been obtained over the pH range 4-9 and temperature range 25-45°C for all four enzymes (Jones et al., 1997). Results for the characterisation of commercial enzymes were determined using the modifications reported by Thompson et al. (2000) as described in section 3.2.1.2.

3.1.3. *An assay for protease activity on collagen*. This assay involves hydrolysis of azo-dye impregnated collagen (Azocoll) (#A4341, SIGMA). On hydrolysis of Azocoll, azo dye is released (in proportion to hydrolysed peptide bonds) and can be measured spectrophotometrically (Chavira et al., 1984) as described in section 3.2.1.3.

3.1.4. *Simple protease spot test plate assays* have been established using gelatine and milk-agar. This is simple assay for quick screening of plant protease hydrolytic activity. These assays can be used to check the presence of proteolytic activity of commercial compounds quickly and cheaply; however, they were not suitable for quantitative estimation of the activity under different pH and temperatures. As a result, this method was not used further.

3.1.5. *A small scale preparation of meat myofibril, collagen and elastin extracts* has been prepared as described by Geesink et al. (2001) and Davis and Mackle (1981) to enable examination of the protease hydrolysis of protein in these fractions under different time course conditions of pH and temperature. The protease hydrolysis was monitored by mini-gel (Invitrogen, Novex) one dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3.1.5.1. *Preparation of meat myofibril*

Meat myofibrils were prepared according to Culler et al. (1978). Beef LD muscle, free of any fat or connective tissue, was minced and then 2 g aliquots were homogenised in 20 mL cold 'MFI' buffer (20 mM potassium phosphate, pH 7.0, containing 100 mM KCl, 1 mM EGTA, 1 mM MgCl2 and 1 mM NaN3) with a Polytron for 20 sec. The homogenate was transferred to a 50 mL Falcon tube and centrifuged at 1000 x g for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 20 mL MFI buffer. The low speed centrifugation was repeated and the supernatant was discarded, followed by the pellet being resuspended by vortexing in 10 mL MFI buffer. The suspension was filtered through a polyethylene strainer to remove larger material and any connective tissue, and the strainer was rinsed with an additional 10 mL MFI buffer. The meat myofibril extract was lyophilised and then uniformly dissociated as a suspension at concentration of 5 $mg.mL^{-1}$ in 0.2 mM sodium formate buffer (pH 3.3) mixed with 0.35% acetic acid (1:1), to enable reproducible dispensing of meat myofibril aliquots for subsequent proteolysis assays.

3.1.5.2. *Separation of collagen and elastin*

Sample preparation

All operations were carried out at 4°C unless stated otherwise. Connective tissue from meat sections and tendons from cow were washed extensively with water and blotted dry. The tissue was cut into fine pieces and extracted for 16 hrs in 0.02 M phosphate buffer containing 0.2 M NaCl (pH 7.4) at a ratio of 1g/100 ml. The mixture was centrifuged at 22,000 × g for 30 min and the pellet was subjected to re-extraction until the protein concentration in the supernatant was less than 0.2% of the original tissue wet weight. The extraction procedure was carried out on the pellet using 0.02 M sodium phosphate (pH 7.4) containing 0.5 M NaCl and then with 0.01 M Tris-HCl (7.4) containing 0.167M EDTA. After centrifugation, the residue was extracted three times with chloroform:methanol (2:1) for 16 h each at a concentration of 0.2 mg tissue/ml

mixture. The tissue was centrifuged for 30 min at 3,000 × g, and then the pellet was washed twice with acetone and dried with diethyl ether.

Separation of collagen and elastin
The process followed for the separation of collagen and elastin is shown in Figure 1.

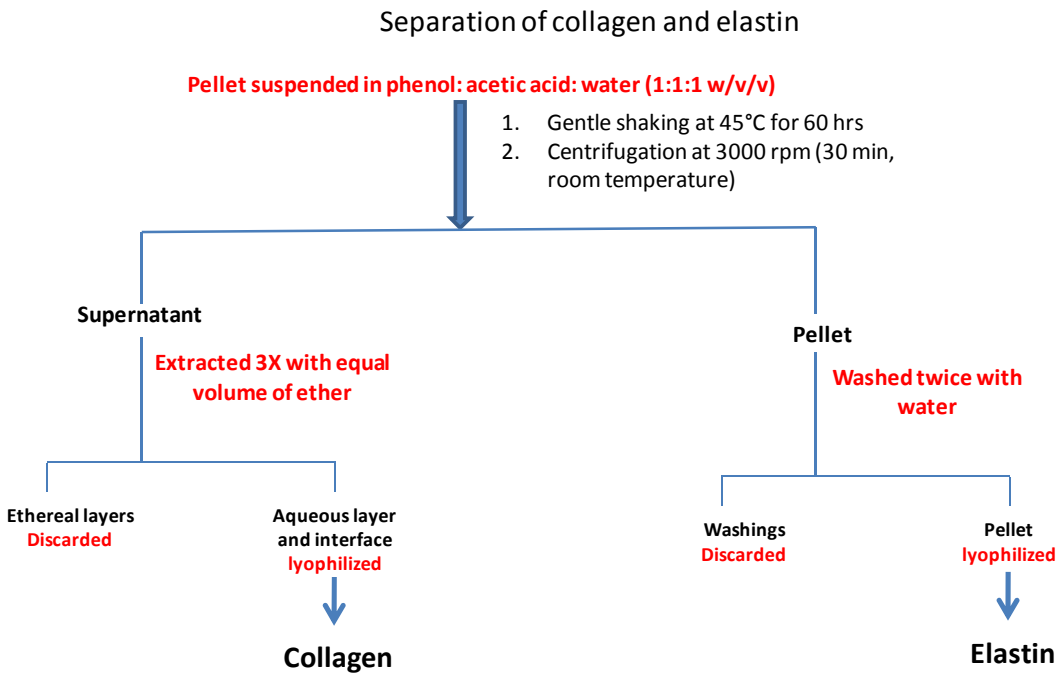

Figure 1. Schematic representation of the separation of collagen and elastin from a sample preparation of cow tendons. (After Davis and Mackle, 1981).

3.2 Characterisation of commercial proteases and in-house preparations of asparagus and Kiwifruit extracts

For the characterisation of the proteolytic activity of commercial and in-house preparations (Table 1), the *p*-nitrophenol, BODIPY-FL and Azocoll assays were used as they are the most suitable for kinetic studies and regular lab use. Proteolytic activity was measured in pH range of 4.5-9.0 and temperature range of 5-85°C. Furthermore, all of the enzymes were examined for their ability to degrade myofibrillar proteins, collagen and elastin during an *in vitro* digestion assay.

Table 1. A summary of the commercial and in-house enzymes preparations used in the present studies.

Enzyme	source
Kiwifruit (in-house)	Prepared as described by Bekhit et al. (2007)
Asparagus (in-house)	
Papain 25,000 MG	Supplier A
Bromelain	Supplier A
Kiwifruit PE (Actinidin)	Supplier B
DigestEasy (Zingibain)	Supplier C
Fungal protease 60,000	Supplier A
Fungal protease 31,000	Supplier A
Protease G	Supplier A

3.2.1 Biochemical Assays

3.2.1.1 Esterase activity using CBZ-Lys-*p*-nitrophenol

Protease activity was measured by comparing the release of *p*-nitrophenol from CBZ-Lys-*p*-nitrophenol in the presence of the protease, with the spontaneous release of *p*-nitrophenol as a control, according to the method of Boland and Hardman (1973) and the modifications of Tsuruhami et al. (2006).

The enzyme assay buffer was 0.07M potassium phosphate (0.2mM EDTA, 1.6% acetonitrile, pH 6.95). The buffer was made weekly and stored at 4°C. A 0.02M p-nitrophenol stock solution was prepared in distilled water for the standard curve using concentrations from 0 – 0.5 µmol. Freshly made 0.01M substrate solution was prepared by dissolving 10.5 mg of N-α-benzyloxycarbony-L-lysine p-nitrophenol ester (CBZ-Lys-ONP) in 0.1 mL of distilled water and 1.9 mL of acetonitrile was kept on ice during the assay. To assay the proteolytic activity of the enzymes, a spectrophotometer (UNICAM UV) was zeroed using the enzyme assay buffer. A 2.98 mL aliquot of buffer was added to 50 µL of the CBZ-Lys-ONP substrate solution in a 3 mL plastic cuvette. The initial degradation rate of the substrate was measured at a wavelength of 348nm for 30 seconds (background non-enzymatic hydrolysis of substrate). Then 50 µL of the enzyme solution was added, and the rate of substrate degradation was measured for a further 30 seconds. Enzyme activity was determined over a pH range of 5.0 to 7.0 and a temperature range of 25 to 85°C by incubating the assay mixture at the required temperature (assay mixture was incubated for 3 min at the required temperature before the reactions were stopped by addition of 100 µL of 1 M sodium carbonate). A standard curve of absorbance vs. concentration of p-nitrophenol was described by a linear equation (y = mx + c) generated using SigmaPlot. The initial nonenzymatic hydrolysis rate of substrate was subtracted from the enzyme activity rate to calculate the enhanced proteolytic activity induced by the proteases. One unit of proteolytic activity was the amount of enzyme (mL or gram depending on the original supplied form of the enzyme) that produced 1µmol of product (p-nitrophenol) per minute. Commercial actinidin had solubility problems which were an obstacle for determining enzyme activity using this assay. Bacterial and fungal proteases did not exhibit esterase activity up to concentrations of (100 mg/ml).

3.2.1.2 Casein proteolytic activity using BODIPY-FL Casein.

The proteolytic activity of the enzymes was determined as described by Thompson et al. (2000). BODIPY-FL was purchased from Molecular Probes (Eugene, OR, USA, obtained through Invitrogen) and the BODIPY-FL casein was prepared as described by Thompson et al. (2000). Microtiter plates were used in this assay. 50µL of enzyme solution (the enzyme concentrations were adjusted to provide adequate activity and fluorescence generation) was combined with 50 µL buffer (20 mM phosphate buffer, pH 7.5) and 100 µL BODIPY-FL casein substrate (5000 µL dilution buffer (40 mM Tris, pH 7.5; 2 mM EDTA; 200 mM EDTA; 200 mM KCl); 3965 µL DDH2O; 10 mM $CaCl_2$; 50 µL BODIPY-FL casein stock; 0.1% β-mercaptoethanol). The blank reference pool contained 100 µL buffer and 100 µL BODIPY-FL casein substrate. Sample fluorescence was read immediately and after every 15 s for 10 minutes in a BMG ELISA plate reader (Polarstar, BMG Labtechnologies GmbH. Offenburg, Germany) with an excitation wavelength of 485 nm, emission 520 nm and a gain of 60. The software used was version 3.02-0.

The fluorescence changes caused by enzyme activity were calculated and expressed as Units (change in fluorescence, Δ F) per mL or per gram of enzyme/minute. Enzymes activity was determined over pH range of 4.5 to 9.0 and temperature range of 5 to 55 °C. The assay temperatures 25, 35 and 45 °C were set by equilibrating the assay mixture at the required temperature before adding the enzyme and measuring the activity.

3.2.1.3 Collagen proteolytic activity using Azocoll

The ability of the enzymes to hydrolyze Azocoll (collagen-dye conjugate) was determined as described by Chavira et al. (1984). Protease enzymes were assayed using azo dye-impregnated collagen (Azocoll, Sigma A4341). Trial experiments with Azocoll resulted in the development of the following protocol.

Processing of Sigma Azocoll for use as substrate in protease assay.

1. Azocoll (240 mg) was suspended in 120 mL 50mM Tris HCl, pH 6.0, and the suspension stirred for 2 h at room temperature.
2. The supernatant containing leached azo dye-labelled peptides was decanted and discarded.
3. Steps 1 and 2 above were repeated.
4. The washed Azocoll was resuspended in 120 mL Tris.HCl, pH 6.0 and homogenised in small batches for 2 min with a Polytron to improve uniformity of the Azocoll fragment suspension, for more consistent substrate hydrolysis in the assay.
5. While maintaining a homogeneous suspension, 1 mL aliquots of the Azocoll were dispensed into 1.5 mL capped microfuge tubes and were then stored at -20°C until required for assay. Each protease assay reaction reproducibly contained almost 2 mg Azocoll, which was in excess of enzyme hydrolysis requirements.

Protease assay with Azocoll

Azocoll substrate tubes were equilibrated to either 55°C (for papain, ginger and bromelain assay) or 45°C (for all other enzymes) before 200 µl of protease extract (bacterial protease G, fungal protease 31, fungal protease 60 and actinidin at a concentration of 100 $mg.mL^{-1}$; bromelain and papain at a concentration of 10 $mg.mL^{-1}$; commercial ginger and in-house Kiwifruit and asparagus preparations in solution form) were added and the tubes were incubated at the respective temperatures and tumbled (Mini hybridization oven MKII, Hybaid Limited, UK) to keep the Azocoll suspended.

At 1 h, 2 h, 3 h, and 6 h time intervals, the tubes were removed from tumbled incubation, the Azocoll was pelleted by brief centrifugation (5 min at 13000 rpm, Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) and the absorbance of the supernatant measured was at 520 nm.

The supernatant was then returned to the assay tube, the Azocoll was resuspended and the tube incubation continued at the selected temperature with tumbling until the next time point reading.

3.2.2 Characterisation of commercial proteases and in-house preparations of Asparagus and Kiwifruit extracts using meat myofibril extract and connective tissue proteins (collagen and elastin).

SDS-PAGE for Myofibrillar proteins

Freeze dried meat myofibril suspension uniformly dissociated (5 $mg.mL^{-1}$) in 0.2 mM sodium formate, pH 3.3 buffer, mixed 1:1 with 0.35% acetic acid, was dispensed as 200 µL aliquots into 1.5 mL capped microfuge tubes. The pH was adjusted to 6.0 with 1 M NaOH solution (8 µL per aliquot) and the aliquots stored frozen until required for proteolysis assay.

The capability of protease extract samples to hydrolyse meat myofibril extract was initially analysed by adding up to 40 µL of protease extract to a 208 µL meat myofibril (pH adjusted) aliquot at 45°C. Aliquots (20 µL each) were removed at various times during incubation and snap frozen in dry ice. Ten µL of each time course sample was mixed with 5 µL of SDS-PAGE sample buffer and analysed by SDS-PAGE mini-gel (4-12% Bis-Tris Novex gel, 12 well, Invitrogen). After electrophoresis, gels were stained with 'Simply Blue' colloidal Coomassie (Invitrogen) to visualise protein bands. In subsequent assays, the amount of enzyme in each assay was adjusted to generate an appropriate display of meat myofibril proteolysis on SDS-PAGE.

SDS-PAGE for connective tissue proteins

Sample preparation

Aliquots (100µL) of the stock collagen/elastin were pre-heated in a Hybaid mini MK II oven at either 45°C or 55°C. Following this, 20µL of crude juice extract or commercial enzyme solution was added to the tube, which was then returned to and tumbled in the oven. Time course hydrolysis assay samples were obtained by retrieving 4µL of the tumbled mix at various time points during the incubation. The hydrolysis assay samples were immediately mixed with 3µL of sample buffer, 1µL of reducing agent and 6µL of Milli-Q water and frozen on dry ice. The samples were then kept at -20°C until analysed by 1 dimension SDS-PAGE.

SDS-PAGE

The hydrolysis of connective tissue proteins by the plant crude juice extracts and commercial enzyme solutions was followed over various time points. A 4µL of each of the collagen/elastin hydrolysis assay samples was heated at 100°C for 10 minutes with 6µL of Milli-Q water, 3µL of Invitrogen NuPAGE® LDS sample buffer (4X) (#NP0007) and 1µL of Invitrogen NuPAGE® sample reducing agent (10X) (#NP0004). Electrophoresis was performed on Invitrogen NuPAGE® 4-12% Bis-Tris gels (#NP0322BOX) at 160V, 4°C for 100 minutes. Subsequently, the gels were washed three times in Milli-Q water for at least 5 minutes each time and stained overnight in 20mL of Invitrogen SimplyBlue™ SafesStain (#LC6060). The gels were then de-stained with Milli-Q water and images were scanned the following day.

3.2.3 Determination of V_{max} and K_m of commercial proteases and in-house preparations of Asparagus and Kiwifruit extracts using meat myofibril extract.

Background for the determination of V_{max} and K_M

The kinetic constant K_M is defined as the substrate concentration required to achieve half the maximum velocity ($V_{max}/2$) in the enzyme catalysed reaction. K_M is effectively a measure of the binding affinity of a substrate to an enzyme (the formation of ES transition complex, governed by rate constants k_1 and k_2). A high K_M indicates low binding affinity and vice versa.

The kinetic constant V_{max}, maximum velocity, of an enzyme is achieved when all enzyme binding sites are fully occupied by substrate i.e., substrate is saturating the enzyme. V_{max} is a measure of the maximum catalytic rate of conversion of the ES transition complex to E + P product, which is governed by rate constant k_3.

Michaelis-Menten kinetics provides a means by which to process first order kinetic data to derive values for V_{max} and K_M. To achieve this, the initial velocity (V_o) should be determined for a range of substrate concentrations, where the highest substrate concentration is recommended to be at least 10 times the K_M for the enzyme, so as to ensure substrate saturation conditions are achieved. In practice, this is often not possible due to substrate solubility limitations or other factors. Much of the enzyme kinetic data reported in the literature has been obtained at relatively low substrate concentrations. The amount of enzyme used in the assays should normally be at a sufficiently low level so as to generate linear velocity data over the (initial) duration of the assay. This in turn results in minimal conversion of substrate to product, resulting in little change in the concentration of the substrate during the assay.

The Michaelis-Menten (M/M) data plot of V_o vs. [S] generates an asymptotic hyperbolic curve, where at high [S], V_o approaches V_{max}. This provides a way to obtain an approximation to the value for K_M in that $V_{max}/2$ can be estimated and then K_M derived from the graph. Various methods have been developed to linearise the M/M data (e.g. Lineweaver-Burk L/B and Eadie-Hofstee E/H plots) so that a more accurate estimate of V_{max} and K_M can be obtained.

Determination of V_{max} and K_M for the commercial enzymes

BODIPY-casein (Molecular Probes) was chosen as the substrate for enzyme assay kinetic data collection after Thompson et al. (2000) and Park et al. (2009). This substrate was used previously in this project for enzyme optimal temperature and pH data collection. The BODIPY-casein used is a standardised and validated commercial product, the fluorescent label provides good sensitivity and the casein component provides a large polymer proteolysis substrate that more closely approximates meat protein proteolysis than does the use of low molecular weight substrates such as labelled peptides. The use of BODIPY-casein hydrolysis assays to obtain kinetic data for other enzymes is also reported in the literature.

The standard validated BODIPY-casein hydrolysis assay protocol uses 1 microgram of BODIPY-casein. For determination of V_{max} and K_M for the commercial enzymes in this project, the highest BODIPY-casein substrate amount used per assay was increased 10-fold to 10 micrograms, and initial velocities (V_o) were determined over the substrate range of 0.5-10 micrograms. Based on the assumption of an average molecular weight for caseins of 25 kDa (as used by others and reported in the literature), the highest substrate amount used (10 micrograms) equates to 2 micromole.L^{-1} in the assay. This is equivalent to that used by others as reported in the literature (Thompson et al., 2000; Park et al., 2009).

The optimal temperature for the enzymes determined in previous experiments was over the range 45-65°C. The Polarstar fluorescent micro titre plate reader used for these experiments has a maximum incubation temperature of 45°C. The kinetics assays were therefore incubated at 45°C and buffered at pH 6.0, this being in the vicinity of the pH range of meat (5.5-6.0) and also near the optimal pH of most of the enzymes.

Preliminary experiments were conducted to determine the amount of enzyme used in each assay, so as to achieve linear velocity during the assay. In doing this, the amount of product formed in the assay was also minimised, so that effectively there was little change in substrate concentration during the assay, but sufficient fluorescence signal generated for measurement.
The enzyme reaction velocity data were obtained as arbitrary fluorescence units generated/time. As there is no information available about the specific activity of fluorescent labelling of the casein for the commercial BODIPY-casein substrate, it was not possible to express the velocity data in molar/time units. Other reports in the literature have expressed velocity and V_{max} as arbitrary fluorescence units/time, or have presented data as V_{max}/K_M.
All BODIPY-casein hydrolysis fluorescence kinetic assays were carried out in triplicate. Initial velocity (V_o) was determined from line slope over the first 30 s of assay for each replicate and then averaged to generate one data point for each substrate concentration, respectively. Data were plotted initially as V_o vs. [S] Michealis-Menten plots, followed by generation of linearised L/B and E/H plots to provide estimation of V_{max} and K_M for each enzyme.

3.2.4 Effect of ascorbic acid (L and D-Iso forms) and cysteine on the proteolytic activity of commercial proteases and in-house preparations of Asparagus and Kiwifruit extracts using meat myofibril extract.

The method described above in section 3.2.2 was used to determine the effects of ascorbic acid and cysteine (in the range of 0.8 mM to 100 mM) on meat myofibril degradation with the commercial proteases and in-house preparations of Asparagus and Kiwifruit extracts.

3.3 Effect of commercial proteases and in-house preparations of Asparagus and Kiwifruit extracts on the eating and keeping qualities of hot-boned beef

3.3.1 Sample preparation
Hot-boned topsides from dairy cows (>5 years old) were randomly selected on the day of slaughter (approximately 2-3 hours following slaughter) from Alliance Group Ltd (Pukeuri Plant, Oamaru). For logistic reasons, topsides from both sides of 22 carcasses (44 topsides in total) were collected over 3 consecutive days. Some basic information of the hot carcass weight, the carcass grade and the topsides weights are shown in Table 2. The topsides were packed in Styrofoam boxes and the temperature of samples was recorded using temperature loggers. The topsides were transported to University of Otago within 1.5-2 hours of boning and sample treatment was performed 2-3 hours after arrival at the Lab. Topsides were halved and cut into steaks (average weight $\pm$ SD was 305.1 $\pm$ 58.8 g) that were assigned to 1 day post-mortem (PM) treatment, and meat blocks of about 9x9x20 cm (average weight $\pm$ SD was 1677.6 $\pm$ 488.5 g) that were assigned to a 21 day PM vacuum packed storage period. All of the samples (steaks and blocks) were subjected to needle injection (to 10% of the original weight) with a series of enzyme tenderisers according to the manufacturers' recommendations (Table 3) or experimental level for the two in-house extracts prepared as described earlier (Bekhit et al., 2007). The samples were vacuum-packed, stored at 2°C for the designated PM time (1 or 21 days) and sampled for shear force, compression force, colour stability during simulated retail display, and analysis of myofibrillar protein and connective tissue. The samples for protein gels and lipid oxidation analysis were frozen in liquid nitrogen, vacuum packed and stored at -30°C until analysis. Sample position for the different analyses was randomized within each sample block.

Table 2. Carcass grade, hot carcass weight and the weight of topsides used in the present study.

animal	Tag No.	Slaughter date	Wt (kg)		Grade	Hot carcass weight (kg)	Topsides weight (kg)
			Left side	Right side			
1	500001	4/05/2010	97	96.6	CWM	193.6	8.77
2	500002	4/05/2010	107.4	109.1	CWM	216.5	8.41
3	500003	4/05/2010	98.5	96.1	CWM	194.6	7.32
4	500004	4/05/2010	77.9	76.6	CWM	154.5	5.65
5	500005	4/05/2010	122.8	117	CWM	239.8	9.22
6	500006	4/05/2010	93.7	94.2	CWM	187.9	6.16
7	500007	4/05/2010	85.3	86.3	CWM	171.6	6.50
8	500008	4/05/2010	79.7	81	CWM	160.7	5.72
9	500001	5/05/2010	92.9	93	CWM	185.9	6.35
10	500002	5/05/2010	66.6	67.8	CWM	134.4	4.60
11	500003	5/05/2010	105.8	109.4	CWM	215.2	8.28
12	500004	5/05/2010	123.1	127.9	HL2	251	8.62
13	500005	5/05/2010	79.2	81.7	CWM	160.9	6.20
14	500006	5/05/2010	85.1	87.1	CWM	172.2	6.22
15	500007	5/05/2010	64.2	64.9	CWM	129.1	4.44
16	500021	6/05/2010	106.3	104	HL3	210.3	7.13
17	500022	6/05/2010	85	84.6	CWM	169.6	6.26
18	500023	6/05/2010	82.5	82.5	HA3	165	5.36
19	500024	6/05/2010	71.6	71.8	CWM	143.4	4.69
20	500025	6/05/2010	88	86.2	CWM	174.2	6.65
21	500026	6/05/2010	122	127.4	CWM	249.4	9.77
22	500027	6/05/2010	69.8	71	CWM	140.8	5.30

Table 3. Description of proteases used in the present trial and their sources.

Code	Product/Treatment	Concentration	Manufacturer
C	None (control)	-	-
T1	Water	-	-
T2	Papain 25,000 MG	0.01g/L	Supplier A
T3	Bromelain	0.05g/L	Supplier A
T4	Kiwifruit PE (Actinidin)	10g/L	Supplier B
T5	DigestEasy (Zingibain)	100 mL/L	Supplier C
T6	Fungal protease 60k	0.01g/L	Supplier A
T7	Fungal protease 31k	0.02g/L	Supplier A
T8	Bacterial protease G	0.08g/L	Supplier A
T9	Kiwifruit crude juice	20%	In house prepared
T10	Asparagus crude juice	50%	In house prepared

3.3.2 pH and weight change measurements
Measurements were carried out at 1 day and after 3 weeks of vacuum packaging using Hanna pH electrode and meter (model HI 98150). Sample weights before injection and after vacuum packaging for the designated PM period were used to calculate the % weight change due to treatments (weight gain).

3.3.3 Cooking and cooking loss measurements
Fast cooking
The samples for shear force and compression from 1 day and 21 day vacuum packed samples were thawed overnight at 2°C, weighed and cooked individually in plastic bags immersed in a water bath at 80°C until they reached an internal temperature of 75°C (10-14 min) as measured individually using Fluke type K temperature probes attached to Fluke 52 meters. The cooked meat was cooled on ice, patted dry with paper towels and re-weighed. The difference in weight before and after cooking was used to calculate the cooking loss. The cooked meat was then sampled for determination of shear force and analysis of protein degradation.

Slow cooking (Roasting)
After 21 days of vacuum packaging meat blocks were thawed for 24 hours at 2°, transferred to aluminium baking trays and placed in a fan forced oven set at 180°C. Samples were roasted till a temperature of 75-78°C in the centre of the meat was achieved (about 1.5-2 hours). Roasted samples were stored overnight at 2°C and subsequently sampled for determination of shear force, compression and analysis of protein degradation.

3.3.4 Colour stability
Objective colour measurements were obtained for steaks (20 mm thick) placed in polystyrene trays which were covered with O_2 permeable polyvinyl chloride film (O^2 permeability >2000 mL m^{-2} atm^{-1} $24h^{-1}$ at 25°C, AEP FilmPac(Ltd), Auckland, NZ). The steaks were exposed to fluorescent cool light (1,076 lux) and colour measurements were carried out daily over 12 days (1-day PM samples) and 6 days (21 day PM samples) of retail display at 2°C using MiniScan XEPlus (Hunter Associates Laboratory Inc., Reston, VA). The unit was calibrated using black and white standard plates. Measurements were Hunter L^*, a^* and b^* values and spectral reflectance (400–700 nm) using illuminant *C* and a 10° observer with an aperture size of 3.5 cm. The chroma ($C = [a^{*2}+b^{*2}]^{[1/2]}$), hue angle ($HA = \tan^{-1} b^*/a^*$) and browning index (630nm/580nm) were calculated (AMSA, 1991).

3.3.5 Shear force and compression
Shear force was determined as described by Chrystall and Devine (1991) and compression values were determined as described by Perry et al. (2001) using TA Plus texture analyzer (TA.XT plus, Stable Micro Systems, UK). A flat cylinder probe (TA-520A) with a diameter of 20mm was used as, according to the manufacturer guidelines, only flat probes larger than the sample should be used for compression testing. http://www.stablemicrosystems.com/frameset.htm?http://www.stablemicrosystems.com/taxtplus2.htm.

3.3.6 Analysis of protein degradation
Samples were taken from all the samples (1 and 21 days PM), before and after cooking for shear force and compression, for the analysis of muscle protein degradation during the aging period and during cooking.

Sample preparation of muscle myofibrillar and sarcoplasmic proteins for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS -PAGE)

A 0.5 g cube of meat free of any fat or connective tissue was cut from the centre of the raw or the cooked samples. Samples were homogenised for 1 min using a Polytron (bursts of 30 sec each) in a mixture containing 5 mL cold myofibril fragmentation index (MFI) buffer (20 mM potassium phosphate, pH 7.0, containing 100 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM NaN_3), 5µL 1% Triton X100 and 5 µL 100 mM PMSF. The homogenised material was centrifuged at 1500 xg for 20 min at 4°C. The centrifuged supernatant was stored at -20°C for SDS PAGE. The pellet was resuspended in Tris HCl (pH 6.8) and then stored at -20°C for SDS PAGE. Myofibrillar meat protein (MMP) and sarcoplasmic meat protein (SMP) from raw meat samples and MMP from cooked meat (SMP was denatured during the cooking) were used for muscle protein analysis. Protein sample (5 µL of MMP or 10 µL of SMP) was mixed with 5 µL of SDS-PAGE sample buffer (0.01 M sodium phosphate buffer (pH 7.0) containing 5% SDS and 1% 2-mercaptoethanol) and heated in boiling water for 2 min followed by centrifugation at 10,000 g for 15 min. Protein concentration was determined using a Pierce BCA protein assay kit (Pierce Laboratories, Rockford, IL). Samples were diluted to 2.0 mg/mL of total protein using SDS-PAGE sample buffer containing 0.5% (vol/vol) 2-mercaptoethanol and bromophenol blue (0.04%; vol/vol). The proteins were separated using SDS-PAGE mini-gels (4-12% bis-tris Novex gel, Invitrogen). After electrophoresis, gels were stained with 'Simply Blue' colloidal Coomassie (Invitrogen) to visualise protein bands. Unfortunately, some of the enzyme preparation seems to interfere with the protein determination and this resulted in some of the protein bands being under loaded.

3.3.7 Lipid oxidation (Thiobarbituric Acid Reactive Substances) Analysis

Thiobarbituric acid reactive substances (TBARS) at 0 and at the end of the display time (12 and 6 days for 1 day and 21 days PM, respectively) were determined as reported by Bekhit et al. (2005) using the method of Witte et al. (1970), with the modification of Siu and Draper (1978). Thiobarbituric acid reactive substances were calculated as milligrams of malondialdehyde/ kilogram of sample, and the mean of the 3 measurements per sample was used for the statistical analyses. Only 3 samples per time point x treatment were analysed.

3.4 Statistical analysis

Data were analysed using the REML routine in GenStat (GenStat Release 12.2, Lawes Agricultural Trust, VSN Int. Ltd., Rothamsted, U.K.), and the significance of treatment terms and their interactions was determined by Wald tests. In the REML analysis, treatment for change in weight, cooking loss, shear force, compression force and pH, or treatment and display time for colour measurements (L*, a*, b*,C, h and browning index) were set as fixed factors, whereas animal and slaughter day, side, cut and slice were set as random factors using the VCOMPONENTS directive. Model terms were sequentially added to the fixed model to test for fixed effects. Data were subjected to transformation and regression analysis to adjust correlation coefficients for confounding effects of animal, treatment, display time, and their interactions (Welham and Thompson, 1997). Means and SEM were those estimated by the REML routine. For TBARS data, due to differences in display time to reach off colour, the data were analysed as a split-plot repeated measurements design for 1 day and 21 days PM individually, with the meat sections as the whole plot and post-mortem time (1 day vs. 3 wk of vacuum-packaged storage) as the subplot. An α level of 0.05 was used to determine statistical significance.

4 Results and Discussion

4.1 Protein profile of commercial and in-house enzyme preparations

The enzymes were prepared as stock solutions in MilliQ water as follows (papain, bromelain 10 $mg.mL^{-1}$ stock solution; protease G, fungal proteases 31 and 60, commercial actinidin, 100 $mg.mL^{-1}$ stock solution; all other enzyme extracts were as received in liquid form). Ten µL of each enzyme solution was used in the SDS-PAGE analysis. All of the enzyme preparations were more or less crude preparations as a mixture of protein bands were exhibited by all preparations, with the exception of commercial actinidin (Figure 2, lane 5). Bacterial and fungal protease 31K preparations had several proteins in the molecular weight (MW) range of 4-56 kDa with the majority of the proteins at MW <35 kDa (Figure 2, lanes 2 and 3). A less extensive protein profile was found with fungal 60K preparation, with the main protein fraction having a MW of 41 kDa. The specification sheets for bacterial and fungal enzymes do not state the molecular weight of the enzymes; however, the majority of the microbial proteases have a MW between 15 to 40 kDa (Gupta et al., 2002).

Commercial actinidin (lane 5) had some solubility problems, which required separating the enzyme from the other bulking agents. Commercial actinidin had 2 main bands at about 24 and 22 kDa. In addition to these two bands, Kiwifruit juice (lane 6) had another major band at 15 kDa. Kiwifruit may contain several different isoforms of actinidin depending on the variety (Nishiyama, 2007). Papain preparation (lane 7) exhibited an extensive protein profile with the major bands <10 kDa. It is well known that commercial papain preparations contain a mixture of papain, chymopapain and caricain (Adler-Nissen, 1993). A band at about 23 kDa is the most reported for the proteases. Papain, chymopapain and caricain have been reported to have a wide range of MW (23-40 kDa) but differ in their biochemical properties. Asparagus crude extract from the stem and the tip of asparagus (lanes 8 and 9, respectively) exhibited a complex protein profile. Asparagus contains several polymorphism endopeptidases (Brettin and Sink, 1992) which appear to be cysteine proteases (Yonezawa et al., 1998). The enzyme was reported to have a MW of 28 kDa, to have maximum activity at pH 7-7.5 and half maximum activity at pH 5 and pH 10.5, and to be stable up to 50°C (Yonezawa et al. 1998). A very small band corresponding to this MW is visible on the gel, but other isoforms may also be important in any proteolytic activity. The N-terminal sequence of the protease from asparagus had high similarity with that of papain (Yonezawa et al., 1998). The commercial zingibain (lane 11) had 4 bands (approximately 31, 12, 9 and 7 kDa), while crude juice from ginger (lane 12) exhibited a less defined profile, with one band at 31 kDa and the majority of the bands < 10 kDa. The MW of zingibain has been reported to be 34.8 kDa (Bhaskar et al., 2006), which is slightly higher than the values found in the commercial and crude extracts in the present study.

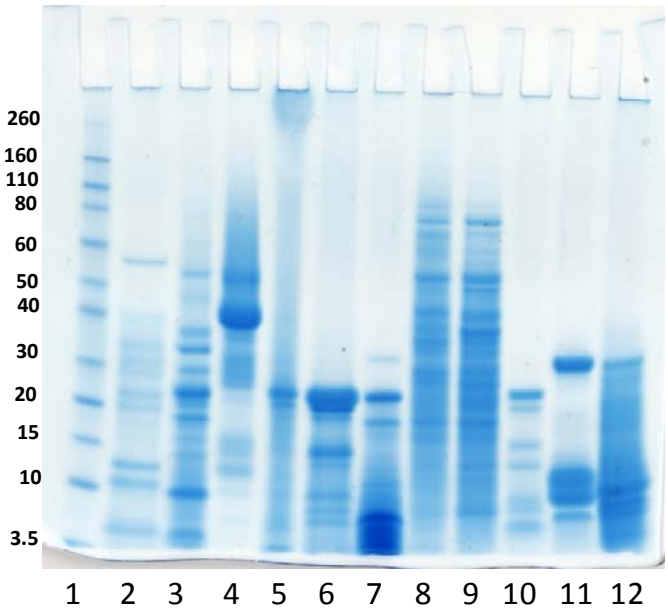

Figure 2. A summary SDS-PAGE of detectable protein components in each protease preparation.

4.2 Characterisation of esterase activities of crude extracts from ginger, pineapple, asparagus and Kiwifruit using CBZ-Lys-p-nitrophenol

Effect of pH

The optimal pH of crude extracts obtained from the plants was 6, 6.5, 7 and 7.5 for actinidin, bromelain, asparagus and zingibain, respectively (Figure 3). Bromelain from stem (EC 3.4.22.32) or from fruit (EC 3.4.22.33) can exist in two isoforms (Ota et al., 1985; Harrach et al., 1998). The reported optimal pH for activity varies (range 5-10) and appears to depend on the substrate, assay temperature and other assay conditions (Murachi, 1976; Harrach et al., 1998; 1995; Khan et al., 2003; Mahmood and Saleemuddin, 2007). Bromelain, using the esterase assay, exhibited maximum activity at pH 6.5 and about 50% of this activity was found at pH 5.0 and 7.0.

Zingibain had an optimum activity at pH 7, which corresponds to the range of pH (6-7) for maximum activity range (Adulyatham and Owusu-Apenten, 2005; Bhaskar et al., 2006).

There are two isoforms for actinidin in Kiwifruit juice, with maximum activities at pH 5 and 7.5 (Figure 3). Actinidin obtained from the green variety (*Actinidia chinensis*) has been reported to have maximum activity at pH 5, while that purified from the golden variety (*Actinidia deliciosa*) exhibited the highest activity at pH 6 (Tuppo et al., 2008). It is worth mentioning that collagenolytic activity of actinidin is pH-dependent with higher collagen hydrolysis ability at pH 7-8.5 compared to 5.5 (Mostafaie et al., 2008). This may be related to the different isoforms that exist in the actinidin extracts.

Asparagus crude extract exhibited reasonably high proteolytic activity and had neutral and basic isoforms that exhibited maximum activities at pH 7 and 8, respectively. Protease from asparagus is a new protease that has not been investigated in detail. A cysteine protease was purified from asparagus and demonstrated to be highly similar to papain. That protease had a maximum activity at pH 7.0 at a temperature of 30°C (Yonezawa et al., 1998). There is no information available in the literature on the effects of this protease on meat quality. The high relative pH for maximum activity (pH= 7.0) compared to normal meat pH (5.5-5.8 for beef) would likely lead to a lower proteolytic activity and the unique flavour of asparagus can be problematic.

However, several processing options are available to de-odour extracts and the lower activity at normal meat pH may be a positive characteristic for controlled protein degradation.

Effect of Temperature

The effect of temperature on the activities of plant crude extracts is shown in Figure 4. The hydrolytic activity (as esterase) in both Kiwifruit juice and asparagus juice increased with the increase in temperature in the range 25-45°C; about a 40% reduction in protease activity was found by increasing to 55°C. Therefore, the optimal temperature for the crude extract should be somewhere between 45°C and 55°C. Purified actinidin exhibits maximum activity at a temperature of 58-62°C (Yamaguchi, 1982) and purified asparagus exhibited maximum activity at 30°C (Yonezawa et al., 1998) using casein as a substrate.

The proteolytic activity in pineapple juice was maximal at pH 6 and a temperature 55°C, but the activity at that temperature was substantially decreased at pH 7. Purified bromelain had maximum activity at 50 - 60°C at pH 5 (Khatoon et al., 2007), which is in agreement with our results, however, higher activity could be obtained at pH 6.

Ginger crude extract exhibited the highest proteolytic activity at pH 7 and 25°C. The maximum proteolytic activity of purified zingibain was reported to be at 60°C (Thompson et al., 1973; Adulyatham and Owusu-Apenten, 2005; Bhaskar et al., 2006) and the activity was decreased to about 75% of the initial activity by heating at 70°C (Thompson et al., 1973). The extract that was obtained from ginger seems to be missing the main zingibain band (the 34.8 kDa band, Figure 2), which probably related to differences in the raw materials used in the present study; therefore, deviation from the conditions that are reported for purified zingibain was expected. As mentioned earlier, several reports documented the change in optimal pH with the change in assay temperature and vice versa (Murachi, 1976; Harrach et al., 1998; 1995; Khan et al., 2003; Mahmood and Saleemuddin, 2007). The obtained data confirm the differences that can be expected during the characterisation of proteases at different purity levels and using different substrates.

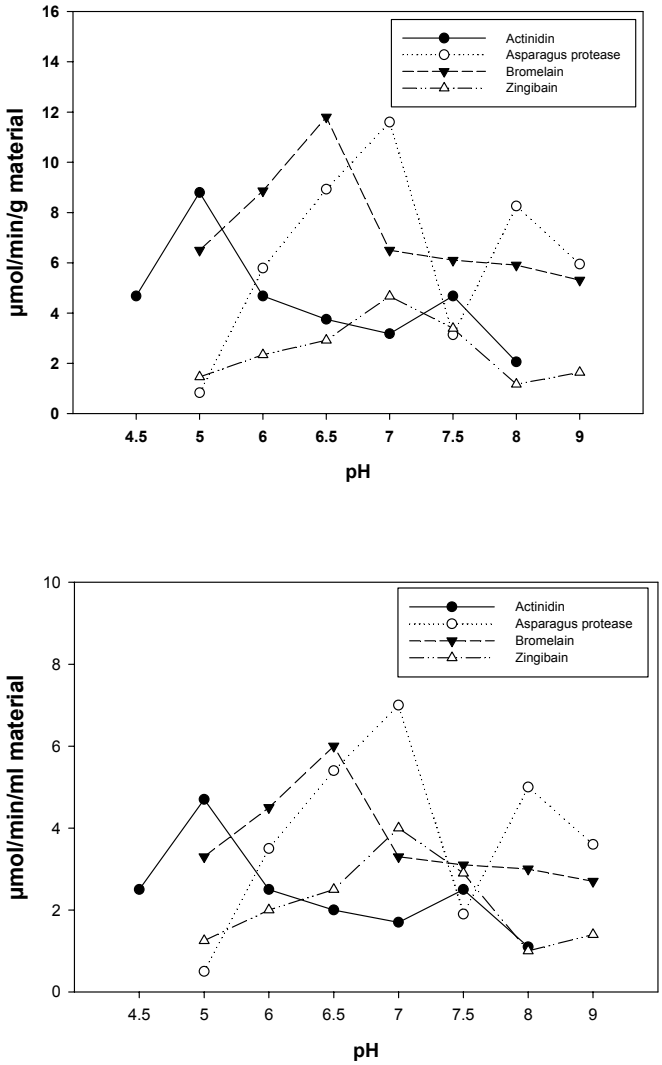

Figure 3. Hydrolytic activity (as esterase) of crude extracts obtained from fresh Kiwifruit, asparagus, pineapple and ginger in pH range of 4.95-8.95. The enzymatic activity was measured at 20°C as outlined in section 3.2.1.

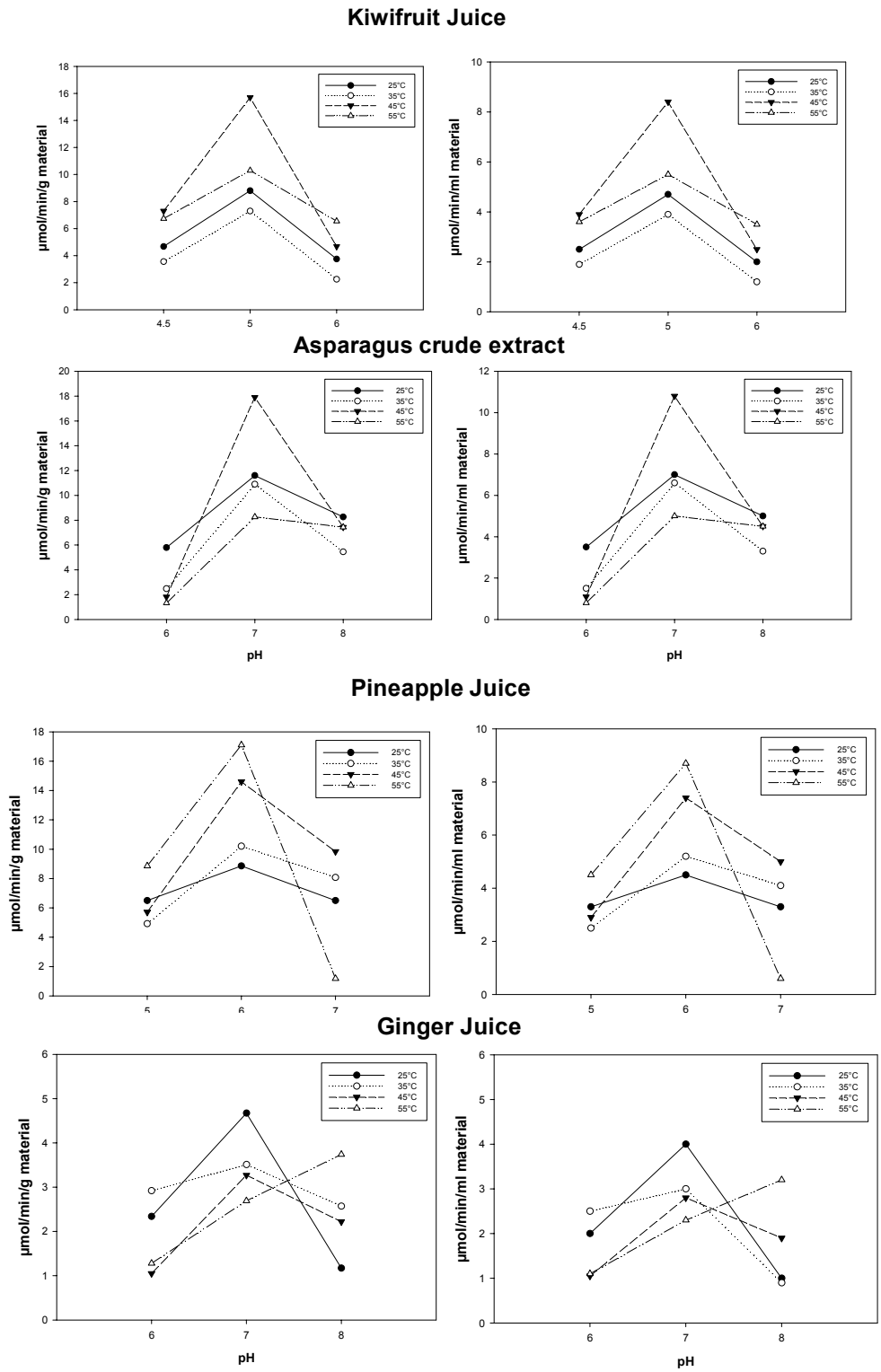

Figure 4. Hydrolytic activity (as esterase) of crude extracts obtained from fresh Kiwifruit, asparagus, pineapple and ginger at pH range of 4.5-8 and temperature range of 25-55°C.

4.3 Characterisation of the Hydrolytic activity (as esterase) of commercial enzymes and in-house preparations of asparagus and Kiwifruit using CBZ-Lys-p-nitrophenol

The hydrolytic activities of papain, bromelain, zingibain, in-house Kiwifruit crude extract, and in-house asparagus crude extract under different pH (5.0-7.0) and temperature (25-85°C) conditions are shown in figures 5. This range encompasses the realistic limits for meat pH and various temperatures that the meat is exposed to during the early stages of cooking. The bacterial, fungal and commercial actinidin did not work well with this assay (up to 100mg/mL). The optimal activity of papain was observed at pH 6 and 75°C. Several proteases may exist in commercial papain preparations. For example, papain (EC 3.4.22.2), chymopapain (EC 3.4.22.6) and caricain (EC 3.4.22.30) can be found in *Carica papaya* and have different optimal temperatures and pH for maximum activities (Brenda, 2010; Diaz-Mochon et al., 2009; Schwimmer, 1981). The hydrolytic activity was the same at 25°C across the pH range examined, and increased about 4 fold at 75°C but was not detectable at 5°C.

Bromelain maximum hydrolytic activity was found to occur at pH 6 and 55°C using CBZ-Lys-p-nitrophenol as a substrate (Figure 5). Per mass unit, bromelain had about 7% of the hydrolytic activity of papain.

The maximum activity of zingibain was found at pH 6.0 and 65°C and there was a tendency toward another peak at pH ≤ 5.0. The crude extract of ginger is reported to contain 2 cysteine protease enzymes with a molecular mass of 29 and 31 kDa (Choi et al., 1999; Su et al., 2009) or 3 enzymes displaying hydrolytic activity with the same molecular mass (29 kDa) (Ohtsuki et al., 1995).

The in-house Kiwifruit crude extract demonstrated two peaks of optimal activity at pH 5.5 and 55°C, and pH 6.5-7 at 75°C, which indicates the presence of at least two different enzymes that are activated under different conditions. There are 6 possible actinidin proteases, all of which have similar molecular weights (23.5 kDa). The actinidin pH stability range is 7-10 and the optimal activity is in the pH range of 7.3-7.6 and a temperature range of 58-62°C (Yamaguchi, 1982). However, pH 5-7 has also been reported (Boyes et al., 1997), reflecting differences in the cultivar and the assay conditions used for estimating hydrolytic activity. The activities in Figure 5 for in-house Kiwifruit crude extract demonstrate the existence of two proteases, which are activated differently and may be related to the differential collagenolytic activity of actinidin at pH 7-8.5 compared to 5.5 reported by Mostafaie et al. (2008).

The in-house crude asparagus extract exhibited a double peak curve with maximum activities at 45 and 75°C. The activities of this fraction were much lower than the initial plant sample (Figure 2), possibly due to oxidation during the extraction of large batches. An optimization of the extraction process would require further research. A summary of the optimal pH and temperature conditions for maximum activities of the examined preparations is shown in table 4. The hydrolytic activity of papain was the highest and it was approximately 15 times that of bromelain.

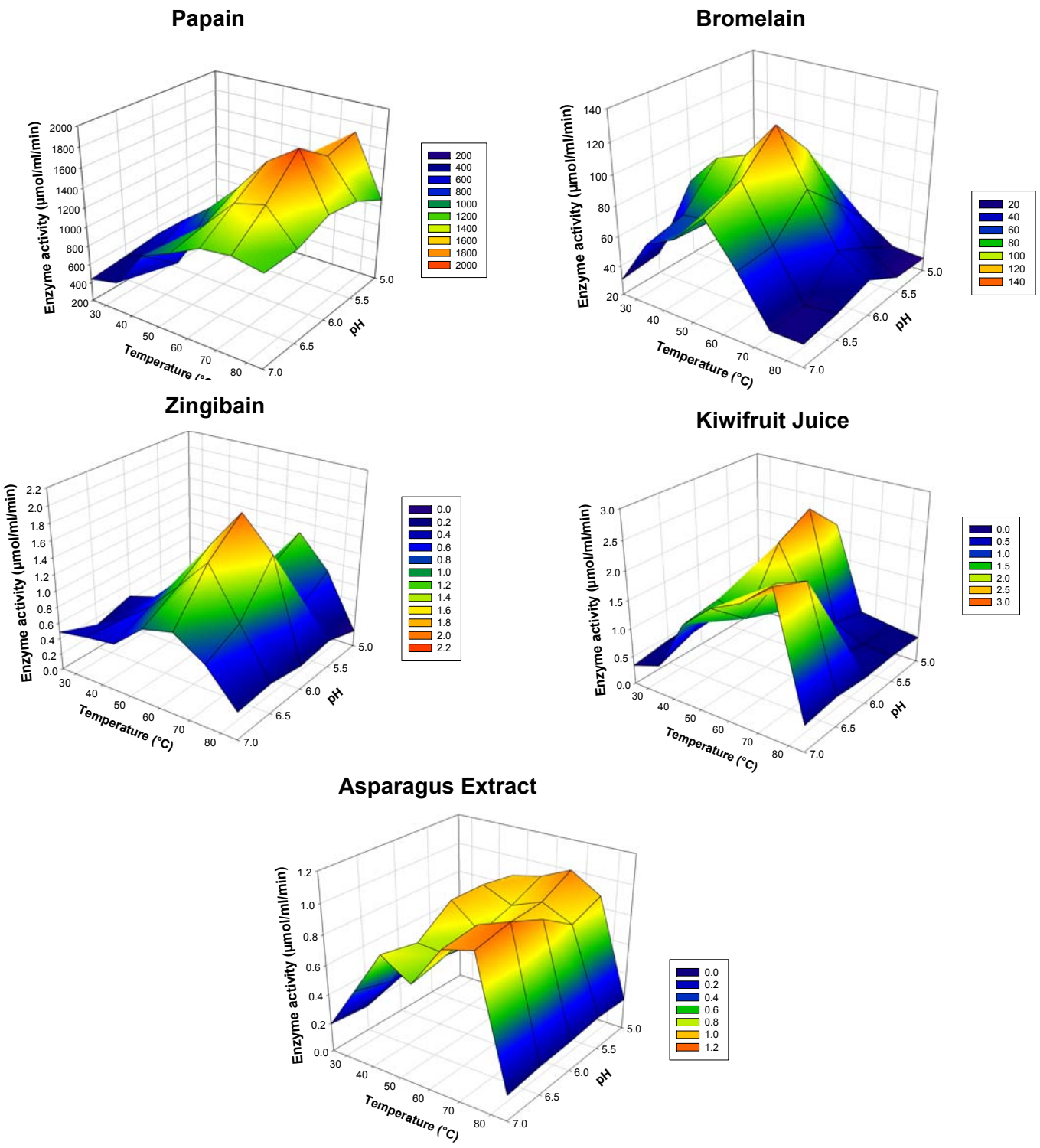

Figure 5. Hydrolytic activity (as esterase) of commercial papain, bromelain, commercial zingibain and in-house preparation of Kiwifruit juice and Asparagus extract at pH range of 5.0- 7.0 and temperature range of 25-85°C.

Table 4. Summary of optimum pH and temperature conditions for maximum hydrolytic activity of commercial papain; bromelain; and zingibain; and in-house crude extracts of Kiwifruit and asparagus investigated under different pH (5.0-7.0) and temperature (25-85°C) conditions using CBZ-Lys-p-nitrophenol. The hydrolytic activity is expressed as µM/g/min for papain and bromelain or µM/ml/min for zingibain and in-house preparations. The activity of these enzymes over the pH range commonly found in meat (5.5-6.0) is also reported.

Protease source	pH and temp	Activity	Activity in meat pH range (5.5-6.0) and (temperature)
Papain	6 and 75°C	1855.6	1210-1855.6 (55-85°C)
Bromelain	6 and 55°C	124.8	90- 124.8 (35-65°C)
Zingibain	6 and 65°C	1.9	1.0-1.9 (55-75°C)
Kiwifruit crude extract	5.5 and 55°C 6.5-7.0 and 75°C	2.6 2.5	2.6 (55°C)
Asparagus crude extract	5.5-6.0 and 45°C 6.0-7.0 and 75°C	0.91 1.02	0.8- 0.95 (45- 75°C)

4.4 Characterisation of the caseinolytic activity of commercial proteases and in-house preparations of asparagus and Kiwifruit using BODIPY-FL Casein

The plate reader has a maximum incubation temperature of 45°C for controlled fluorescence measurement. The 55°C readings were carried out by incubating the plates for different times at 55°C and inhibiting the enzyme and then measuring the fluorescence of the samples. Papain had the highest measured caseinolytic activity at pH 7.5 and 55°C and this trend continued at higher temperatures where maximum activity could be expected (Figure 6). Bromelain exhibited the highest measured caseinolytic activity at pH 5.5 and 55°C and this trend, similar to papain, continued, indicating an optimum temperature for maximum activity of > 55°C (Figure 6).

Fungal 31K exhibited the highest caseinolytic activity at pH 5.5 and 45°C (Figure 6). Similarly, fungal 60K exhibited the highest activity at pH 6.5-7.0 and 35-45°C (Figure 6). Bacterial protease G exhibited maximum caseinolytic activity at pH range 7.5- 9.0 at 45°C.

While maximum caseinolytic activity of zingibain was achieved at pH 6.5 and 7.0 and at a temperature of 55°C, the protease activity at other pH values appears to require > 55°C for maximum activity (Figure 6).

Commercial actinidin had maximum caseinolytic activity at pH range of 4.5-5.5 and at a temperature of 45°C (Figure 7); whereas, in-house Kiwifruit crude extract exhibited a more complex trend, possibly due to having different isoforms of actinidin (Figure 7). It appears that 45°C is the optimal temperature for the proteases in the crude extract, with higher activities being commonly found in two pH zones (5.0-6.5 and 7.5-8.0). The Kiwifruit enzyme preparation was the only one that showed significant activity at 5°C (about 30% of the maximum activity).

The caseinolytic activity of asparagus crude extracts is shown in Figure 7. The highest activities were found at pH >7.5 and at >45°C. A cysteine protease from asparagus has been reported to have an optimum pH of 7.0-7.5 and was stable up to 50°C (Yonezawa et al., 1998).

The results from these assays (Table 5) indicate that most the enzyme activation occurs at temperatures >35°C and that most proteases are inhibited by 55°C, with the exception of papain, bromelain, zingibain and asparagus protease. Furthermore, the data shows that, relative to the maximum activity under optimal conditions, caseinolytic activity of these preparations at 5°C was negligible, with the exception of the in-house Kiwifruit preparation.

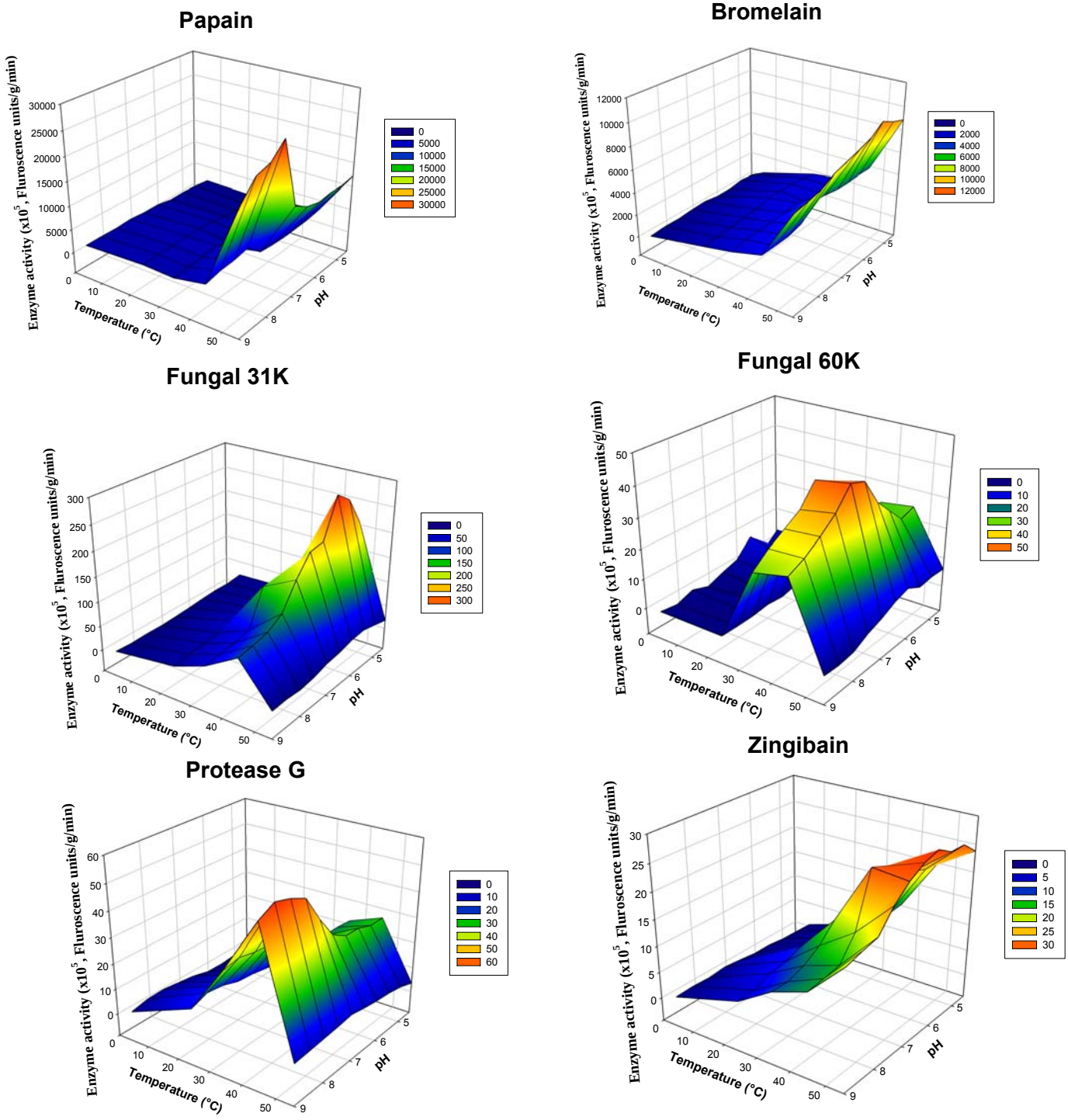

Figure 6. Proteolytic (as caseinolytic) activity of commercial papain, bromelain, fungal 31K protease, fungal 60K protease, bacterial protease G and zingibain at pH range of 4.5-9.0 and temperature range of 5-55°C.

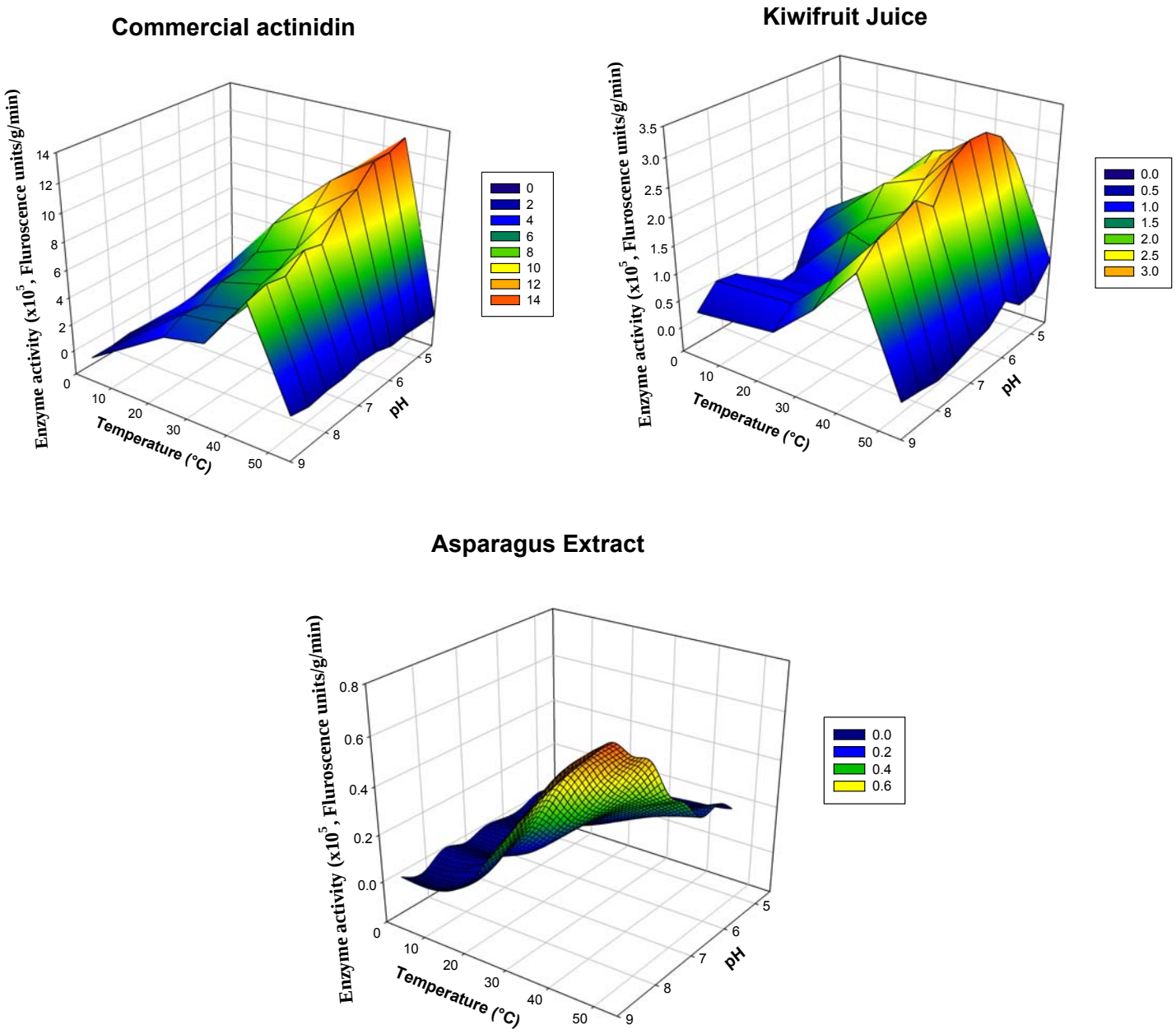

Figure 7. Proteolytic activities (as caseinolytic) of commercial actinidin, in-house Kiwifruit juice and in-house asparagus extract preparation at pH range of 4.5-9.0 and temperature range of 5-55°C.

Table 5. Summary of optimum pH and temperature conditions for maximum proteolytic activity of commercial papain; bromelain and zingibain; and in-house crude extracts of Kiwifruit and asparagus investigated under different pH (4.5-9.0) and temperature (5-55°C) conditions using BODIPY-FL Casein. Protease activity is expressed as fluorescence units/g/min ($\times 10^5$) for papain and bromelain or fluorescence units/g/min ($\times 10^5$) for zingibain and in-house preparations. The activity of these enzymes over the pH range commonly found in meat (5.5-6.0) is also reported.

Protease source	pH and temp	Activity	Activity in meat pH range (5.5-6.0) and (temperature)
Papain	7.5 and >55°C	28593.1	2331-12011.5* (45-55°C)
Bromelain	5.5 and >55°C	9826.7	3818.2-9826.7* (45-55°C)
Fungal 31K	5.5 and 45°C	283.6	208.7-283.6 (45°C)
Fungal 60K	6.5-7.0 and 35-45°C	39.5-44.0	27-33 (35-45°C)
Bacterial protease G	7.5- 9.0 and 45°C	51.5- 56.1	21.8- 28.6 (35-45°C)
Zingibain	6.5- 7.0 and 55°C Other pH, > 55°C	25.6-27.2 > 21.0- 28.1	21.3 -28.1* (45-55°C)
Commercial actinidin	4.5-5.5 and 45°C	12.4- 12.9	10.4- 12.4 (35- 45°C)
Kiwifruit crude extract	5.0- 6.5 and 55°C 7.5- 8.0 and 555°C	2.9- 3.2 2.5- 2.7	3.2 (45°C)
Asparagus crude extract	>7.5 and > 45°C	0.62- 0.72	0.19- 0.30* (45°C)

*** = enzyme did not reach its optimal temperature for maximum activity**

4.5 Characterisation of the collagenolytic activities of commercial proteases and in-house preparations of asparagus and Kiwifruit using Azocoll

The ability of the tested enzymes to hydrolyse connective tissue (specifically collagen) was investigated using the Azocoll assay. The higher the absorbance at wavelength 520nm indicates a higher collagenolytic activity. With the exception of zingibain, all the enzymes achieved near maximum hydrolysis of Azocoll by the first hour of incubation at 45°C (Figures 8 and 9). The activity at 5, 55, 65 and 75°C at 3 and 12 hours was further investigated (Figure 10). Incubation temperature increased the collagenolytic activity of the enzymes with maximum activity for bromelain, fungal 31K and zingibain occurring at 65°C, and maximum activity for papain and commercial actinidin occurring at 75°C. Generally, the incubation time did not show any significant effect on the activity of the enzymes, with the exception of fungal 31K at 55°C. The collagenolytic activity of the enzymes was in the following order; bromelain = fungal 31K = papain > zingibain> commercial actinidin = fungal 60K = Kiwifruit crude extract = asparagus crude extract.

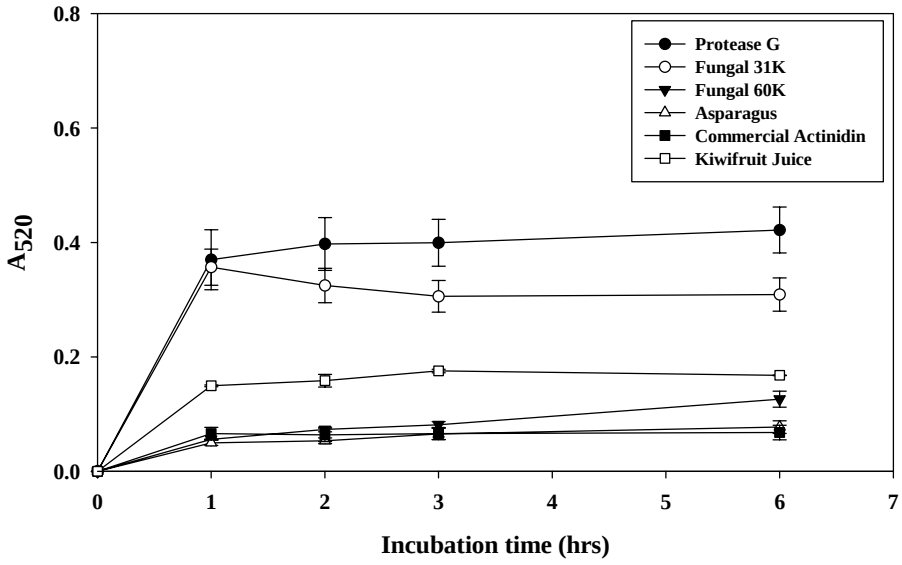

Figure 8. Proteolytic activity (as collagenolytic) of commercial enzymes (protease G, Fungal 31K, Fungal 60K and actinidin) and in-house preparations of asparagus and Kiwifruit over 6 hrs of incubation at 45°C. Error bars are the standard deviation of the means.

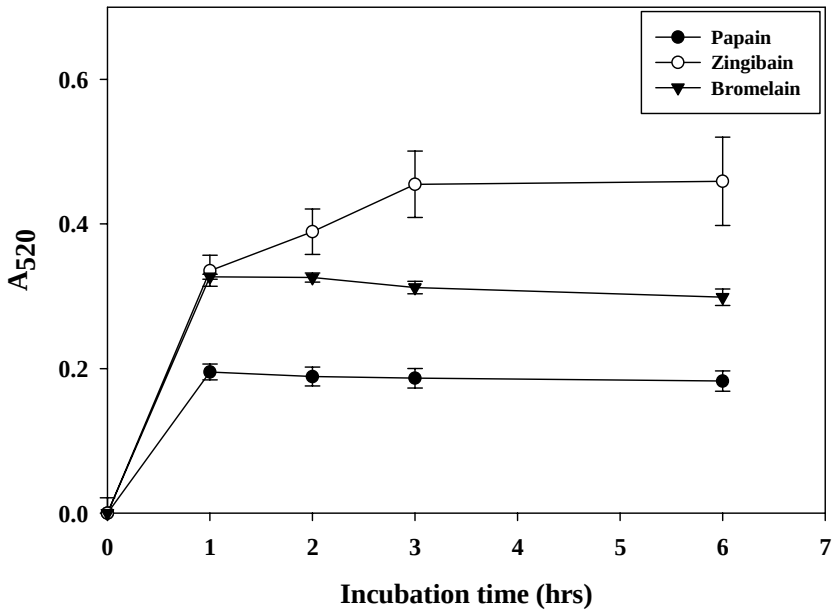

Figure 9. Proteolytic activity (as collagenolytic) of commercial enzymes (bromelain, papain and zingibain) over 6 hrs of incubation at 55°C. Error bars are the standard deviation of the means.

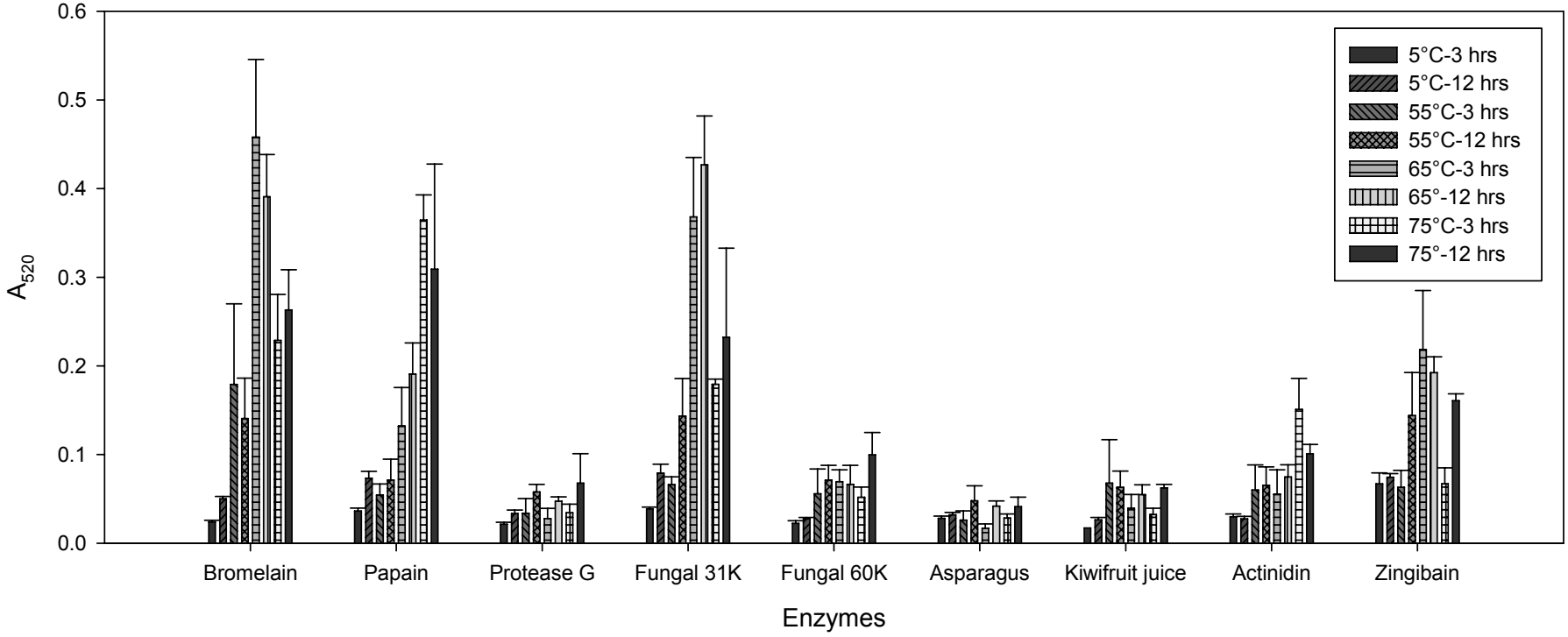

Figure 10. Proteolytic activity (as collagenolytic) of commercial enzymes (bromelain, papain, zingibain, protease G, Fungal 31K, Fungal 60K and actinidin) and in-house preparations of asparagus and Kiwifruit after 3 and 12 hrs of incubation at 5, 55, 65 and 75°C. Error bars are the standard deviation of the means

4.6 Determination of V_{max} and K_m kinetic data for the protease enzyme extracts

Assays were performed with a range of BODIPY-casein substrate concentrations and with incubation at 45°C at pH 6.0 to obtain initial velocity data to construct Michaelis-Menten, Lineweaver-Burke and Eadie-Hoffstee plots (Appendix A). A summary of the calculated V_{max} and K_m values is shown in (Table 6). The V_{max} and K_M data relate specifically to casein hydrolysis. The interpretation of these values in relation to the observed activities with other assays is reported below for individual enzymes.

Papain – relatively high V_{max} and low K_M, indicating high binding affinity to casein and rapid hydrolysis – supported by SDS-PAGE meat myofibrillar (MM) hydrolysis data (please see section 4.8 below), and also Azocoll data where there was an indication that papain efficiently hydrolysed the collagen.

Bromelain - relatively very low V_{max} but with a K_M significantly lower than that of papain indicating very high binding affinity to casein but the conversion of the substrate is much lower than papain – this may explain the lower but still significant hydrolysis of MM as detected by SDS-PAGE – bromelain also hydrolysed Azocoll relatively efficiently.

Fungal protease 31K - relatively very high V_{max} and relatively very high K_M, indicating low binding affinity to casein but with very good hydrolysis (conversion of the substrate) which overall indicates poor hydrolysis unless very high concentrations used – supported by SDS-PAGE MM hydrolysis data indicating poor hydrolysis at relatively high E/S ratios- Azocoll data showed a relatively significant hydrolysis of collagen.

Fungal protease 60K - relatively low V_{max} and relatively high K_M, indicating modest binding affinity to casein and slow hydrolysis – this is supported by SDS-PAGE MM hydrolysis data and also by Azocoll data which showed a relatively slow but continuous hydrolysis of collagen.

Protease G - relatively high V_{max} and relatively high K_M, indicating lower binding affinity to casein and but with relatively good hydrolysis – supported by SDS-PAGE MM hydrolysis data indicating significant hydrolysis but not as extensive as the protein fragmentation seen with other enzymes - and also by Azocoll data which showed a relatively significant hydrolysis of collagen.

Commercial zingibain – relatively high V_{max} and relatively low K_M, indicating modest binding affinity to casein and but with relatively good hydrolysis – SDS-PAGE MM hydrolysis data indicate relatively poor hydrolysis - Azocoll data showed a relatively significant hydrolysis of collagen.

Commercial actinidin – relatively low V_{max} and modest K_M, indicating lower binding affinity to casein and relatively slow hydrolysis – supported by SDS-PAGE MM hydrolysis data requiring relatively high amount of enzyme to achieve hydrolysis and also by Azocoll data which showed relatively slow hydrolysis of collagen.

In-house Kiwifruit extract – relatively high V_{max} and modest K_M, indicating lower binding affinity to casein and relatively slow hydrolysis – supported by SDS-PAGE MM hydrolysis data and also by Azocoll data which showed a relatively slow hydrolysis of collagen but at about twice the rate of the commercial actinidin.

'In house' asparagus extract - relatively low V_{max} and relatively low K_M, indicating higher binding affinity to casein and but relatively slow hydrolysis – supported by SDS-PAGE MM hydrolysis data

and also by Azocoll data which showed a relatively slow hydrolysis of collagen but which continued for some time.

'In house' ginger extract – V_{max} and K_M were not determined. SDS-PAGE MM hydrolysis data indicate relatively poor hydrolysis - Azocoll data showed a relatively significant hydrolysis of collagen.

Table 6. Summary of V_{max} and K_m kinetic data for the protease enzyme extracts obtained using Michaelis-Menten, Lineweaver-Burke and Eadie-Hoffstee plots.

Enzyme preparation	V_{max} (ΔFU/sec)			K_m (μM)		
	LW	MM	EH	LW	MM	EH
Papain	172.41	180.0	162.3	0.190	0.180	0.166
Bromelain	5.562	6.0	5.50	0.012	0.012	0.012
Fungal 31K	344.83	100.0	204.25	7.241	1.0	3.614
Fungal 60K	19.72	18.0	21.29	1.448	1.25	1.618
Protease G	106.38	70.0	106.47	2.234	1.0	2.22
Zingibain	178.57	180.0	182.34	0.518	0.50	0.518
Commercial actinidin	24.9	25.0	26.5	0.761	0.70	0.839
Kiwifruit crude extract	147.06	120.0	140.34	1.353	0.90	1.272
Asparagus Crude extract	8.84	12.0	9.35	0.115	0.25	0.128

LW = Lineweaver-Burke MM = Michaelis-Menten EH = Eadie-Hoffstee

V_{max} derived from Michaelis-Menten plots is an approximation to the hyperbolic asymptote which can have some error. An error is then associated with $K_m = V_{max}/2$. V_{max} and K_m derived from double reciprocal Lineweaver-Burk plot also can result in error. Eadie-Hofstee plot is often a more accurate representation of V_{max} and K_m.

4.7 Determination of Arrhenius constant (k_0), activation energy (E_A) and temperature coefficient (Q_{10}) of commercial proteases and in-house preparations of asparagus and Kiwifruit using esterase and caseinolytic activity

The ability to describe the rate of reactions through the Arrhenius model enables estimation of the effects of temperature on the rate of reactions. For enzymes, the relationship is applicable within a limited temperature range due to enzyme susceptibility to denaturation at higher temperatures, and the requirement to reach a certain temperature before activation. Arrhenius equation (below), is a relationship between rate of reaction (K,s^{-1}) and the reaction temperature (T, K) that can easily be used to predict the potential rates of reaction at different temperatures once the constants of the equation are established. K_0 is Arrhenius constant (also known as frequency factor, s^{-1}); E_A is the activation energy (kJ mol^{-1}) and R is the constant gas coefficient (8.314 x 10^{-3} kJ $mol^{-1}K^{-1}$).

$$k = k_0 e^{-\left(E_A / RT\right)}$$

An important condition for applying Arrhenius equations to enzyme kinetics is to determine and use the equations within a valid range of temperatures where enzyme activity is not compromised by the temperature (not below the level required for providing the activation energy and not too high to cause denaturation). The rates of reaction for the enzymes (using both assays 3.2.1.1 and 3.2.1.2, M & M section) were determined within their valid range of temperatures (35-55°C, unless the enzyme is denatured at 55°C) and used to generate the Arrhenius plots shown in Figures 11 and 12. The rates of reaction were also used to determine the temperature coefficients (Q_{10}), which are shown in Figures 13 and 14. Because of the nature of enzymatic reactions which involve change in molecular configuration, inactivation of enzymes and dissociation of enzyme-substrate complex as energy is added to the system; Q10 cannot be used over wide temperature range. Furthermore, most of the enzyme preparations investigated contained more than one protease which can complicate the reaction kinetics. For simplicity, the assumption of one reaction rate is used for the calculations since the end product was the key point of determining the kinetics. Generally speaking, Q_{10} in biological systems decreases with the increase in temperature and the decrease in E_A. Using the BODIPY-FL casein assay, Q_{10} of all the enzymes decreased with the increase in temperature, with the exception of bromelain and papain as they move toward their optimum temperatures. It is widely assumed that Q_{10} for enzymatic reactions is generally between 1.2 and 2.5, except when denaturation is occurring where it could be between 6 and 36. The large decrease in Q_{10} of fungal 60K is possibly reflecting a denaturation of the enzyme. Using CBZ-Lys-p-nitrophenol, a high Q_{10} for Kiwifruit crude extract, commercial actinidin, and asparagus crude extract, was found within the temperature range 25-45°C, followed by rapid decrease when the temperature was increased to 55°C.

Arrhenius constants (K_0) and E_A from BODIPY-FL casein and CBZ-Lys-p-nitrophenol assays are shown in table 7. The feasibility of a reaction is governed thermodynamically. This means that for a reaction to be able to occur, the energy changes must be favourable and this energy is reflected by the minimum energy required for the reaction (E_A). The lower the E_A, the easier for the reaction to occur but in a biochemical sense this interpretation may have certain limits that may affect the outcome. For example, by increasing the temperature, the average increase in particle kinetic energy caused by the absorbed heat means that a much greater proportion of the reactant molecules now have the minimum activation energy to react and thus, a lower E_A will be required for reactions. The results from both assays produced conflicting outcomes in terms of activation energy and Arrhenius constants (Table 7A and 7B). While low E_A values were found for Kiwifruit crude extract, asparagus crude extract, and commercial actinidin, and higher E_A values were required for papain, bromelain and zingibain with BODIPY-FL casein, the opposite was found with CBZ-Lys-p-

nitrophenol (with the exception of zingibain). Hydrolysis reactions of proteins are dictated by several factors (e.g. % protein in the reaction mixture, enzyme/substrate ratio per mass unit of protein, pH and temperature, as well as the specificity and properties of the enzyme and substrate (Nielsen and Olsen, 2002). While some differences in the Arrhenius equation constants were expected due to the different substrates used, the current results using synthetic substrates (which are commonly used in characterizing enzymes) don't give clear indications. Furthermore, the complex structure of meat proteins compared with these synthetic substrates may limit the interpretation of the results in the context of substrate used. Therefore, the use of meat protein components (e.g. myofibrils and collagen) was used in subsequent analyses.

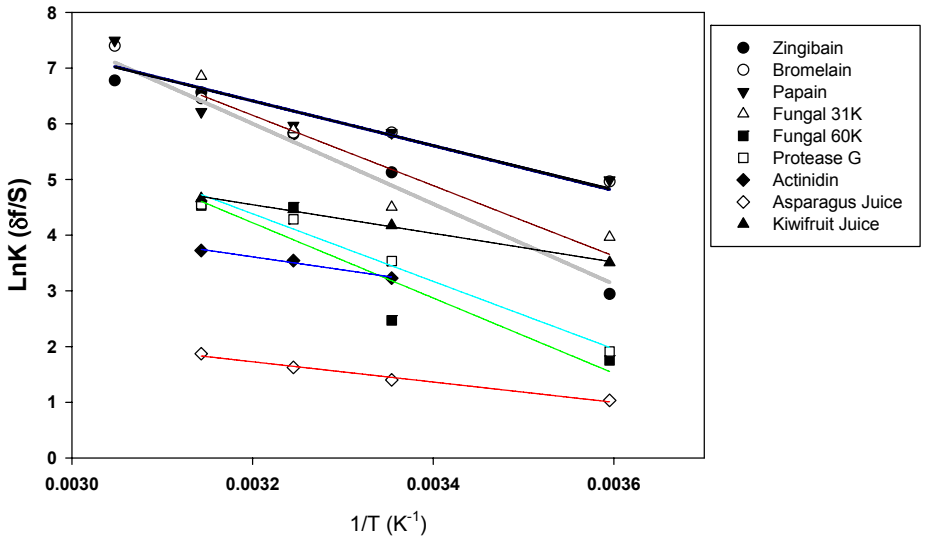

Figure 11. Arrhenius plots of commercial enzymes (bromelain, papain, zingibain, protease G, Fungal 31K, Fungal 60K and actinidin) and in-house Kiwifruit and asparagus preparations at temperature range of 5-55°C using BODIPY-FL-Casein system.

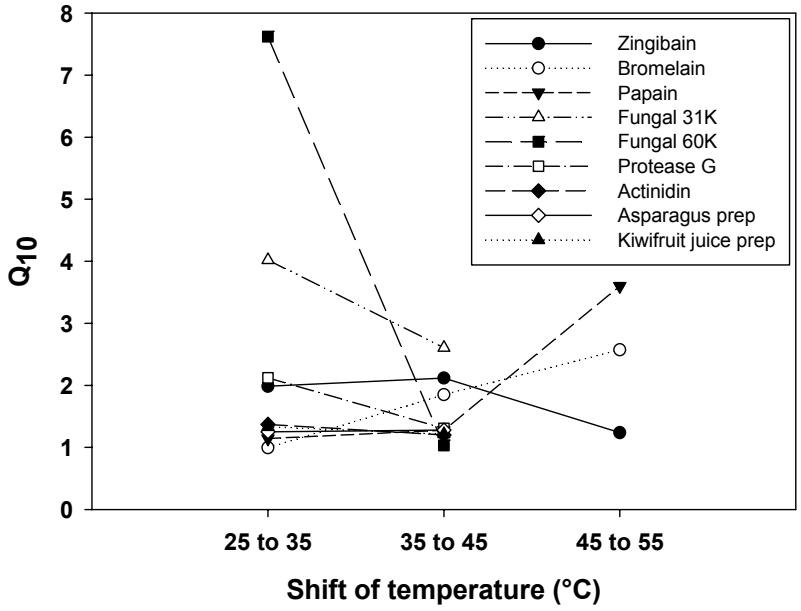

Figure 12. Effect of temperature on the temperature coefficient (Q_{10}) of commercial enzymes (bromelain, papain, zingibain, protease G, Fungal 31K, Fungal 60K and actinidin) and in-house preparations of Kiwifruit and asparagus at temperature range 5-55°C using BODIPY-FL-Casein system.

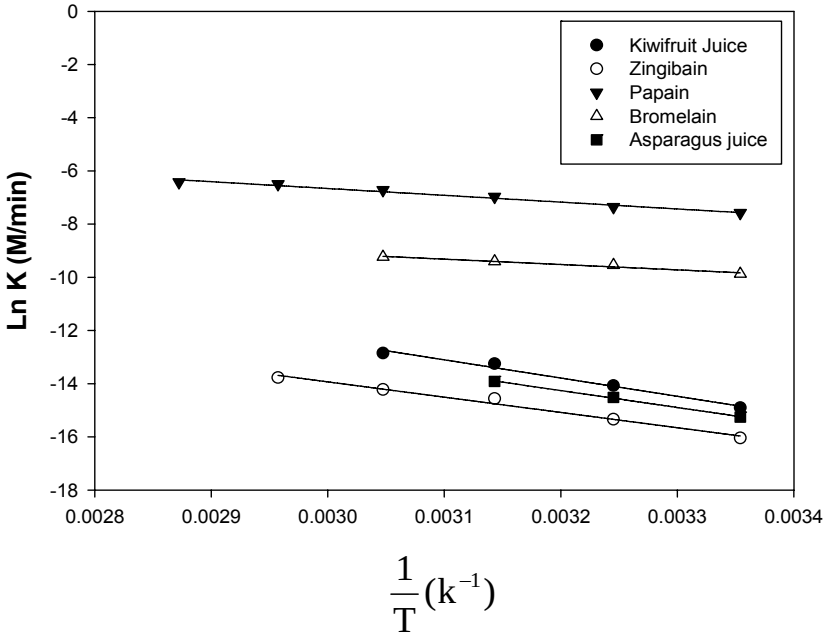

Figure 13. Arrhenius plots of commercial enzymes (bromelain, papain and zingibain,) and in-house preparations of Kiwifruit and asparagus at temperature range 25-75°C using CBZ-Lys-p-nitrophenol system.

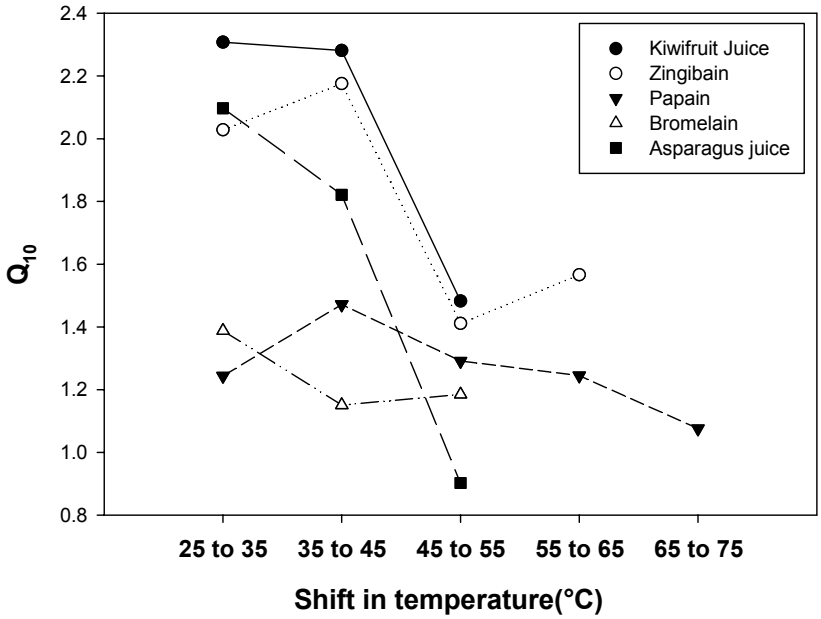

Figure 14. Effect of temperature on the temperature coefficient (Q10) of commercial enzymes (bromelain, papain and zingibain) and in-house preparations of Kiwifruit and asparagus at temperature range 5-55°C using CBZ-Lys-p-nitrophenol system.

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Table 7. Arrhenius coefficient (K_o) and Activation energy (E_A) values of commercial enzymes (bromelain, papain, zingibain, protease G, Fungal 31K, Fungal 60K and actinidin) and in-house preparations of Kiwifruit and asparagus. The values were generated using Arrhenius plots for the enzymatic activity using BODIPY-FL casein (A) or CBZ-Lys-p-nitrophenol system (B).

A

Enzyme prep	Temperature(°C)	K_o (1/s)	E_A kJ
Papain	5-55	$1.84x10^8$	32.8
Bromelain	5-55	$2.4x10^8$	33.5
Fungal 31K	25-45	$2.84x10^{11}$	52.5
Fungal 60K	5-45	$1.68x10^{11}$	56.18
Protease G	5-45	$2.17x10^{10}$	50.45
Zingibain	25-55	$2.6x10^{10}$	46.61
Kiwifruit crude extract	25-45	$6.92x10^4$	19.57
Asparagus crude extract	5-45	$1.9x10^3$	15.13
actinidin	5-45	$3.6x10^5$	21.43

B

Enzyme prep	Temperature(°C)	K_o (mol/min)	E_A (kJ/mol)
Papain	**25-75**	**2.8**	**21.3**
Bromelain	**25-55**	**21.4**	**16.8**
Zingibain	**25-65**	**27.4**	**47.8**
Kiwifruit juice	**25-55**	**$3.5x10^3$**	**57.1**
Asparagus	**5-45**	**442.0**	**52.9**

4.8 Determination of proteolytic activity of commercial proteases and in-house preparations of asparagus and Kiwifruit using meat myofibril degradation and SDS-PAGE

This research project is directed at characterising the ability of protease enzyme extracts to tenderise meat. Meat myofibrils are a useful material which can provide insight into the proteolytic capability of enzymes prior to analysing their function in injected whole meat samples. Initially, 45°C was chosen for the assay incubation as this was near the optimum temperature for most of the enzymes, based on the aforementioned enzyme assay data. The hydrolysis assays were performed at pH 6.0 as it is reported that the pH of meat is typically in the vicinity of pH 5.5 - 6.0. It is also anticipated that protease meat tenderising may be affected by the cooking method and temperature (fast vs. slow), which will determine the temperature-time window for the enzyme action.
The initial focus was directed at generating limited time-course meat myofibril hydrolysis displays that would provide information about the kinetics of proteolysis of individual meat myofibril proteins for each protease preparation. It is intended that subsequent analysis of meat protein hydrolysis fragments will be performed by mass spectrometry to provide molecular-level information with regard to the time-course and specificity of meat protein hydrolysis achieved by each protease enzyme (part of the MSc thesis supported by this project; due to be submitted in March 2011).

4.8.1 *Degradation of meat myofibrils (MM) with commercial and in-house proteases*

The efficacy of the proteases in degrading meat myofibrils is shown in Figure 15. The enzyme concentrations used initially were: papain at 40 µg/mg MM; protease G, fungal 31K, fungal 60K and commercial actinidin at 400 µg/mg MM, and zingibain and in-house preparations were at 40 µl/ mg MM.
The protein profile, without heating (lane 2) (Figure 15) and after heating at 45°C for 2 hours (lane 3), shows the expected myosin heavy chain (MHC), α-actinin, actin, troponin, β-tropomyosin, α-tropomyosin, myosin light chain (MLC) and troponin C . No apparent change in the protein profile was found after 2 h of incubation at 45°C (lane 3), indicating that any change in the profile with subsequent treatments will be solely due to the exogenous proteases used. Lanes 4-6 and 8-12 show major changes in MM protein profile due to protease hydrolysis after 2 h incubation for protease G (lane 4), fungal 31K (lane 5), in-house preparation Kiwifruit crude extract (lane 8) and papain (lane 9). It is evident that some proteases appear to be more active towards hydrolysis of MM than others (protease G > papain > in-house Kiwifruit crude extract > fungal 31K) under the experimental conditions (stated concentration used and incubation temperature and time). These enzymes were effective in hydrolysing all the major structural proteins. Limited hydrolysis was found with fungal 60K and commercial actinidin, with the main action targeting myosin. Asparagus crude

extracts (extracted by two different methods) showed very limited or no effect on MM proteins. The efficacy of fungal 60K, commercial actinidin, and asparagus crude extracts, may be limited due to the time of incubation and a longer time may be required for maximum activity. This suggestion was further investigated in section 3.6.1.1. below.

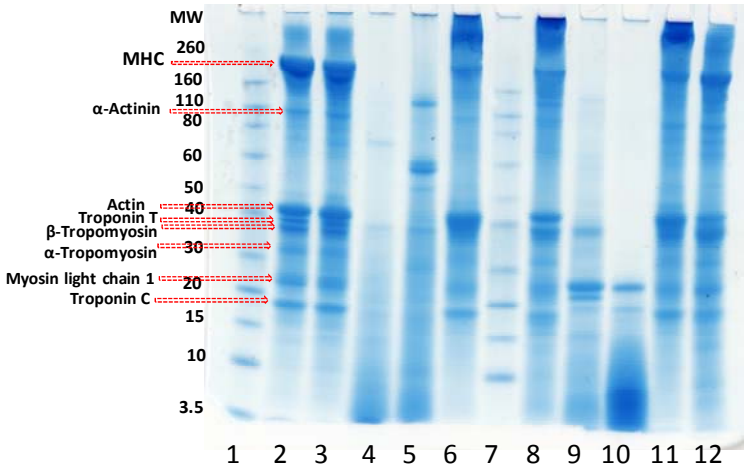


Figure 15. Meat myofibrils (MM) degradation profile with papain at a concentration of 40 µg/mg MM (E/S=0.1), protease G, fungal 31K, fungal 60K and commercial actinidin at 400 µg/mg MM (E/S=1), and zingibain and in-house preparations at 40 µl/ mg MM. The enzyme preparation and incubation condition are those in section 2.3. E/S is enzyme to substrate ratio

4.8.1.1. Effect of incubation time on the degradation of meat myofibrils (MM) with fungal 60K and commercial actinidin and in-house asparagus crude extracts.

The time-course for the degradation of meat myofibrils (MM) with fungal 60K and commercial actinidin (Figure 1A, Appendix A) and in-house asparagus crude extracts (Figure 1B, Appendix A) reveal that these enzymes have different target proteins compared with other tested proteases. Fungal 60K, commercial actinidin, and asparagus proteases initiate their protein hydrolysis by fast degradation of myosin (both MHC and MLC) with slow hydrolysis on α-actinin and actin (slightly better with commercial actinidin and worse with Asparagus crude extract). After 16 hours of incubation, commercial actinidin was very effective in degrading all MM proteins (figure 1A, Appendix A). These actions of fungal 60K and asparagus indicate that they produce limited hydrolysis, which can be beneficial in meat tenderisation in terms of avoiding over-tenderisation and mushy textures.

4.8.1.2. Effect of incubation time on the degradation of meat myofibrils (MM) with in-house asparagus crude extract 1 and commercial bromelain.

The time-course for the degradation of meat myofibrils (MM) with in-house asparagus crude extract and commercial bromelain is shown below (Figure 2, Appendix A). Within the first two hours of incubation at 45°C, bromelain degraded all the protein except actin, which was subsequently degraded at slower rate. On the other hand, gradual and limited degradation of myosin occurred over 16 hours of incubation with asparagus extract while no major changes occurred with actin. This selective degradation of proteins at different rates may offer an opportunity to tailor a mixture of enzymes to target the protein degradation for optimal outcome in terms of tenderness.

4.8.1.3. Effect of incubation time and enzyme concentration on the degradation of meat myofibrils (MM) with papain and bromelain.

Papain at enzyme to substrate ratio of 0.04 produced complete degradation of MM (Figure 15, above) after incubation of 2 hrs at 45°C, which consequently can produce the mushy texture in meat at this concentration level. Because the incubation time used is normally higher than what is normally encountered in fast cooking styles (e.g. grilling) and the incubation temperature is lower than those used for cooking, the activity of the papain is expected to be higher (optimum temperature for maximum activity is 70-75°C). A lower concentration of papain and shorter incubation time may afford a better control over papain. However, a similar MM degradation profile was found at E/S of 0.02 (Figure 3A, Appendix A). A better controlled degradation was achieved at E/S of 0.0004 (Figure 3B, Appendix A) with clear hydrolytic effects on MHC and actin proteins after only 5 min with no further changes with longer incubation times. These results demonstrate the fast proteolytic nature of papain and suggest that the concentration, and possibly the temperature (rather than cooking time) will dictate the suitability of papain as a meat tenderiser.

Bromelain at E/S of 0.02 did not show any effect on MM protein (Figure 3A, Appendix A). Slight to moderate effects on MHC and actin were observed with E/S of 0.06 (Figure 3B, Appendix A). There were no further changes with longer incubation times. The above results demonstrate that papain and bromelain are fast acting proteases as no further proteolysis occurred with longer reaction periods, possibly due to a by-product inhibition mechanism as sufficient substrate remained in the reaction mixture. Papain is by far the most effective protease toward MM.

4.8.1.4. Effect of incubation time and enzyme concentration on the degradation of meat myofibrils (MM) with protease G and fungal 31K.

Protease G at E/S of 0.2 (Figure 4A) caused significant degradation of MM proteins but not at E/S ratio of 0.02 (Figure 4B). Significant protein degradation caused by protease G occurred after 5 min of incubation, but seemed to continue over the 30 min incubation period with complete disappearance of the MHC at the end of the incubation period. Fungal 31k at E/S 0.2 did not cause any changes in MM protein profile over the 30 min incubation period (Figure 4A). At an E/S ratio of 1, fungal 31k produced a complete degradation of MM proteins after 5 min (Figure 4B) without any further degradation over 30 min of incubation. It was observed earlier that at an E/S of 1 and a longer incubation, more degradation took place (Figure 15, above), which suggests that a threshold concentration of protease is required to initiate the protein degradation. It is possible that higher proteolysis could be achieved with longer reaction times.

4.8.1.5. Effect of incubation time and enzyme concentration on the degradation of meat myofibrils (MM) with commercial zingibain and ginger crude extract.

The effect of commercial zingibain and ginger crude extracts on protein degradation of MM over 30 min of incubation at 45°C is shown in Figure 5 (Appendix A). Commercial zingibain was more effective in hydrolysing MM protein than ginger crude extract at the amounts of enzyme used, which suggests more activity in the commercial zingibain. It was clear from the protein profiles of commercial zingibain and ginger crude extract that a higher amount of zingibain was present in the commercial preparation, as evidenced by the intense large band at 34 kDa (Figure 2). Varietal or processing conditions could be responsible for these differences. Several interesting observations can be found in Figure 5. Firstly, zingibain seems to work in a progressive controlled fashion as is indicated by the progressive disappearance of the band intensity of the native proteins (Figure 5). Secondly, unlike the other proteases, zingibain seems to be hydrolysing α-actinin, actin, troponin, α-tropomyosin, β-tropomyosin, myosin light chain (MLC) and troponin C faster than MHC. Finally, this regimented degradation did not cause severe degradation of MM proteins after extended periods of incubation at 45°C, contrary to the actions of papain. This could have beneficial consequences in terms of structure coherence and texture of the meat.

4.8.2 Effect of ascorbic acid (L and D-Iso forms) and cysteine on the degradation of meat myofibrils (MM) with papain.

While a few reports have described the effects of ascorbic acid, cysteine and some other additives on the sensory attributes (Ockerman et al., 1993) or the kinetics of a few proteases (Ozawa et al., 1962; Fukal et al., 1986), investigation of the effects of ascorbic acid and cysteine on the protein degradation of MM has not been reported. This type of investigation could pinpoint the actions of the additives and the mechanism of inhibition, as well as generate new information that could potentially lead to better application of these additives to activate or limit the activity of proteases.

4.8.2.1. Effect of ascorbic acid (L and D-Iso forms)

Papain

Both L-ascorbic acid and L-isoascorbic acid demonstrated inhibitory effects on MM protein degradation by papain. Overall, ascorbic acid and its isoform affected the degradation of the hydrolyzed protein subunits not the native structural proteins (i.e. MHC, MLC, Troponin and actin), with the exception of actin when MM is treated with isoascorbic acid at a concentration of 1.6 mM (Figure 1, Appendix B). Generally speaking, isoascorbic acid was more effective as an inhibitor compared with ascorbic acid. Highest inhibition of protein degradation by papain was observed at concentrations of 2.5mM and 1.6mM for L-ascorbic and D-isoascorbic acids, respectively, and inhibition level appears to decrease above and below these values. These observations mean that a controlled degradation of MM may be possible using ascorbic acid. Earlier studies by Ockerman et al. (1993) demonstrated the inhibition of papain activity in meat by ascorbic acid at 2.5×10^{-3} M but that level induced an off flavour that was unacceptable in a sensory evaluation. The authors suggested a 10-fold lower concentration of ascorbic acid to decrease the activity of papain while maintaining an acceptable product. The present data suggest a possible better control of papain activity by the use of different isoforms of ascorbic acid.

Bromelain

The proteolytic activity of bromelain on MM is inhibited by both L-ascorbic and D-isoascorbic acids at concentrations in the range of 0.8 to 3.3 mM. The main effects appear to be direct inhibition of bromelain ability to degrade native structural proteins (MHC, MLC and actin) (Figure 2, Appendix B).

In-house Kiwifruit crude extract

Minimal and moderate inhibitory effects were observed for L-ascorbic and D-isoascorbic acids, respectively, on MM protein degradation by in-house Kiwifruit crude extract (Figure 3, Appendix B). D-Isoascorbic acid is a more effective inhibitor. The effective isoascorbic acid concentration range was 0.8 to 2.5 mM and the inhibition level was decreased at a concentration of 3.3 mM. The inhibition pattern was similar to that observed with papain and the main effects were the inhibition of the hydrolysis of the subunits generated from MHC.

In-house asparagus crude extract

Contrary to the above enzymes, L-ascorbic acid and D-isoascorbic acid activated the protease(s) in asparagus crude extract. Ascorbic acid (effective at concentration of 2.5mM) was more effective than isoascorbic acid (effective at concentrations of 3.3mM) in promoting the degradation of MM protein by asparagus crude extract (Figure 4, Appendix B). The activation enhances the degradation of MHC and actin by asparagus protease(s).

Fungal 31K

Both L-ascorbic and D-isoascorbic acids are activators of fungal 31K protease with better activity with isoascorbic acid, as indicated by the higher protein degradation at lower concentration (Figure 5, Appendix B). As a threshold, total activation requires $\geq$ 1.6 mM of L-ascorbic acid or $\leq$ 0.8 mM of

D-isoascorbic acid. Ascorbic acid appears to promote the degradation of the protein subunits by stabilizing the enzyme as evidenced by the maintenance of the two bands corresponding to the protease at about 34 and 26 kDa in lane 2.

Protease G

Both L-ascorbic acid and D-isoascorbic acid are activators of bacterial protease G at the highest concentrations used (3.3 mM) (Figure 6, Appendix B). Both isoforms of ascorbic acid help to stabilize the enzyme(s) at this temperature as it can be seen that most of the dominant bands on lane 2 were not present in lane 4 when the incubation was carried out without addition of either of the isoforms. These two bands, however, can be seen in the presence of a high concentration of ascorbic acid (lanes 7, 8 and 10-12).

Fungal 60K

Both L-ascorbic and D-isoascorbic acids appear to have the same effect on fungal 60K , possibly activating the degradation of large proteins (> 260 kDa). This may deserve to be considered for future research with appropriate gels that separate these protein efficiently (Figure 7, Appendix B).

Commercial Zingibain

There were no inhibitory effects for ascorbic acid isoforms on the proteolytic activity of zingibain. It was not clear whether there was an activation effect of ascorbic acid isoforms at the enzyme concentration used as total protein degradation was attained (Figure 8, Appendix B).

4.8.2.2. Effect of cysteine

***Papain*.** Cysteine, at a concentration of 25 mM, acts as an activator of papain and this activating effect is lost proportionally with the increase in cysteine content > 25 mM (Figure 9, Appendix B).

***Bromelain*.** Cysteine increased the proteolysis of MM protein with 5 mM being sufficient to cause enough activation to degrade all of the structural proteins (despite the fact that the bromelain concentration used was not effective in degrading these proteins (Figure 10, Appendix B).

***In-house Kiwifruit and asparagus extracts*.** These proteases were activated by cysteine at a concentration of ≥ 5 mM. Asparagus was more activated at that level of cysteine than actinidin. The activation seems to be through the enhanced proteolysis of the large proteins (> 250 kDa), MHC and actin, rather than stabilizing the proteases (Figure 11, Appendix B).

***Protease G*.** This enzyme appears to be slightly activated at low concentrations of 5 mM (lane 4), but was inhibited at a high concentration of 50 mM (lane 6) (Figure 12, Appendix B).

***Fungal 31K*.** Activation of fungal 31K occurred at a concentration of ≥ 5mM (Figure 12, Appendix B).

***Fungal 60K*.** Fungal 60K required a higher concentration than fungal 31K (25 mM) for activation and complete degradation of MM protein. Most of the degradation targeted MHC but at a cysteine concentration of 100 mM both MHC and actin were completely degraded (Figure 13, Appendix B).

***Commercial Zingibain*.** Similar to ascorbic acid, there were no inhibitory effects for cysteine on the proteolytic activity of zingibain. Again, it was not clear whether there was an activation effect at the enzyme concentration used as total protein degradation was achieved in the experiment (Figure 13, Appendix B).

4.9 Determination of proteolytic activity of commercial proteases and in-house preparations of asparagus and Kiwifruit using beef connective tissue degradation and SDS-PAGE

Protease G at a concentration of 0.4mg/0.07mg protein connective tissue (E/S ratio = 5.7), was very effective in hydrolysing collagen but not elastin (Figure 1, Appendix C). Complete hydrolysis of the collagen bands (heavy molecular weight = HMW, γ, β11, β12, α1 and α2) occurred after 5 minutes of incubation. Further degradation in the produced subunits occurred with an increase in incubation time which coincided with increased intensity of bands at approximately 38, 35, 32 and 24 kD (Figure 1, Appendix C). The elastin band, however, remained intact even after thirty minute of incubation with the enzyme.

Asparagus extract exhibited a much slower degradation of the HWM band, and β and α collagen dimers. On the other hand, the asparagus extract rapidly hydrolysed both β12 and elastin (Figure 1, Appendix C). Complete hydrolysis of collagen and elastin was achieved after 24 hours of incubation.

Commercial actinidin, at a concentration of 0.4mg/0.07mg protein connective tissue (E/S ratio = 5.7) was effective in hydrolysing elastin but not collagen (Figure 2, Appendix C). The elastin band was completely hydrolysed after 30 min of incubation, whereas the collagen bands α1 and α2 remained perfectly intact. Moderate activity toward the HMW and β11 bands was found after 30 min of incubation.

In-house kiwifruit juice was very effective in hydrolyzing both collagen and elastin even in diluted form (5-fold) (Figure 2, Appendix C). Both protein species were completely hydrolysed after just five minutes of incubation. Incubation with in-house kiwifruit juice at an even lower concentration (20-fold dilution) was effective in hydrolyzing all connective tissue proteins (Figure 5, Appendix C).

Commercial fungal 31K protease, at a concentration of 0.04mg/0.07mg protein connective tissue (E/S ratio = 0.57), was very effective against both collagen and elastin (Figure 3, Appendix C). Both protein species were completely hydrolysed after thirty minutes of incubation. Fungal 31K protease exhibited a greater affinity toward hydrolyzing β and HMW bands compared to elastin and α collagen bands.

Commercial fungal 60K protease, at a concentration of 0.2mg/0.07mg protein connective tissue (E/S ratio = 2.85), was only partially effective in hydrolysing collagen, with only HMW, β and α11 bands hydrolyzed after 30 min. Fungal 60 was ineffective against elastin (Figure 3, Appendix C).

Commercial ginger protease was very effective against both collagen and elastin. In a diluted form (30-fold), the protease was able to completely hydrolyze both collagen and elastin after five minutes of incubation (Figure 4, Appendix C). The commercial ginger extract seems to have been processed in such a way to optimize the activity of zingibain, as fresh ginger extracts at higher concentrations (20-fold), were unable to hydrolyse either collagen or elastin. This was also evident from the high efficacy in of commercial ginger extracts in hydrolyzing the connective tissue after 5 minutes of incubation, even at a100-fold dilution levels (Figure 5, Appendix C).

Papain, at a concentration of 0.002mg/0.07mg protein (E/S ratio = 2.86 $x10^{-2}$), completely degraded connective tissue proteins after 5 minutes of incubation (data not shown). At a

concentration of 0.0001mg/ 0.07mg protein (E/S ratio = 1.43 x10^{-3}), more organised degradation of collagen and elastin was observed (Figure 6, Appendix C). Papain demonstrated strong affinity toward hydrolyzing HMW, γ and β12, with these bands completely hydrolyzed after 5 minutes of incubation at this low concentration (E/S ratio = 1.43 x10^{-3}). Papain, at a concentration of 0.0001mg/ 0.07mg protein, had moderate affinity towards hydrolyzing β11 and elastin and weak ability to hydrolyze α1 and α2 bands.

Bromelain, at a concentration of 0.01mg/0.07mg protein (E/S ratio = 1.43), completely degraded the connective tissue proteins after 5 minutes of incubation (data not shown). At a concentration of 0.001mg/ 0.07mg protein (E/S ratio = 1.43 x10^{-2}), complete degradation of only γ and slight hydrolysis of β12 was observed (Figure 6, Appendix C).

The size of protein subunits generated from the hydrolysis process varied widely depending on the enzyme preparation used (Figures 1-6, Appendix C). For example, while asparagus resulted in major degraded protein bands at about 55, 45 and < 20 kD, protease G had its major degraded protein bands at approximately 38, 35, 32 and 24 kD (Figure1, Appendix C). Smaller bands (< 20kD) were generated by kiwifruit juice and ginger extract (Figures 2, 4 and 5, Appendix C) with the main degradation band at about 37-38 kD at lower concentrations of kiwifruit juice (Figure 5, Appendix C). While fungal protease 31K generated main degradation protein bands at 40 and 30kD, fungal 60K had its main degraded proteins at 45 and about 37 kD (Figures 3, Appendix C). Collectively, these observations demonstrate that the different enzymes studied have different affinities toward hydrolyzing the proteins of connective tissue, target different amino sequences for hydrolysis, and produce different hydrolysis products of variable sizes. This will have important consequences for the tenderisation process in terms of the level of tenderness, the flavour of the tenderised meat, and the potential use of the hydrolysis products for other functional meat-derived products.

4.10 Effect of commercial proteases and in-house preparations of Asparagus and Kiwifruit extracts on the eating and keeping qualities of hot-boned beef

4.10.1 pH, weight change due to treatment and cooking loss

There was no effect of treatments on the pH. All treated samples had higher weight gain compared to controls at 1 day post-mortem (Table 8) but not after 3 weeks of post-mortem (Table 9). A higher weight gain (%) was found with water and Zingibain (≈4 %) at 1 day PM. Fungal 60K, protease G and kiwifruit juice-treated samples had the lowest weight gain (1.7-1.8%). Cooking loss (%) at 1 day PM was higher in all injected meat samples except for Zingibain-treated samples, which exhibited the lowest cooking loss (28.5%). Therefore, any weight gain due to treatments was lost as cooking loss upon cooking 1 day PM meat samples, with the exception of the Zingibain-treated samples (Table 8). After 3 weeks of vacuum packaging storage at 2°C, papain-treated samples were the only samples with a positive gain that was significantly ($P < 0.05$) higher than control samples (Table 9). Higher numerically purge (%) was found with Protease G and Asparagus juice treated samples.

4.10.2 Shear force and compression

Shear force was higher than the tender level of 79 N (equivalent to 8 kgF) for all of the meat samples, with the exception of meat samples treated with the in-house kiwifruit juice preparation (Table 8). Some of the meat samples from this treatment had extensive breakdown of their structure, indicating the need to apply a less concentrated kiwifruit preparation. There was no tenderising effect for papain, bromelain, commercial actinidin, fungal 60K, bacterial protease G or asparagus crude extract, whereas zingibain and fungal 31K tended to reduce the shear force. Similarly, the

compression values indicate a clear tenderising effect for the in-house kiwifruit preparation and the tendency of commercial actinidin and zingibain to tenderise the SM muscle.

As expected, significant tenderisation occurred in the meat samples during post-mortem storage due to the actions of endogenous enzymes on the myofibrillar proteins. Only Kiwifruit juice-treated samples were significantly more tender than the control samples, as assessed by shear and compression forces (Table 4). Fast cooking (10-14 min of cooking in a bag as described by Chrystall and Devine, 1991) or slow cooking (1:30-2 hrs roasting at 180°C) did not have a significant effect on the shear and compression forces, with the exception of increased compression values of kiwifruit treated samples with slow cooking.

The tenderising effect of proteases is a function of enzyme concentration and the level of activity that is dictated by the activation temperature and the time of effective activation. This means that the cooking temperature/method (fast or slow) and the size of the meat (temperature gradient within the meat cut during cooking) will have an impact on the level of enzyme activation and hence the tenderising effect achieved by an enzyme if it is available in sufficient concentrations. While it may appear that the protease concentration seems to be the main reason for the tenderising effect found with kiwifruit juice compared to the commercial actinidin (since the samples were of similar size and all the samples were cooked to the same temperature), this may not be the case. The results from the protein profile of both preparations (Figure 2) and their actions on meat myofibrils (Figure 15) and on connective tissue proteins (Figures 2 and 5, Appendix C) indicate the presence of a 15kD band in kiwifruit juice that may potentially be responsible for the differential effects on meat proteins. Kiwifruit juice is normally subjected to an ultra-filtration step during the preparation of the commercial actinidin with a cut off limit of 10-15kD. This could potentially result in the loss of the 15kD protein, as well as sugars and polyphenols (important factors for keeping qualities of meat)with the filtrate. Commercial actinidin, used at the recommended concentration, tended to reduce the shear force and compression values after 3 weeks of vacuum packaging. This suggests the tenderising effects of actinidin in the commercial preparation are slow due to either low concentration of the enzyme and the absence of the 15kD protein (Figure 2) or due to inhibitory effects of the additives used in the preparation of the commercial actinidin. A higher concentration of the enzyme is required to achieve a reasonable level of tenderisation. However, the commercial preparation can sometimes cause problems during the injection process and is difficult to use in several biochemical assays.

Fungal 31K protease tended to act on the myofibrillar fraction only, and potential combinations of commercial actinidin and fungal 31K may be used to synchronize the breakdown of different muscle proteins.

Zingibain-treated SM muscles tended to have a tenderizing effect, as assessed by shear force and compression measurements. It is possible that a higher concentration of zingibain could achieve a more acceptable level of tenderisation. Zingibain seems to act on both the myofibrillar and collagen fractions of the muscle proteins.

There was no tenderising effect for papain, bromelain, fungal 60K and bacterial protease G under the described experimental conditions. The proteolytic activities of these proteases have been reported, and the lack of tenderising action in our study is probably due to the low protease concentration used.

Table 8. Effect of treatment of hot boned beef topsides with enzyme tenderisers on % change in weight, pH, cooking loss (%), the shear force and compression values at 1 day post-mortem.

Treatments	Change in weight due to treatment (%)	pH	Cooking loss (%)	Shear force (N)	Compression (N)
Control	-2.0^{d}	5.57	29.3^{bc}	121.2^{abc}	123.4^{ab}
Water	3.9^{a}	5.58	34.5^{a}	134.0^{ab}	123.8 ^{ab}
Papain	2.6^{abc}	5.56	32.5^{ab}	127.9^{abc}	127.4^{a}
Bromelain	3.3^{ab}	5.54	34.5^{a}	138.0^{a}	132.0^{a}
Actinidin	3.0^{abc}	5.54	33.3^{a}	125.2^{abc}	113.5^{ab}
Zingibain	4.1^{a}	5.59	28.5^{c}	105.0^{c}	105.0^{b}
Fungal 31K	2.5^{abc}	5.55	31.5^{abc}	110.7^{bc}	127.4^{a}
Fungal 60K	1.3^{c}	5.53	31.5^{abc}	120.9^{abc}	124.6^{ab}
Protease G	1.6^{bc}	5.53	29.4^{bc}	121.2^{abc}	122.1^{ab}
Kiwifruit Juice	1.8^{bc}	5.53	29.5^{bc}	70.4^{d}	62.2^{c}
Asparagus	3.1^{abc}	5.55	31.3^{abc}	114.3^{abc}	113.6^{ab}
SED	0.9	0.05	1.7	12.2	9.8

Table 9. Effect of treatment of hot boned beef topsides with enzyme tenderisers on % change in weight, pH, cooking loss (%), the shear force and compression values of boiled (fast cooking) and roasted (slow cooking) beef at 3 weeks post-mortem.

Treatments	Change in weight due to treatment (%)	Cooking loss (%)	Shear force (N)	Compression (N)	Roast Shear force (N)	Roast Compression (N)
Control	-2.6^{bc}	33.9	83.4^{abcd}	135.0^{a}	75.3^{ab}	138.8^{abc}
Water	-1.2^{ab}	36.5	68.9^{cde}	136.1^{a}	73.8^{ab}	147.9^{abc}
Papain	1.3^{a}	36.7	71.4^{bcd}	122.0^{a}	75.9^{ab}	127.5^{abc}
Bromelain	-0.7^{ab}	36.9	84.0^{ab}	138.7^{a}	70.2^{bc}	119.2^{cd}
Actinidin	-2.7^{bc}	33.6	67.9^{de}	124.2^{a}	74.9^{ab}	117.1^{cd}
Zingibain	-1.2^{ab}	36.1	79.6^{abcd}	126.5^{a}	74.1^{ab}	122.0^{bcd}
Fungal 31K	-0.9^{ab}	36.0	85.5^{ab}	142.9^{a}	81.4^{ab}	156.6^{a}
Fungal 60K	-2.8^{bc}	37.5	87.9^{a}	144.2^{a}	82.1^{ab}	151.9^{ab}
Protease G	-4.0^{c}	37.1	84.1^{ab}	136.1^{a}	87.6^{a}	153.5^{ab}
Kiwifruit Juice	-0.7^{ab}	34.8	58.1^{e}	76.0^{b}	58.6^{c}	94.2^{d}
Asparagus	-4.0^{c}	33.3	87.3^{ab}	150.7^{a}	84.6^{a}	144.8^{abc}
SED	1.35	2.4	8.9	13.0	7.1	16.1

4.3.10.3 Colour and colour stability

All of the injected 1 day post-mortem samples exhibited a slightly lighter colour compared with the control samples due to the addition of liquid solutions (Figure 16) [Note: this was not statistically significant; for full statistical analyse of results see Appendix D]. One day post-mortem fungal 60K and bacterial proteases treated samples exhibited a redder colour compared with control meat samples. Despite initial lower a*-values with asparagus treated samples, these samples had good colour stability and higher a*-values after 12 days of display at 2°C. In terms of acceptable display time based on a*-value of 15 (Farouk et al., 2007), bacterial protease and asparagus treated meat had 10 and 11 days of display time, compared with control samples that exhibited around 5 days of display time. Both kiwifruit juice and commercial actinidin had 9 and 5 days of display time, highlighting the advantage of the natural antioxidants in the juice. Higher yellowness values were found with zingibain and asparagus compared to control samples but not to the water injected samples (Appendix D). The treatments had no effect on lightness (L*), redness (a*) and yellowness (b*) values after 3 weeks of vacuum packaging storage (Figure 17).

The overall effects of these treatments on the colour can be better visualized in terms of colour intensity (Chroma, C) and the shifting of the colour from the red toward yellow (increase in the hue angle, h). Both, a decrease in C values or an increase in h values can reduce the acceptability of fresh meat colour. The results in Figure 16 indicate that papain, bromelain, and actinidin promoted browning (higher h-values) compared with the control meat samples. This effect was more obvious with the increase in display time. The most obvious negative impact on colour was found with commercial actinidin, where the increase in h value was in conjunction with low C values. This was confirmed with browning index values (630nm/580nm values) shown in Appendix D. A similar trend was found after 3 weeks of vacuum packaging at 2°C (Figure 17 and Appendix E).

Figure 16. Effects of treatment of hot boned beef topsides with enzyme tenderisers on colour parameters (L*, a*, b*, C and h) at 1 day post-mortem.

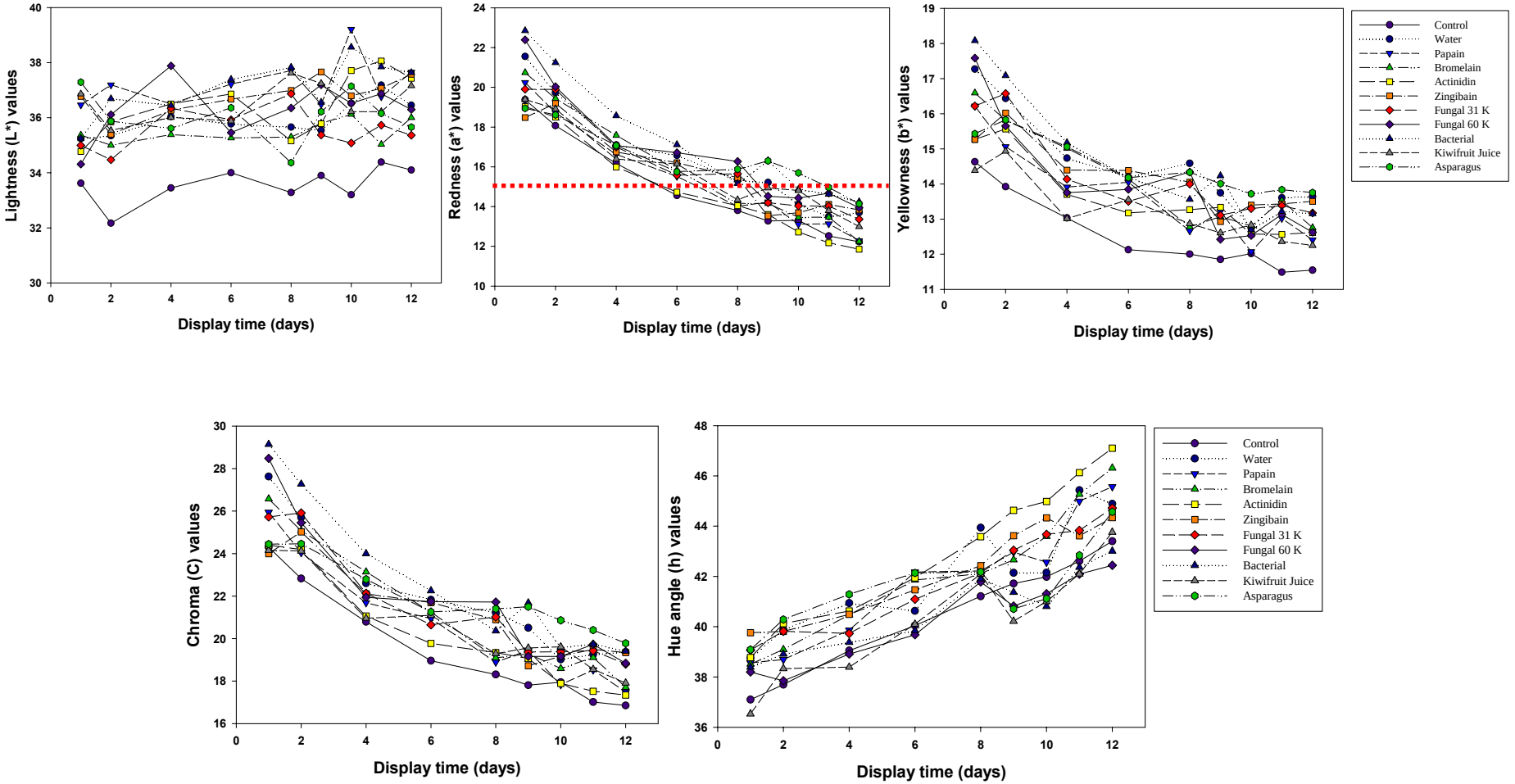
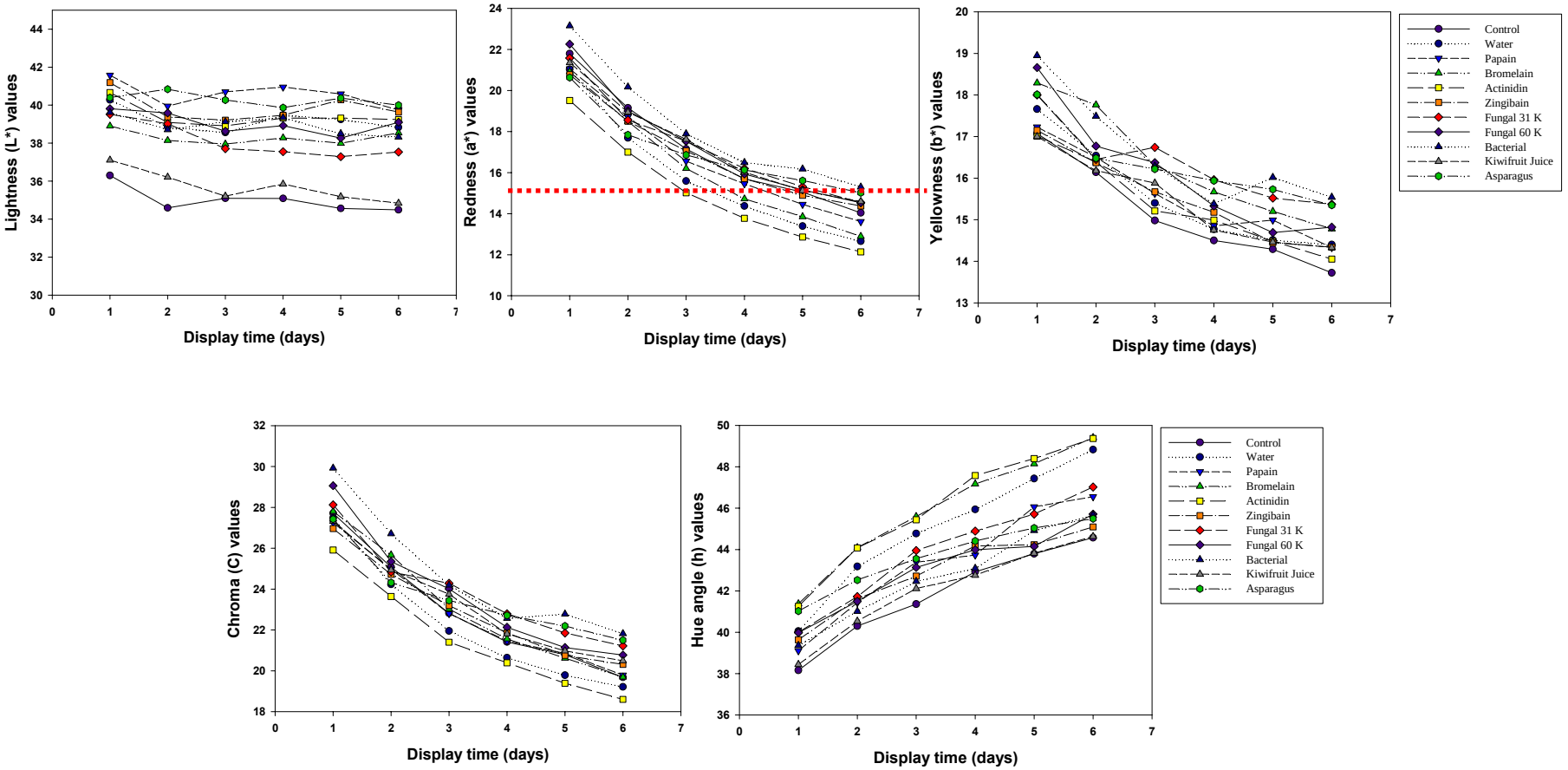


Figure 17. Effects of treatment of hot boned beef topsides with enzyme tenderisers on colour parameters (L*, a*, b*, C and h) at 3 weeks post-mortem.



4.10.4 Protein degradation

4.10.4.1 24 h post mortem

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to analyse the SMP of raw, and MMP from raw and cooked, meat samples (Figure 1, Appendix F). The changes in SMP and MMP from treated meat samples and controls were monitored using subunit molecular marker weights ranging from 3.5 kDa to 260 kDa. A representative SDS-PAGE profile is presented in the results (Figure 1, Appendix F).

Sarcoplasmic fraction

Generally, there were extensive visible changes in the protein profile only in T9 (in-house kiwifruit juice preparation) (Figure 1A, Appendix F). A small band > 260 kD was present only in T9, which probably re-localized from the myofibril fraction, as it appears in the sarcoplasmic fraction of all meat samples after 3 weeks of aging (with the exception of the treatment T10) (Figure 2A, Appendix F). Another small band at 110 kD was present only in T9. Compared with the other treatments, SMP of T9 did not have protein bands at ≈180-190 kD; 45 kD; 29 kD and ≈22 kD and had smaller bands at all other protein bands. The effect of exogenous proteases on SMP is not commonly reported in meat studies, with the exception of several studies examining the role of microbial proteases in small goods (Fadda et al., 1999a and 1999b; Rodríguez et al., 1998; Mauriello et al., 2002). Proteases from *Lactobacillus plantarum* CRL681, *L. Casei* CRL705 and *L. curvatus* NCDO904 and *L. Sake* caused complete degradation of proteins at molecular weight ≥ 66 kD and 38 kD (Fadden et al., 2001). The protein degradation profile is influenced by the source of the protease. For example, while most of the changes in sarcoplasmic protein with proteases from lactobacillus occur in protein bands at molecular weight ≥ 66 kD and 38 kD (Fadden et al., 2001), most of the changes by *Staphylococcus xylosus* proteases occur in protein bands at about 90 kD and 35-50 kD (Mauriello et al., 2002). None of the microbial proteases used in the present study (T6, T7 andT8) caused any changes in the SMP.

Myofibillar fraction of raw and cooked meat

Probably the most significant changes in the MMP of raw meat can be observed with Kiwifruit juice (Lane 10, Figure 1B, Appendix F), with the increase in the band size at 130-160 kD (Figure 1B, Appendix F) due to the degradation of myosin heavy chain (MHC). None of the other meat samples, whether injected with protease preparations or the controls, had the same level of change in their protein profiles. The degradation of α-actinin and the appearance of the 70-75 kD protein band in kiwifruit juice-treated samples is evidence of the myofibrillar protein degradation with this treatment. This level of protein degradation is equivalent to the level that is normally found with beef *M. semitendinosus* aged more than 2 weeks at 5 °C (Locker and Daines, 1973).

Complete degradation of myosin light chain 1 and Troponin C was found in kiwifruit juice-treated beef samples. A very slight effect on actin was observed with kiwifruit juice. This is in agreement with the results of Han (2008), who reported extensive degradation in myosin and no change in actin in kiwifruit juice infused lamb carcasses at 6 days post-mortem. Given the observed changes in the present study occurred after only 1 day of injection, compared with 6 days in the Han (2008) study, injection may be better than infusion as a means of delivering proteases into the meat matrix.

The protein profile of 1 day cooked beef topside is shown in Figure 1C (Appendix F). Extensive degradation of MHC, Desmin and Troponin T and a minimal effect on actin was found with kiwifruit juice-treated beef samples. These changes were not observed with the protease treatments or controls, indicating that kiwifruit juice was the only active treatment under the experimental conditions described in the M&M section above.

A 30 kD polypeptide fragment was found in Kiwifruit juice-treated meat. This polypeptide was reported to be the proteolytic product derived from troponin T in post-mortem (Claeys et al., 1995; Ho et al., 1994) and is used as an indicator of proteolysis (Locker and Daines, 1973; Ho et al., 1994; McBride and Parrish, 1977). The desmin band at 55kD was completely degraded upon cooking the

kiwifruit-injected meat. Desmin degradation is regarded as an indicator of post-mortem proteolysis during meat tenderisation (Koohmaraie & Shackelford, 1991; Wheeler & Koohmaraie, 1999).

4.10.4.2 Protein degradation at 3 weeks post-mortem

Sarcoplasmic fraction

Similar to 1 day post-mortem, there were extensive visible changes in the protein profile only in T9 (in-house kiwifruit juice preparation) (Figure 2A, Appendix F). The same small band > 260 kD was present in all meat samples, except for treatment T10. A small band at 110 kD was present and bands at 90 and 28 kD were absent only in T9. Interestingly, unlike in 1 day post-mortem samples, all the other protein bands were not different from controls. The SMP profiles from all other protease preparations were not different from controls.

Myofibillar fraction of raw and cooked meat

As with 1 day post-mortem samples, the most significant changes in the MMP of raw meat can be observed with Kiwifruit juice (Lane 10, Figure 2B, Appendix F), with an increase in the size of the band at 130-160 kD and the formation of the 75 kD polypeptide (Figure 2B, Appendix F) due to the degradation of myosin heavy chain (MHC). Also, the formation of 30 kD and small peptides <15 kD was found only in T9. No change in actin was found in any samples.

Fast cooking did not cause any changes in the protein profile from protease treated samples (lanes 3-11) compared with controls (lanes 2 and 12), with the exception of T9 (Kiwifruit juice treatment, lane 10). The appearance of the protein bands at 130-160 kD and 75 kD indicate the degradation of large structural proteins, especially myosin. The 75 kD peptide band was also visible in all other treatments, which reflect the occurrence of proteolysis by the endogenous proteases.

Slow cooking seems to be less effective in aiding the degradation of desmin, with the exception of papain and kiwifruit juice treated samples (lane 3 and 10, respectively). Desmin does not appear to be affected by fast cooking at 1 day post-mortem (Figure 1C, Appendix F) but is greatly affected after 3 weeks of storage at 2°C (Figure 2C, Appendix F). This may be due to the effects of calpains, the endogenous proteases in muscle, which play an important role in meat tenderisation by weakening/degrading the structural myofibrillar proteins during post-mortem ageing (Geesink et al., 2006; Koohmaraie and Geesink, 2006). The MMP profile of zingibain-treated meat (T5, lane 6) was very similar to kiwifruit juice (T9, lane 10), with the exception of a less intense band at 75 kD and a higher intensity desmin band in Zingibain treated meat (Figure 2D). Given the differences in the shear force values (58.6 and 74.1 N, respectively) found in these samples (Table 9), both desmin degradation and the appearance of the 75 kD peptide can be used as an indicator for tenderness of meat treated with exogenous proteases. This suggestion is supported by the tenderness values for bromelain-treated meat under the fast and slow cooking regime. Under fast cooking conditions, bromelain -treated meat had a mean shear force value of 84 N and almost no detectable 75 kD band (Figure 2C, Appendix F), whereas under slow cooking conditions, that band was clearly present (Figure 2D, Appendix F) and the mean shear force value was 70.2 N. The same tendency can be observed in commercial actinidin where the 75 kD band was more clear and complete degradation of desmin was achieved under fast cooking conditions compared with slow cooking. This resulted in lower shear force values under fast cooking compared with slow cooking (67.9 and 74.9 N, respectively).

4.10.5 Lipid Oxidation

1 day post-mortem

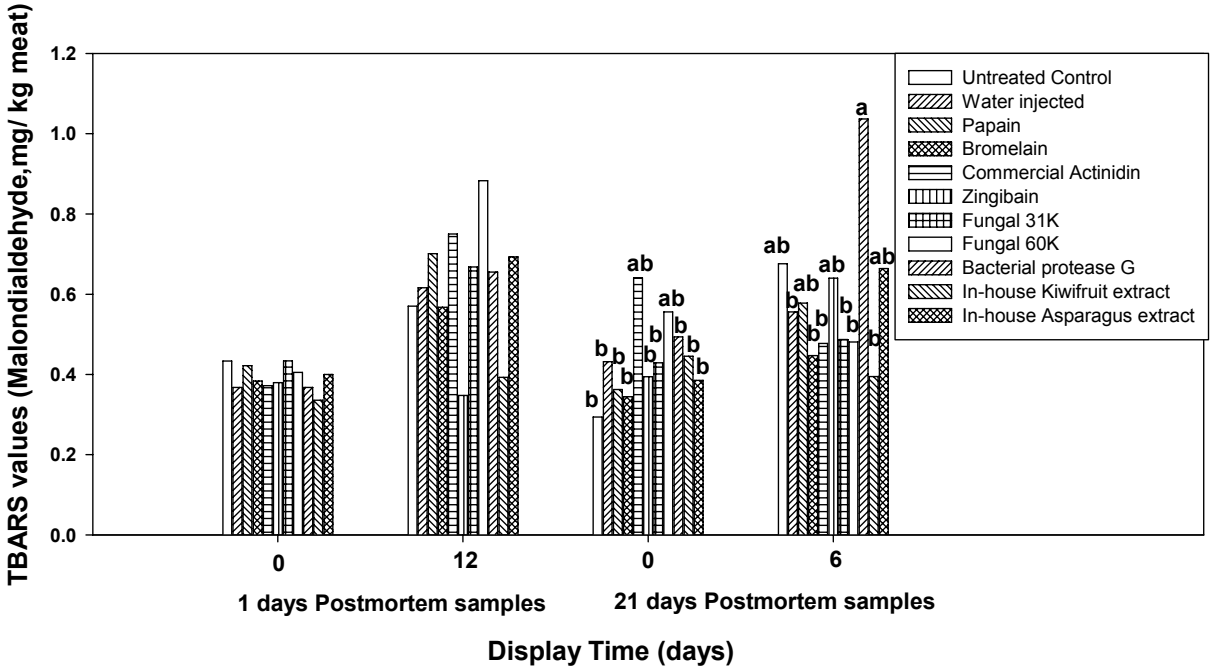
The lipid oxidation in hot boned beef topsides increased with the increase in display time ($P < 0.05$) but there were no effects for the enzyme treatments and the interaction between treatments and display time ($P > 0.05$). The mean TBARS values for all the samples were numerically higher after 12 days of display time, with the exception of Zingibain and in-house kiwifruit treated samples (Figure 18). The addition of ginger extracts to beef and sheep meat (Lee et al., 1986; Mendiratta et

al., 2000) and infusion of lamb with kiwifruit juice (Bekhit et al., 2007) have been shown to decrease lipid oxidation of meat. Both preparations contain polyphenols and other antioxidants (e.g. gingerols in ginger and Vitamin C in kiwifruit) which act as antioxidants and consequently inhibit or delay lipid oxidation. The lack of statistical significance may be due to the large number of treatments in the present study which led to a large standard error of difference.

21 days post-mortem

The lipid oxidation in hot boned beef topsides was affected by enzyme treatment, display time and their interaction ($P < 0.05$). There were no differences among all treated samples and the untreated control at 0 display time (Figure 18). After 6 days of display, beef samples treated with bacterial protease G had higher TBARS values compared with water, bromelain, commercial actinidin, fungal 31K, fungal 60K and in-house Kiwifruit-treated beef samples ($P < 0.05$). Untreated control, papain, zingibain and in-house asparagus extract-treated samples were not different from the other treatments. While protease G and in-house asparagus extract-treated beef exhibited better colour stability, they appear to have high lipid oxidation. The ability of different antioxidants (especially phenolics) to inhibit the oxidation of lipids without an effect on the oxidation of meat colour pigments and *vice versa* has been observed with several natural plant and muscle antioxidants (Bekhit et al., 2003 and 2004), and grapeseed extracts (Bekhit et al., 2008).

Figure 18. Effects of treatment, post-mortem time (1 day and 21 day) and time of display (0 and 12 days for 1 day post-mortem and 0 and 6 days for 21 days post-mortem samples) on lipid oxidation (TBARS-values) during simulated aerobic display of hot boned beef topsides treated with enzyme tenderisers. [a-b] means with 21 day post-mortem samples



The current results show that the recommended concentrations of the commercial enzymes were not sufficient to tenderise the meat at 1 day PM or to cause a significant tenderising effect after 3 weeks of vacuum packaging, except for the in-house kiwifruit juice. The analysis of fast cooked 1 day PM samples and the fast cooked and slow roasted 21 days PM samples indicated that the

cooking process/time was not a factor in manipulating the tenderisation process of injected proteases. Since most of the enzymes demonstrated varying degrees of protein degradation capability using meat myofibrillar and connective tissue proteins as substrates, and the energy required for activation should have been provided during cooking, the concentration used seems to be the limiting factor for achieving a desirable level of tenderisation. The E/S ratio based on the recommended concentrations of the commercial enzymes was too low and/or the cooking time was to fast to achieve sufficient tenderisation. Therefore a higher concentration will be required to achieve a tangible tenderising effect. Several enzymes, such as zingibain and kiwifruit in-house preparation, or a combination of enzymes (e.g. actinidin and fungal 31K) have been shown to have promising meat tenderising effects. [Note: Currently we are collaborating with a team at UNE to investigate the effect of five concentrations of these preparations in meat; and their biochemical actions will be confirmed with the analysis of the myofibrillar and connective tissue proteins.]
Apart from commercial actinidin, the injected proteases appear to either improve, or not affect the colour stability. Based on a* value of 15 as a threshold for colour acceptability, all the studied proteases, apart from commercial actinidin, offer some extra display time compared with control samples.

5 Success in Achieving Objectives

The primary objective of this project was to characterise available tenderisers in terms of their cardinal temperature and kinetics in order to identify the best for a range of red meat applications. The work undertaken within this project generated novel information on the proteolytic activities of several commercial preparations produced in Australia, as well as two in-house enzyme preparations, using synthetic and meat fractions as substrates.
The results demonstrate, for the first time, the specific affinities of the enzyme preparations toward the meat proteins (myofibrils, collagens and elastin) and show the degree of hydrolysis obtained with each enzyme preparation. Also, the work demonstrated the ability of food grade compounds to regulate the enzyme activity to varying degrees. This information can be used to better inform the way enzyme preparations are produced and utilised, and thereby offer opportunities to regulate their functioning in meat.
Furthermore, the commercial products, when used at their current recommended levels, were not effective in tenderising hot boned beef topsides from dairy cows. This information will be of important value to meat processors. However, the project demonstrated that Kiwifruit juice may represent a better choice as a source of actinidin; possibly due to it containing natural compounds that enhance the keeping qualities of meat (better colour stability and reduced lipid oxidation) and a protein fraction (15kD) which could be responsible for the tenderising action reported here.
Collectively, the information generated from the characterisation of the commercial enzyme preparations and their effects on meat proteins will enable better selection of single or a combination of enzymes that can work in synergy toward controlled hydrolysis of meat proteins and an optimal meat tenderisation process.

6 Impact on Meat and Livestock Industry- now & in five years time

There are obvious direct economic gains (increased return from the meat sales), and indirect advantages (reduced meat waste at home due to inappropriate cooking and increase consumer confidence in red meat) in adding value to lower-grade cuts. The use of exogenous proteases to improve meat tenderness is one of the top research priorities in Europe and USA, as evidenced by increasing research output in this area from these parts of the world.

This project identified the proteins targeted by the enzymes, as well as the optimal conditions for maximum activity and the effects if inhibitors/activators (Ascorbic acid & Cysteine). Several enzyme preparations were identified to have added benefits in terms of meat keeping quality. Therefore, informed decisions can be made with confidence about the use of an enzyme preparation, or a combination of enzymes, to generate a controlled tenderisation process as well as improve other quality attributes. It is expected that the industry with use this information to undertake further trials and developments in order to optimize the processing conditions of their products to their targeted markets (local or international).

The present project also found that different enzymes produce different protein hydrolysates with differing molecular weight profiles. This may enable meat industry to generate tailored polypeptides (other than carnosine, anserine, L-carnitine which have been previously reported), with designed functional properties (e.g., antihypertensive, antioxidative, opioid and Immunomodulating properties) from cheap meat cuts, connective tissue, and off bones or other waste streams. Several markets such as Japan, South Korea and China are interested in functional ingredients (Arihara, 2006) and this could be an opportunity to create significant new value within the industry.

7 Conclusions

- All of the investigated enzymes demonstrated variable proteolytic activities which were dependent on the substrate used and the assay conditions (pH and temperature). Caseinolytic activity, or the use of meat myofibrils, is better methodology than methods rely on synthetic substrates to reflect the expected proteolytic activity of the studied enzymes in meat application.
- The kinetic data obtained from BODIPY-FL casein assay appears to be in agreement with the results obtained using meat proteins (myofibrils and connective tissue) and therefore the kinetic results from that assay would be of value to estimate the binding affinity and the rates of reactions. However, these rates and affinity to different structural proteins is affected greatly, either activated or inhibited, by the use of ascorbic acid and cysteine.
- The isoform of ascorbic acid can manipulate the protease activities in a way that can be used to either use low concentrations to achieve higher rates of protein degradation (such as with fungal and bacterial proteases) or slow down the degradation of proteins (such with Kiwifruit extracts and D-isoascorbic acid) to achieve optimum level of proteolysis.
- Kiwifruit juice consistently resulted in myofibrillar proteins degradation, with its main targets being MHC and desmin. The effects of this treatment were clearly visible in raw and cooked meat at 1 or 21 days post-mortem. None of the other enzyme preparations at the concentration tested demonstrated any significant changes in raw meat. Slight positive changes occurred in myofbrilliar protein from meat treated with protease preparations other than kiwifruit juice during cooking and the cooking effects varied depending on the enzymes. Bromelain appeared to be activated more during slow cooking (roasting), while zingibain appeared to generate more myofibrillar protein degradation during fast cooking (e.g. boiling, broiling). Actin was not affected by any of the examined protease preparations.
- The manufacturers' recommended use level of the commercial proteases were not sufficient to cause significant proteolysis and tenderness in hot boned beef from dairy cows. The level of the enzymes used will require optimization to have any meaningful effect in plant.
- The colour and lipid stability of topsides during display and post-mortem storage appeared to benefit from the treatment with the proteases preparations (except commercial actinidin). Therefore other quality attributes, in addition to tenderness, may benefit from the application of the enzyme preparations.

8 Recommendations and Future Opportunities

The present project reveals several research opportunities that could benefit the meat industry through optimisation of the use of proteases to tenderise meat cuts, or through the production of bioactive polypeptides from meat fractions.

- Tenderisation could be optimised by complementary selection of a combination of enzymes that have synergistic effects on the hydrolysis of different meat fractions. The increase in the yield in the current project at injection rate of 10% of the meat weight was negligible, whereas higher injection rates (25%, Toohey et al., 2010) led to a significant increase in the product yield (average of ~20%). Therefore, higher injection rates should be considered.
- The effects of the protease preparations studied in the current project on meat large proteins (>300 kD) appear to be variable depending on the enzyme. The contribution of these proteins to meat tenderization and the contribution of the different proteases in synchronizing the initial events of proteolysis require further investigations.
- Meat enhancement via injection of ionic solutions can improve the tenderness of meat and other quality attributes (e.g., water holding capacity and juiciness). This process could be integrated with an enzymatic strategy to get a synergistic effect. However, there is limited information on the effects of the different ionic compounds on the activity of the proteases in meat. This approach could maximise the use of available interventions for meat tenderness.
- The identified fraction in kiwifruit Juice and the new protease in Asparagus may represent opportunities for property rights, but further fundamental research is required for full elucidation of nature and characteristics of the pure components.
- The enzymes demonstrated the potential of generating polypeptides with variable molecular sizes from meat fractions which are expected to have different amino acid sequences, and, potentially different bioactivities. This is an interesting opportunity because the raw material for such products can be sinew/connective tissue, tough meat cuts or material off the bones.

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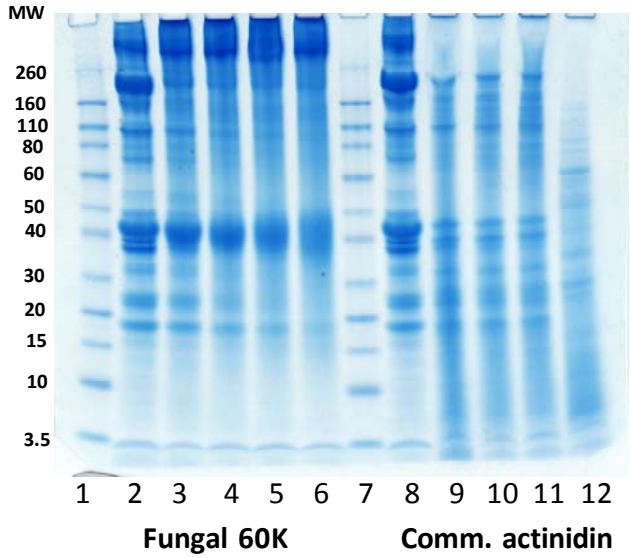
10 Appendices

Appendix A:

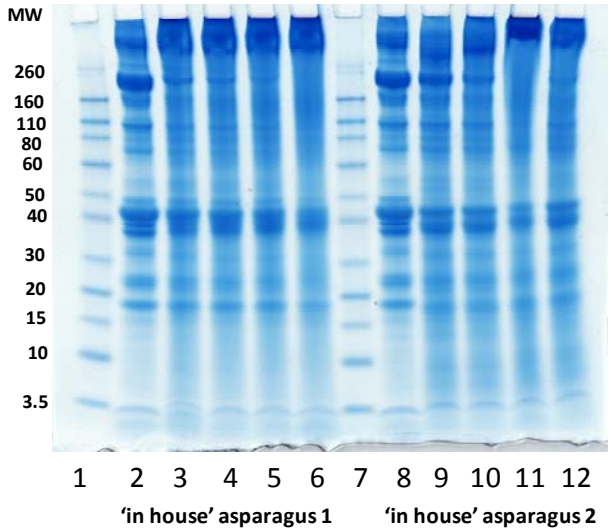
1) Effect of incubation time on the degradation of meat myofibrils (MM) with fungal 60K and commercial actinidin and in-house asparagus crude extracts.

A



Lane 1, molecular weight marker
Lane 2, meat myofibril (MM) untreated control
Lane 3, MM + fungal 60K (2hrs)
Lane 4, MM + fungal 60K (4hrs)
Lane 5, MM + fungal 60K (6hrs)
Lane 6, MM + fungal 60K (16hrs)
Lane 7, molecular weight marker
Lane 8, meat myofibril (MM) untreated control
Lane 9, MM + commercial actinidin (2hrs)
Lane 10, MM + commercial actinidin (4hrs)
Lane 11, MM + commercial actinidin (6hrs)
Lane 12, MM + commercial actinidin (16hrs)

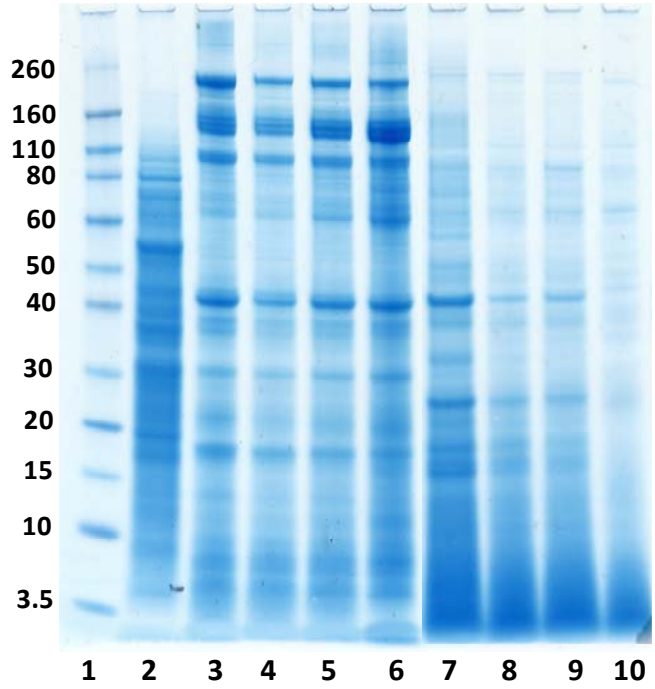
B



Lane 1, molecular weight marker
Lane 2, meat myofibril (MM) untreated control
Lane 3, MM + 'in-house' asparagus 1 (2 h)
Lane 4, MM + 'in-house' asparagus 1 (4 h)
Lane 5, MM + 'in-house' asparagus 1 (6 h)
Lane 6, MM + 'in-house' asparagus 1 (16 h)
Lane 7, molecular weight marker
Lane 8, meat myofibril (MM) untreated control
Lane 9, MM + 'in-house' asparagus 2 (2 h)
Lane 10, MM + 'in-house' asparagus 2 (4 h)
Lane 11, MM + 'in-house' asparagus 2 (6 h)
Lane 12, MM + 'in-house' asparagus 2 (16 h)

Figure 1. Effect of incubation time (2, 4, 6 and 16 hrs) on meat myofibrils (MM) protein degradation profile with fungal 60K and commercial actinidin at 400 µg/mg MM (A), and in-house asparagus preparations at 40 µl/ mg MM(B). The enzyme preparation and incubation condition are those in section 2.2.2.

2) Effect of incubation time on the degradation of meat myofibrils (MM) with in-house asparagus crude extract 1 and commercial bromelain.

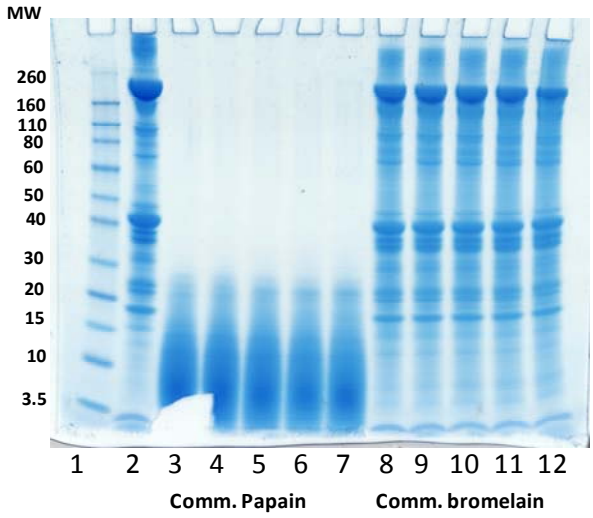


Lane 1, molecular weight marker
Lane 2, asparagus extract
Lane 3, MM + 'in-house' asparagus 1 (2 h)
Lane 4, MM + 'in-house' asparagus 1 (4 h)
Lane 5, MM + 'in-house' asparagus 1 (6 h)
Lane 6, MM + 'in-house' asparagus 1 (16 h)
Lane 7, MM + bromelain(2 h)
Lane 8, MM + 'bromelain (4 h)
Lane 9, MM + bromelain (6 h)
Lane 10, MM + bromelain (16 h)

Figure 2. Effect of incubation time (2, 4, 6 and 16 hrs) on meat myofibrils (MM) protein degradation profile with in-house asparagus crude extract (40 µl/mg MM) and commercial bromelain (0.05mg/mg MM). The enzyme preparation and incubation condition are those in section 2.2.2.

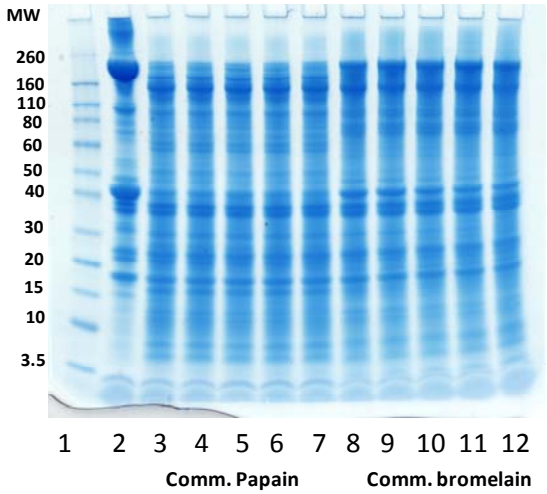
3) Effect of incubation time and enzyme concentration on the degradation of meat myofibrils (MM) with papain and bromelain.

A



Lane 1, molecular weight marker
Lane 2, meat myofibril (MM) untreated control
Lane 3, MM + commercial papain (5 min)
Lane 4, MM + commercial papain (10 min)
Lane 5, MM + commercial papain (15 min)
Lane 6, MM + commercial papain (20 min)
Lane 7, MM + commercial papain (30 min)
Lane 8, MM + commercial bromelain (5 min)
Lane 9, MM + commercial bromelain (10 min)
Lane 10, MM + commercial bromelain (15 min)
Lane 11, MM + commercial bromelain (20 min)
Lane 12, MM + commercial bromelain (30 min)

B



Lane 1, molecular weight marker
Lane 2, meat myofibril (MM) untreated control
Lane 3, MM + commercial papain (5 min)
Lane 4, MM + commercial papain (10 min)
Lane 5, MM + commercial papain (15 min)
Lane 6, MM + commercial papain (20 min)
Lane 7, MM + commercial papain (30 min)
Lane 8, MM + commercial bromelain (5 min)
Lane 9, MM + commercial bromelain (10 min)
Lane 10, MM + commercial bromelain (15 min)
Lane 11, MM + commercial bromelain (20 min)
Lane 12, MM + commercial bromelain (30 min)

Figure 3. Effects of incubation time (5, 10, 15, 20 and 30 min) and concentration on meat myofibrils (MM) protein degradation profile with papain and bromelain [papain 20 µg/mg MM in (A) or 0.4µg/ mg MM in (B); bromelain 20µg/mg MM in (A) or 60µg/mg MM in (B)]. The enzyme preparation and incubation condition are those in section 2.2.2.

4) Effect of incubation time and enzyme concentration on the degradation of meat myofibrils (MM) with protease G and fungal 31K.

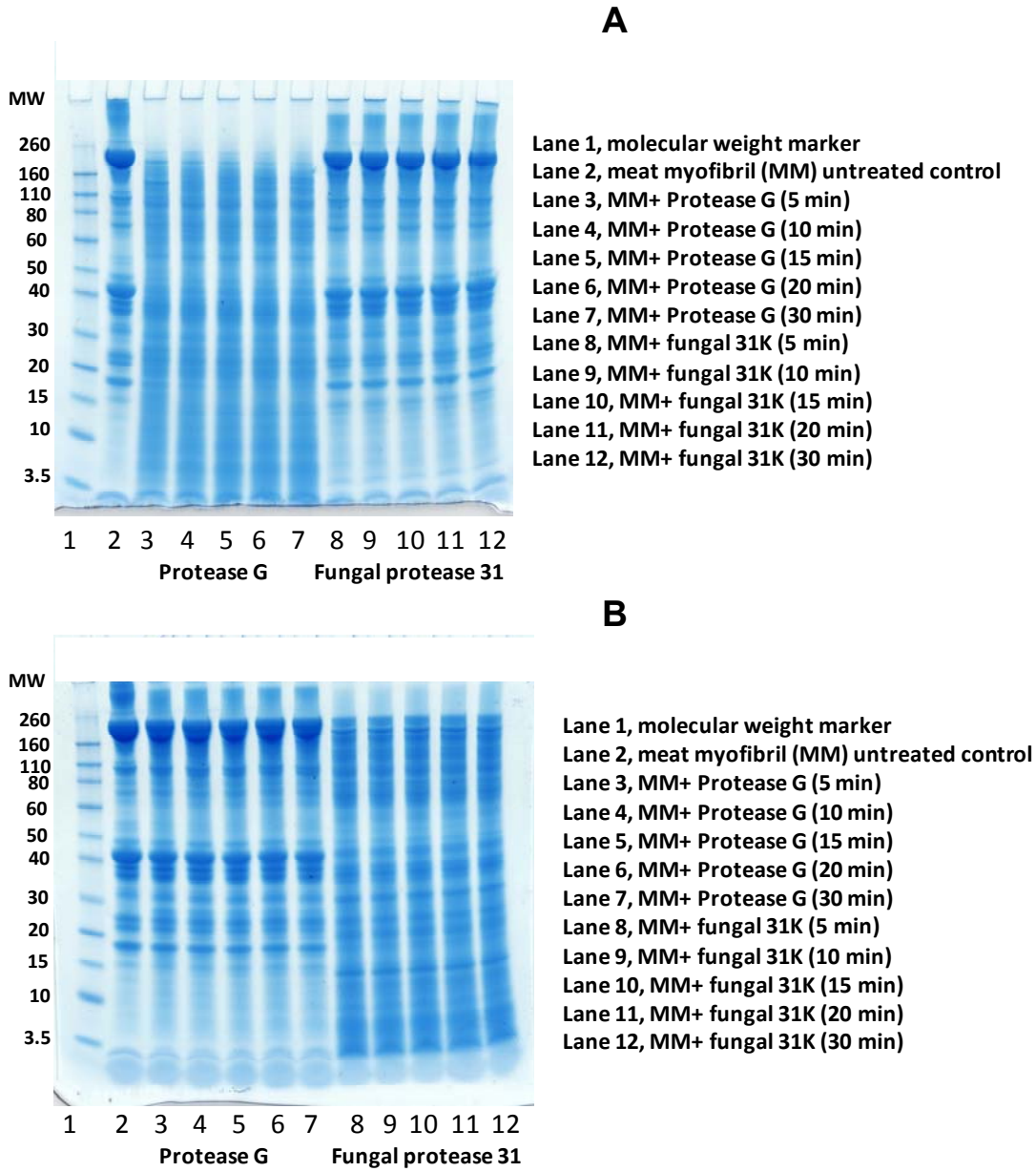
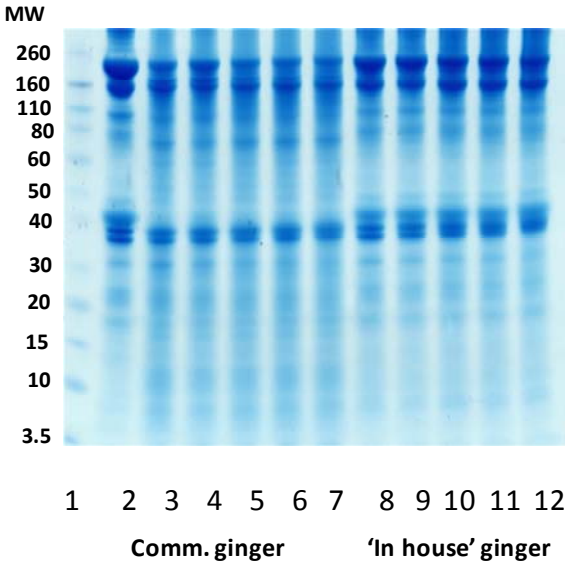


Figure 4. Effects of incubation time (5, 10, 15, 20 and 30 min) and concentration on meat myofibrils (MM) protein degradation profile with protease G and fungal 31K [protease G 0.2 mg/mg MM in (A) or 0.02 mg/ mg MM in (B); fungal 31K 0.2 mg/mg MM in (A) or 1 mg/mg MM in (B)]. The enzyme preparation and incubation condition are those in section 2.2.2.

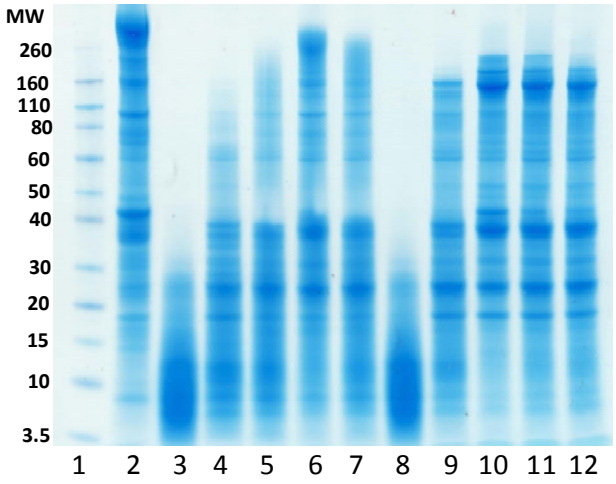
5) Effect of incubation time and enzyme concentration on the degradation of meat myofibrils (MM) with commercial zingibain and ginger crude extract.



Lane 1, molecular weight marker
Lane 2, meat myofibril (MM) untreated control
Lane 3, MM+ commercial Zingibain (5 min)
Lane 4, MM+ commercial Zingibain (10 min)
Lane 5, MM+ commercial Zingibain (15 min)
Lane 6, MM+ commercial Zingibain (20 min)
Lane 7, MM+ commercial Zingibain (30 min)
Lane 8, MM+ in-house ginger crude extract (5 min)
Lane 9, MM+ in-house ginger crude extract (10 min)
Lane 10, MM+ in-house ginger crude extract (15 min)
Lane 11, MM+ in-house ginger crude extract (20 min)
Lane 12, MM+ in-house ginger crude extract (30 min)

Figure 5. Effects of incubation time (5, 10, 15, 20 and 30 min) on meat myofibrils (MM) protein degradation profile with commercial zingibain and ginger crude extract (40 µl/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.2.

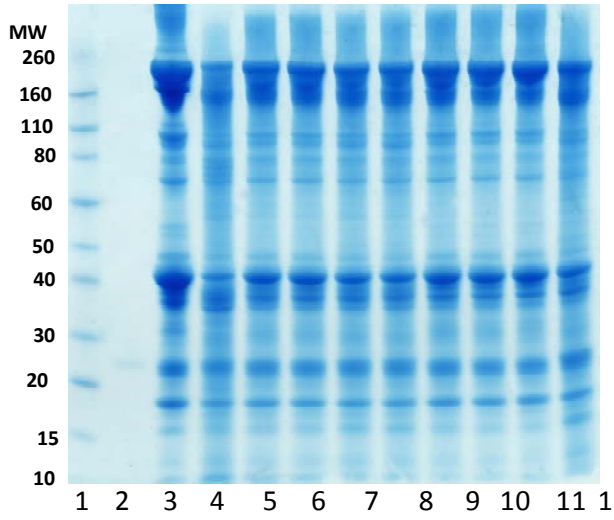
Appendix B:
Effect of ascorbic acid (L and D-Iso forms) and cysteine on the degradation of meat myofibrils (MM) with proteases.
1) Effect of ascorbic acid (L and D-Iso forms)



1	2	3	4	5	6	7	8	9	10	11	12
Marker	Myofibril only	M+E	M+E With 0.8mM L-Abs	M+E With 1.6mM L-Abs	M+E With 2.5mM L-Abs	M+E With 3.3mM L-Abs	M+E	M+E With 0.8mM Iso-Abs	M+E With 1.6mM Iso-Abs	M+E With 2.5mM Iso-Abs	M+E With 3.3mM Iso-Abs

M = Myofibril E = Enzyme Abs = Ascorbic acid Cys = Cysteine

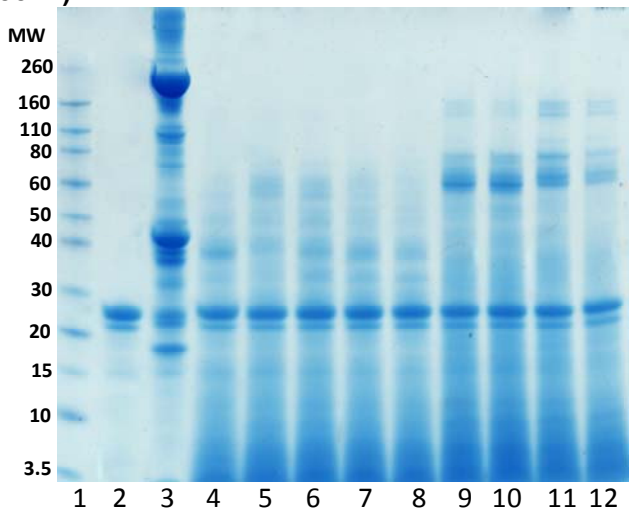
Figure 1. Effects of ascorbic acid (L and D-Iso forms) on meat myofibrils (M) protein degradation profile with commercial papain (0.04 mg/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.4. (except that the incubation temperature was 55°C).



1	2	3	4	5	6	7	8	9	10	11	12
Marker	Enzyme only	Myofibril only	M+E	M+E With 0.8mM L-Abs	M+E With 1.6mM L-Abs	M+E With 2.5mM L-Abs	M+E With 3.3mM L-Abs	M+E With 0.8mM Iso-Abs	M+E With 1.6mM Iso-Abs	M+E With 2.5mM Iso-Abs	M+E With 3.3mM Iso-Abs

M = Myofibril E = Enzyme Abs = Ascorbic acid Cys = Cysteine

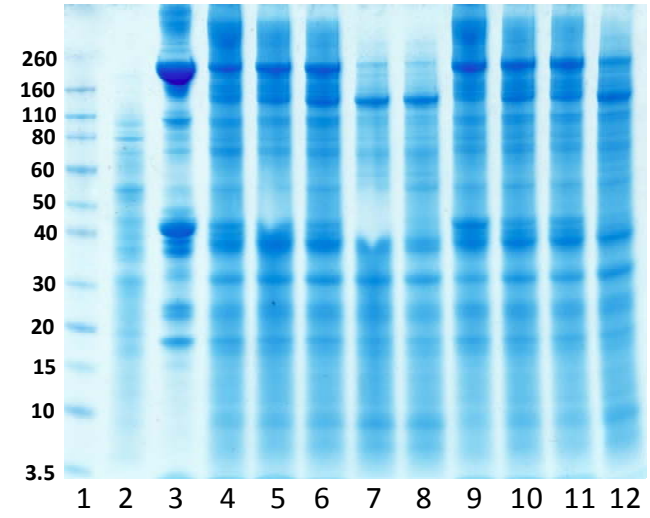
Figure 2. Effects of ascorbic acid (L and D-Iso forms) on meat myofibrils (MM) protein degradation profile with Commercial bromelain (0.4 mg/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.4. (except that the incubation temperature was 55°C).



1	2	3	4	5	6	7	8	9	10	11	12
Marker	Enzyme only	Myofibril only	M+E	M+E With 0.8mM L-Abs	M+E With 1.6mM L-Abs	M+E With 2.5mM L-Abs	M+E With 3.3mM L-Abs	M+E With 0.8mM Iso-Abs	M+E With 1.6mM Iso-Abs	M+E With 2.5mM Iso-Abs	M+E With 3.3mM Iso-Abs

M = Myofibril E = Enzyme Abs = Ascorbic acid Cys = Cysteine

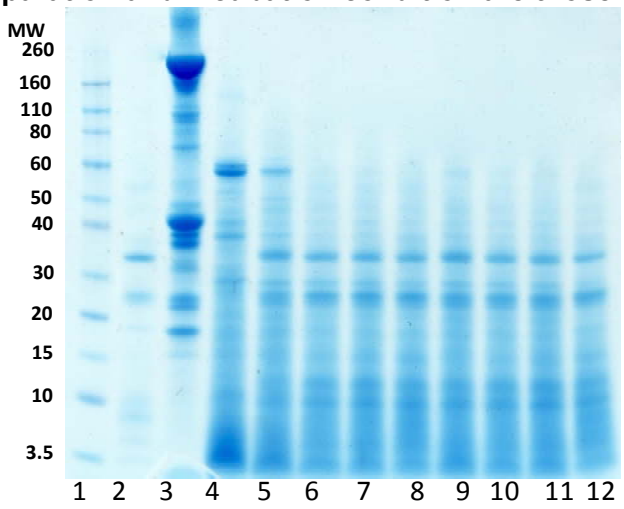
Figure 3. Effects of ascorbic acid (L and D-Iso forms) on meat myofibrils (MM) protein degradation profile with in-house Kiwifruit crude extract (40 µl/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.4.



1	2	3	4	5	6	7	8	9	10	11	12
Marker	Enzyme only	Myofibril only	M+E	M+E With 0.8mM L-Abs	M+E With 1.6mM L-Abs	M+E With 2.5mM L-Abs	M+E With 3.3mM L-Abs	M+E With 0.8mM Iso-Abs	M+E With 1.6mM Iso-Abs	M+E With 2.5mM Iso-Abs	M+E With 3.3mM Iso-Abs

M = Myofibril E = Enzyme Abs = Ascorbic acid Cys = Cysteine

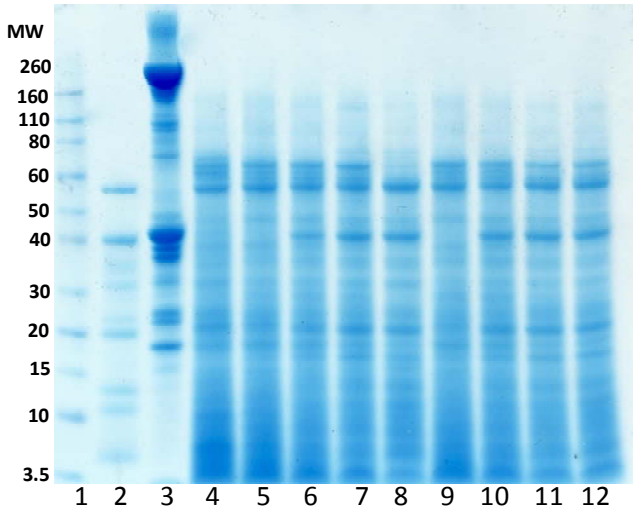
Figure 4. Effects of ascorbic acid (L and D-Iso forms) on meat myofibrils (MM) protein degradation profile with in-house asparagus crude extract (40 µl/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.4.



1	2	3	4	5	6	7	8	9	10	11	12
Marker	Enzyme only	Myofibril only	M+E	M+E With 0.8mM L-Abs	M+E With 1.6mM L-Abs	M+E With 2.5mM L-Abs	M+E With 3.3mM L-Abs	M+E With 0.8mM Iso-Abs	M+E With 1.6mM Iso-Abs	M+E With 2.5mM Iso-Abs	M+E With 3.3mM Iso-Abs

M = Myofibril E = Enzyme Abs = Ascorbic acid Cys = Cysteine

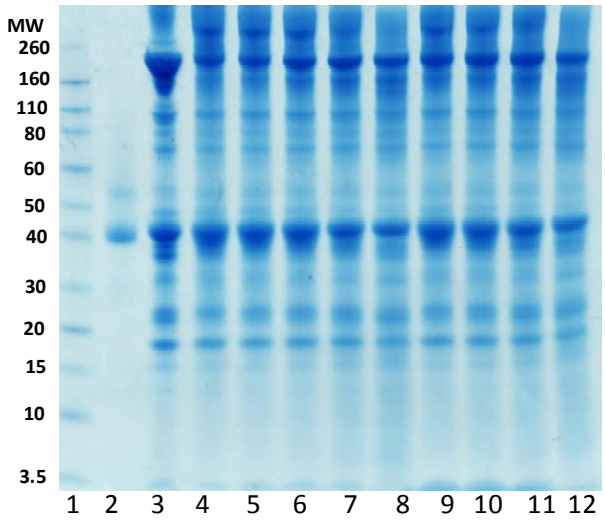
Figure 5. Effects of ascorbic acid (L and D-Iso forms) on meat myofibrils (MM) protein degradation profile with Commercial fungal 31K protease (4.0 mg/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.4.



1	2	3	4	5	6	7	8	9	10	11	12
Marker	Enzyme only	Myofibril only	M+E	M+E With 0.8mM L-Abs	M+E With 1.6mM L-Abs	M+E With 2.5mM L-Abs	M+E With 3.3mM L-Abs	M+E With 0.8mM Iso-Abs	M+E With 1.6mM Iso-Abs	M+E With 2.5mM Iso-Abs	M+E With 3.3mM Iso-Abs

M = Myofibril E = Enzyme Abs = Ascorbic acid Cys = Cysteine

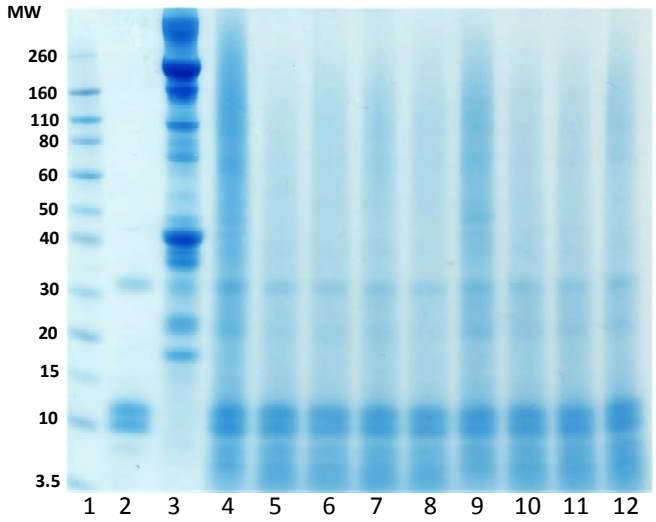
Figure 6. Effects of ascorbic acid (L and D-Iso forms) on meat myofibrils (MM) protein degradation profile with Commercial protease G (4.0 mg/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.4.



1	2	3	4	5	6	7	8	9	10	11	12
Marker	Enzyme only	Myofibril only	M+E	M+E With 0.8mM L-Abs	M+E With 1.6mM L-Abs	M+E With 2.5mM L-Abs	M+E With 3.3mM L-Abs	M+E With 0.8mM Iso-Abs	M+E With 1.6mM Iso-Abs	M+E With 2.5mM Iso-Abs	M+E With 3.3mM Iso-Abs

M = Myofibril E = Enzyme Abs = Ascorbic acid Cys = Cysteine

Figure 7. Effects of ascorbic acid (L and D-Iso forms) on meat myofibrils (MM) protein degradation profile with Commercial fungal 60K (4.0 mg/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.4.

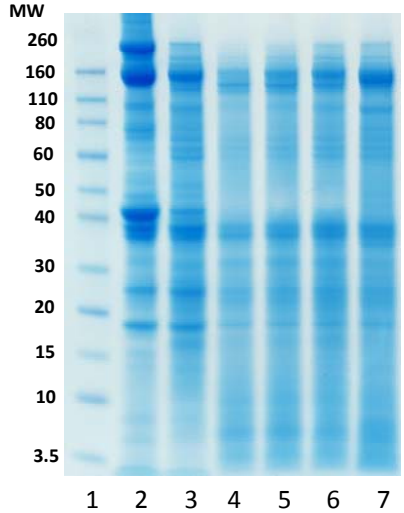


1	2	3	4	5	6	7	8	9	10	11	12
Marker	Enzyme only	Myofibril only	M+E	M+E With 0.8mM L-Abs	M+E With 1.6mM L-Abs	M+E With 2.5mM L-Abs	M+E With 3.3mM L-Abs	M+E With 0.8mM Iso-Abs	M+E With 1.6mM Iso-Abs	M+E With 2.5mM Iso-Abs	M+E With 3.3mM Iso-Abs

M = Myofibril E = Enzyme Abs = Ascorbic acid Cys = Cysteine

Figure 8. Effects of ascorbic acid (L and D-Iso forms) on meat myofibrils (MM) protein degradation profile with Commercial zingibain (40 µl/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.4. (except that the incubation temperature was 55°C).

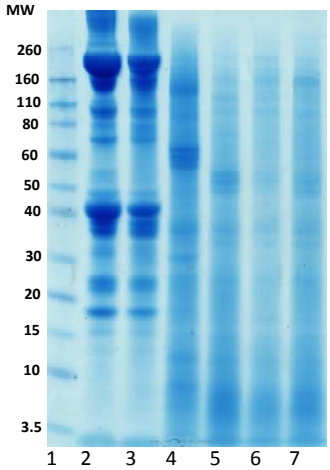
2) Effect of cysteine



1	2	3	4	5	6	7
Marker	Myofibril only	M+E	M+E With 25mM Cys	M+E With 50mM Cys	M+E With 100mM Cys	M+E With 200mM Cys

M = Myofibril E = Enzyme Cys = Cysteine

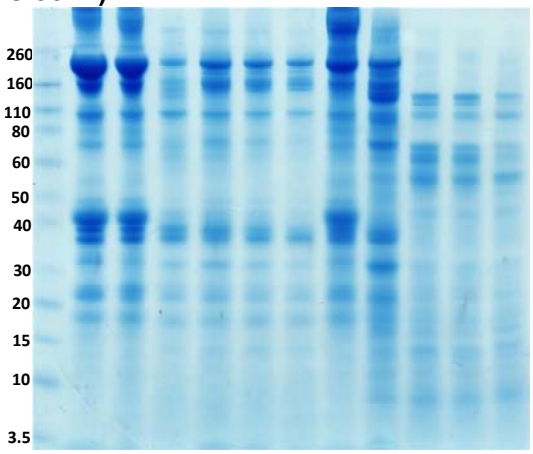
Figure 9. Effects of cysteine concentration (25-200 mM) on meat myofibrils (MM) protein degradation profile with commercial papain (0.0008 mg/ mg MM). The enzyme preparation and incubation conditions are those in section 2.2.4. (except that the incubation temperature was 55°C).



1	2	3	4	5	6	7
Marker	Myofibril only	M+E	M+E With 5mM Cys	M+E With 25mM Cys	M+E With 50mM Cys	M+E With 100mM Cys

M = Myofibril E = Enzyme Cys = Cysteine

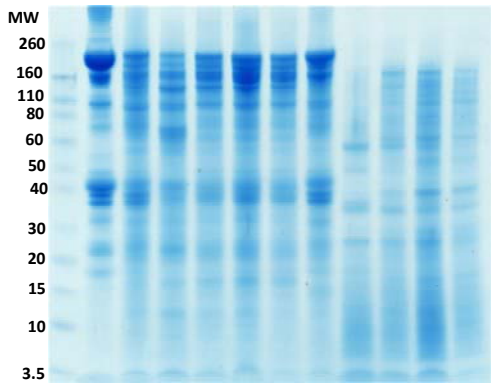
Figure 10. Effects of cysteine concentration (5-100 mM) on meat myofibrils (MM) protein degradation profile with Commercial bromelain (0.12 mg/ mg MM). The enzyme preparation and incubation conditions are those in section 2.2.4. (except that the incubation temperature was 55°C).



1	2	3	4	5	6	7	8	9	10	11	12
Marker	Myofibril only	M+E	M+E With 5mM Cys	M+E With 25mM Cys	M+E With 50mM Cys	M+E With 100mM Cys	M+E	M+E With 5mM Cys	M+E With 25mM Cys	M+E With 50mM Cys	M+E With 100mM Cys
		In-house kiwifruit crude extract					In-house asparagus crude extract				

M = Myofibril E = Enzyme Cys = Cysteine

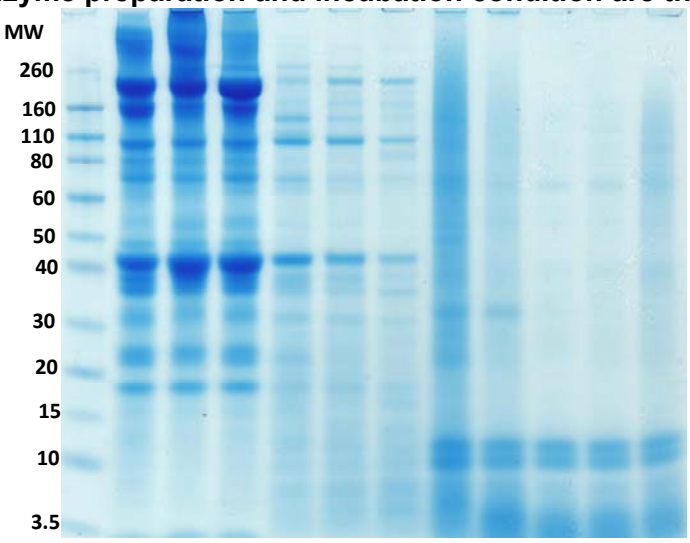
Figure 11. Effects of cysteine concentration (5-100 mM) on meat myofibrils (MM) protein degradation profile with in-house Kiwifruit and asparagus crude extracts (40 µl/ mg MM). The enzyme preparation and incubation conditions are those in section 2.2.4.



1	2	3	4	5	6	7	8	9	10	11	12
Marker	M only	M+E	M+E With 5mM Cys	M+E With 25mM Cys	M+E With 50mM Cys	M+E With 100m M Cys	M+E	M+E With 5mM Cys	M+E With 25mM Cys	M+E With 50mM Cys	M+E With 100m M Cys
		Commercial Protease G					Commercial fungal 31K				

M = Myofibril E = Enzyme Cys = Cysteine

Figure 12. Effects of cysteine concentration (5-100 mM) on meat myofibrils (MM) protein degradation profile with Commercial fungal 31K and protease G (2.0 mg/ mg MM). The enzyme preparation and incubation condition are those in section 2.2.4.



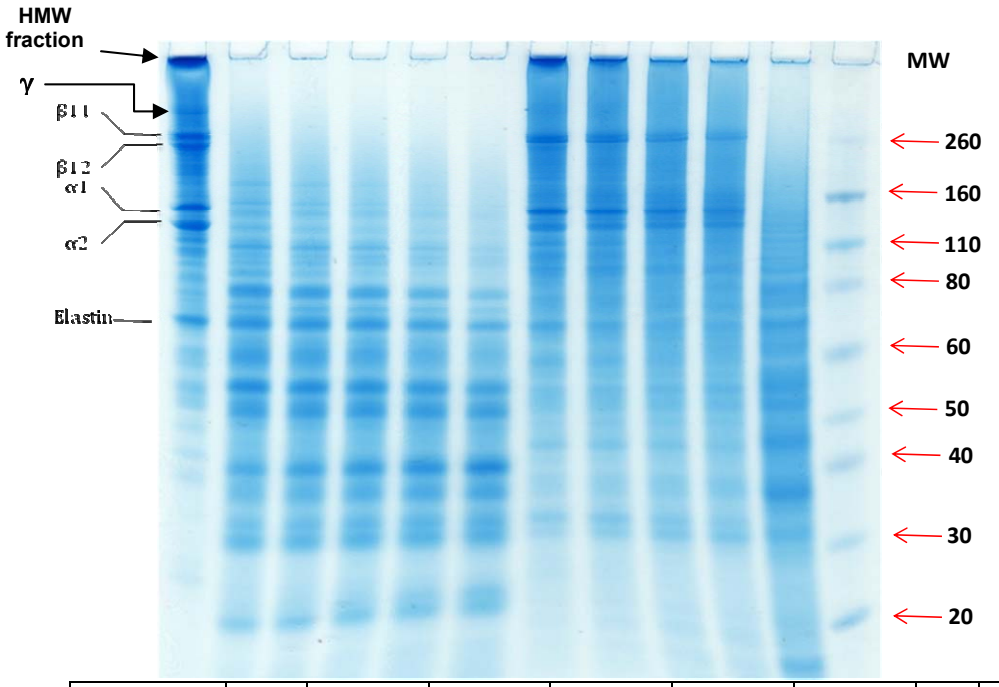
1	2	3	4	5	6	7	8	9	10	11	12
Marker	M only	M+E	M+E With 5mM Cys	M+E With 25mM Cys	M+E With 50mM Cys	M+E With 100m M Cys	M+E	M+E With 5mM Cys	M+E With 25mM Cys	M+E With 50mM Cys	M+E With 100m M Cys
		Commercial fungal 60K					Commercial zingibain				

M = Myofibril E = Enzyme Cys = Cysteine

Figure 13. Effects of cysteine concentration (5-100 mM) on meat myofibrils (MM) protein degradation profile with Commercial fungal 60K and zingibain (4.0 mg/ mg MM and 40µl/mg MM, respectively). The enzyme preparation and incubation condition are those in section 2.2.4.

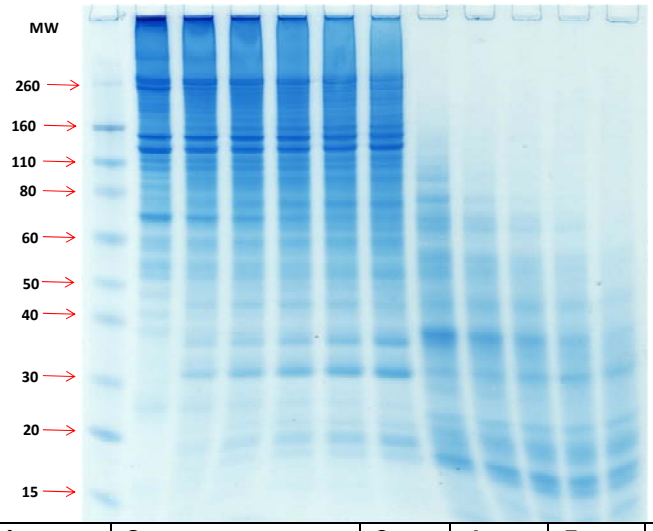
Appendix C:

1) Effect of incubation time on the degradation of meat connective tissue (collagen and elastin) during incubation with commercial proteases and in-house kiwifruit juice and asparagus crude extract.



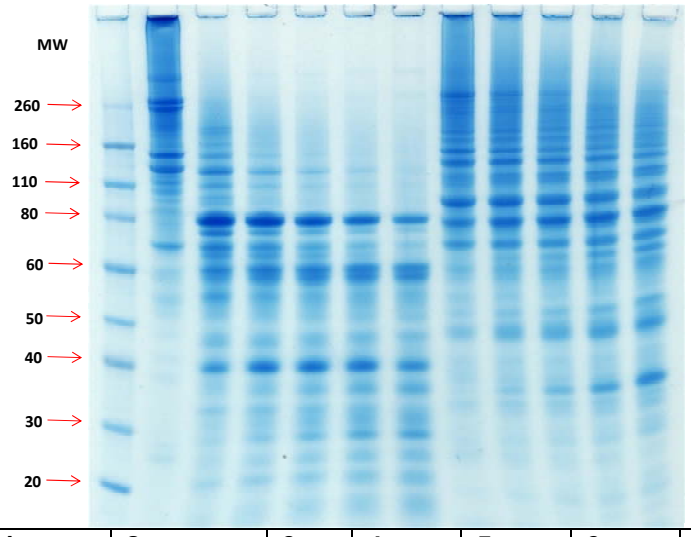
1	2	3	4	5	6	7	8	9	10	11	12
collagen/ elastin	5 min	10 min	15 min	20 min	30 min	30 min	1h	2h	3h	24h	Marker
	Protease G (20mg/mL)					FS Asparagus (undiluted)					

Figure 1. Effect of incubation time (5, 10, 15, 20 and 30 min for Protease G and 0.5, 1, 2, 3 and 24 h for Asparagus extract) on beef collagen and elastin degradation profile with Protease G (0.4mg/0.07mg protein) and in-house asparagus crude extract (20 µl//0.07mg protein). The enzyme preparation and incubation conditions are those in section 2.2.2.
HMW= heavy molecular weight band



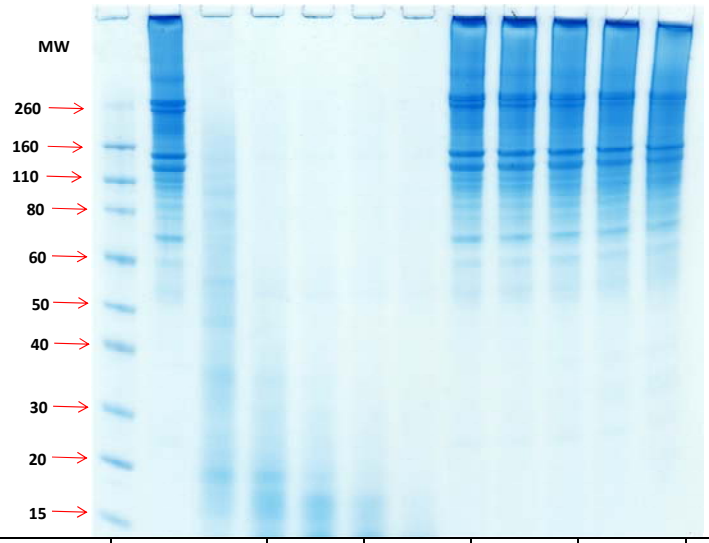
1	2	3	4	5	6	7	8	9	10	11	12
Marker	collagen/elastin	5 min	10 min	15 min	20 min	30 min	5 min	10 min	15 min	20 min	30 min
		Commercial Actinidin (20mg/mL)					FS kiwifruit extract (1:4 dilution)				

Figure 2. Effect of incubation time (5, 10, 15, 20 and 30 min) on beef collagen and elastin degradation profile with commercial actinidin (0.4mg/0.07mg protein) and diluted in-house kiwifruit juice (20 µl/0.07mg protein). The enzyme preparation and incubation conditions are those in section 2.2.2.



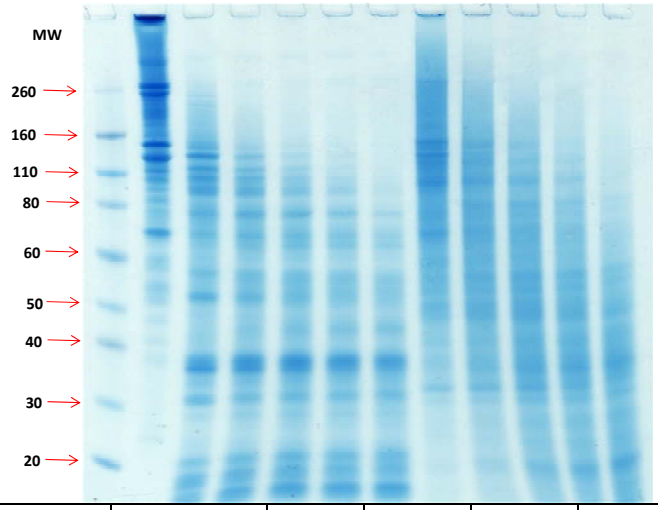
1	2	3	4	5	6	7	8	9	10	11	12
Marker	collagen/ elastin	5 min	10 min	15 min	20 min	30 min	5 min	10 min	15 min	20 min	30 min
		Commercial fungal 31K protease (2mg/mL)					Commercial fungal 60K protease (10mg/mL)				

Figure 3. Effect of incubation time (5, 10, 15, 20 and 30 min) on beef collagen and elastin degradation profile with fungal 31K and fungal 60K (0.4mg/0.07mg protein). The enzyme preparation and incubation conditions are those in section 2.2.2.



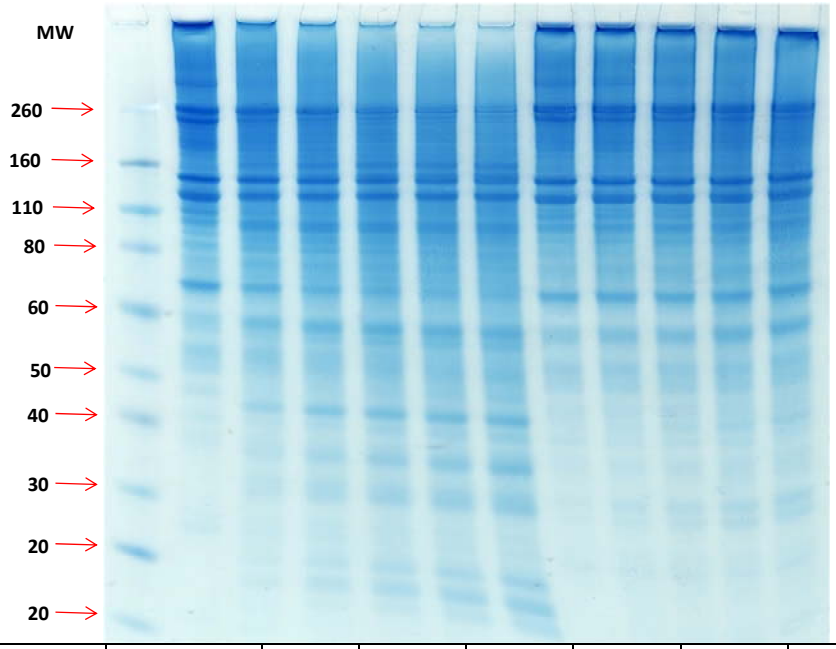
1	2	3	4	5	6	7	8	9	10	11	12
Marker	collagen/ elastin	5 min	10 min	15 min	20 min	30 min	5 min	10 min	15 min	20 min	30 min
		Commercial ginger protease (1:29 dilution)					In house ginger extract (1:19 dilution)				

Figure 4. Effect of incubation time (5, 10, 15, 20 and 30 min) on meat collagen and elastin degradation profile with commercial and in-house preparation of ginger extracts (20µl/0.07mg protein). The enzyme preparation and incubation conditions are those in section 2.2.2.



1	2	3	4	5	6	7	8	9	10	11	12
Marker	collagen/ elastin	5 min	10 min	15 min	20 min	30 min	5 min	10 min	15 min	20 min	30 min
		FS kiwifruit extract (1:19 dilution)					Commercial ginger protease (1:99 dilution)				

Figure 5. Effect of incubation time (5, 10, 15, 20 and 30 min) on beef collagen and elastin degradation profile with commercial ginger and in-house kiwifruit extracts (20µl/0.07mg protein). The enzyme preparation and incubation conditions are those in section 2.2.2.



1	2	3	4	5	6	7	8	9	10	11	12
Marker	collagen/ elastin	5 min	10 min	15 min	20 min	30 min	5 min	10 min	15 min	20 min	30 min
		Commercial papain (5µg/mL)					Commercial bromelain (50µg/mL)				

Figure 6. Effect of incubation time (5, 10, 15, 20 and 30 min) on beef collagen and elastin degradation profile with commercial papain (0.0001mg/0.07mg protein) and bromelain (0.001mg/0.07mg protein). The enzyme preparation and incubation conditions are those in section 2.2.2.

Appendix D.

1) Effects of treatment of hot boned beef topsides with enzyme tenderizers on colour parameters (L*, a*, b*, C and h) at 1 day post-mortem.

L* (SED =3.5)

Treatment	Display time (days)								
	1	2	4	6	8	9	10	11	12
Control	33.6	32.2	33.5	34.0	33.3	33.9	33.2	34.4	34.1
Water	35.2	35.3	36.0	35.9	35.7	35.5	36.5	37.2	36.5
Papain	36.5	37.2	36.5	37.2	37.7	36.5	39.2	36.8	37.7
Bromelain	35.4	35.0	35.4	35.1	35.3	35.8	36.1	35.0	36.0
Actinidin	34.8	35.9	36.5	37.0	35.2	35.8	37.7	38.1	37.4
Zingibain	36.8	35.4	36.3	36.8	37.0	37.7	36.8	37.1	37.6
Fungal 31K	35.0	34.5	36.3	35.7	36.9	35.4	35.1	35.7	35.4
Fungal 60K	34.3	36.1	37.9	35.9	36.4	37.2	36.5	36.9	36.3
Protease G	35.3	36.7	36.5	37.1	37.8	36.6	38.6	37.9	37.6
Kiwifruit Juice	36.9	35.5	36.0	35.9	37.6	37.2	36.2	36.2	37.2
Asparagus	37.3	35.9	35.6	36.3	34.4	36.2	37.1	36.2	35.7

a* (SED = 0.95)

Treatment	Display time (days)								
	1	2	4	6	8	9	10	11	12
Control	19.4^{bc}	18.1^{c}	16.2^{b}	14.6^{c}	13.8^{c}	13.3^{b}	13.3^{bc}	12.5^{b}	12.2^{bc}
Water	21.6^{a}	19.7^{abc}	17.1^{ab}	16.1^{abc}	15.3^{abc}	15.2^{ab}	14.1^{abc}	13.5^{ab}	13.7^{abc}
Papain	20.2^{bc}	18.7^{bc}	16.6^{ab}	15.4^{abc}	14.0^{bc}	14.2^{b}	13.1^{bc}	13.1^{ab}	12.3^{bc}
Bromelain	20.7^{bc}	19.4^{abc}	17.6^{ab}	16.0^{abc}	14.2^{bc}	14.2^{b}	13.5^{bc}	13.5^{ab}	12.2^{bc}
Actinidin	19.0^{bc}	18.5^{bc}	16.0^{b}	14.8^{bc}	14.0^{bc}	13.6^{b}	12.7^{c}	12.2^{b}	11.9^{c}
Zingibain	18.5^{c}	19.2^{bc}	16.8^{ab}	15.8^{abc}	15.5^{abc}	13.5^{b}	13.7^{bc}	14.1^{ab}	13.8^{ab}
Fungal 31K	19.9^{bc}	19.9^{abc}	17.0^{ab}	15.7^{abc}	15.7^{abc}	14.2^{b}	14.0^{abc}	14.0^{ab}	13.4^{abc}
Fungal 60K	22.4^{a}	20.0^{ab}	17.1^{ab}	16.8^{ab}	16.3^{a}	14.5^{ab}	14.4^{abc}	14.7^{a}	13.9^{ab}
Protease G	22.9^{a}	21.2^{a}	18.6^{a}	17.1^{a}	15.2^{abc}	16.3^{a}	14.9^{ab}	14.6^{a}	14.2^{a}
Kiwifruit Juice	19.4^{bc}	18.9^{bc}	16.4^{b}	16.1^{abc}	14.3^{abc}	14.9^{ab}	14.8^{ab}	13.8^{ab}	13.0^{abc}
Asparagus	18.9^{bc}	18.6^{bc}	17.1^{ab}	16.4^{abc}	15.9^{ab}	16.3^{a}	15.7^{a}	15.0^{a}	14.1^{a}

b* (SED = 0.87)

Treatment	Display time (days)								
	1	2	4	6	8	9	10	11	12
Control	14.6^{e}	13.9^{c}	13.0^{b}	12.1^{b}	12.0^{c}	11.9^{c}	12.0	11.5^{b}	11.6^{b}
Water	17.3^{abc}	16.4^{ab}	14.7^{ab}	14.3^{a}	14.6^{a}	13.8^{abc}	12.7	13.6^{a}	13.6^{a}
Papain	16.2^{bcde}	15.1^{b}	13.9^{ab}	13.8^{a}	12.7^{bc}	13.2^{abc}	12.1	13.0^{ab}	12.4^{ab}
Bromelain	16.6^{abcd}	15.8^{ab}	15.0^{a}	14.2^{a}	12.8^{abc}	13.1^{abc}	12.8	13.5^{a}	12.8^{ab}
Actinidin	15.3^{de}	15.6^{abc}	13.7^{ab}	13.5^{ab}	13.3^{abc}	13.3^{abc}	12.6	12.6^{ab}	12.6^{ab}
Zingibain	15.3^{de}	16.0^{ab}	14.4^{ab}	13.8^{a}	14.1^{ab}	12.9^{abc}	13.4	13.4^{a}	13.5^{a}
Fungal 31K	16.2^{bcde}	16.6^{ab}	14.1^{ab}	13.8^{ab}	14.0^{ab}	13.1^{abc}	13.3	13.4^{a}	13.2^{ab}
Fungal 60K	17.6^{ab}	15.7^{abc}	13.8^{ab}	13.7^{ab}	14.3^{ab}	12.4^{b}c	12.5	13.1^{ab}	12.6^{ab}
Protease G	18.1^{a}	17.1^{a}	15.2^{a}	14.3^{a}	13.6^{abc}	14.2^{a}	12.7	13.2^{ab}	13.2^{ab}
Kiwifruit Juice	14.4^{e}	14.9^{b}	13.0^{b}	13.6^{ab}	12.9^{abc}	12.6^{abc}	12.8	12.4^{ab}	11.6^{b}
Asparagus	15.4^{cde}	15.8^{ab}	15.1^{a}	14.4^{a}	14.3^{ab}	14.0^{ab}	13.7	13.8^{a}	13.8^{a}

Chroma (SED = 1.23)

Treatment	Display time (days)								
	1	2	4	6	8	9	10	11	12
Control	24.3^{de}	22.8^{c}	20.8^{b}	19.0^{b}	18.3^{d}	17.8^{d}	18.0^{b}	17.0^{c}	16.9^{b}
Water	27.6^{abc}	25.7^{ab}	22.6^{ab}	21.5^{a}	21.2^{abc}	20.5^{abc}	19.0^{ab}	19.3^{abc}	19.4^{a}
Papain	25.9^{cde}	24.0^{abc}	21.7^{ab}	20.7^{ab}	18.9^{cd}	19.4^{abcd}	17.8^{b}	18.5^{abc}	17.5^{ab}
Bromelain	26.6^{bcd}	25.1^{abc}	23.1^{ab}	21.4^{ab}	19.1^{bcd}	19.3^{abcd}	18.6^{ab}	19.1^{abc}	17.7^{ab}
Actinidin	24.4^{de}	24.2^{abc}	21.1^{b}	20.0^{ab}	19.3^{abcd}	19.1^{bc}	17.9^{b}	17.5^{bc}	17.3^{ab}
Zingibain	24.0^{e}	25.0^{abc}	22.1^{ab}	21.0^{ab}	20.9^{abc}	18.7^{cd}	19.2^{ab}	19.5^{ab}	19.3^{ab}
Fungal 31K	25.7^{cde}	25.9^{ab}	22.1^{ab}	20.9^{ab}	21.0^{abc}	19.4^{abcd}	19.4^{ab}	19.4^{abc}	18.8^{ab}
Fungal 60K	28.5^{ab}	25.5^{ab}	21.9^{ab}	21.7^{a}	21.7^{a}	19.2^{abcd}	19.2^{ab}	19.7^{ab}	18.8^{ab}
Protease G	29.1^{a}	27.3^{a}	24.0^{a}	22.3^{a}	20.4^{abcd}	21.7^{a}	19.6^{ab}	19.7^{ab}	19.4^{a}
Kiwifruit Juice	24.2^{de}	24.1^{abc}	21.0^{b}	21.1^{ab}	19.3^{abcd}	19.6^{abcd}	19.6^{ab}	18.6^{abc}	17.5^{ab}
Asparagus	24.4^{de}	24.5^{abc}	22.8^{ab}	21.8^{a}	21.4^{ab}	21.5^{ab}	20.9^{a}	20.4^{a}	19.8^{a}

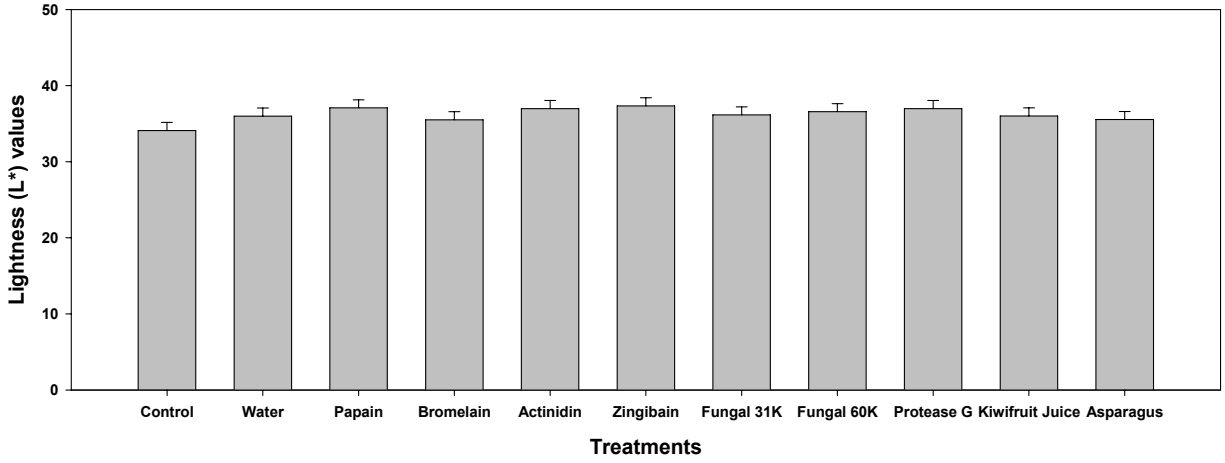
Hue (SED =1.13)

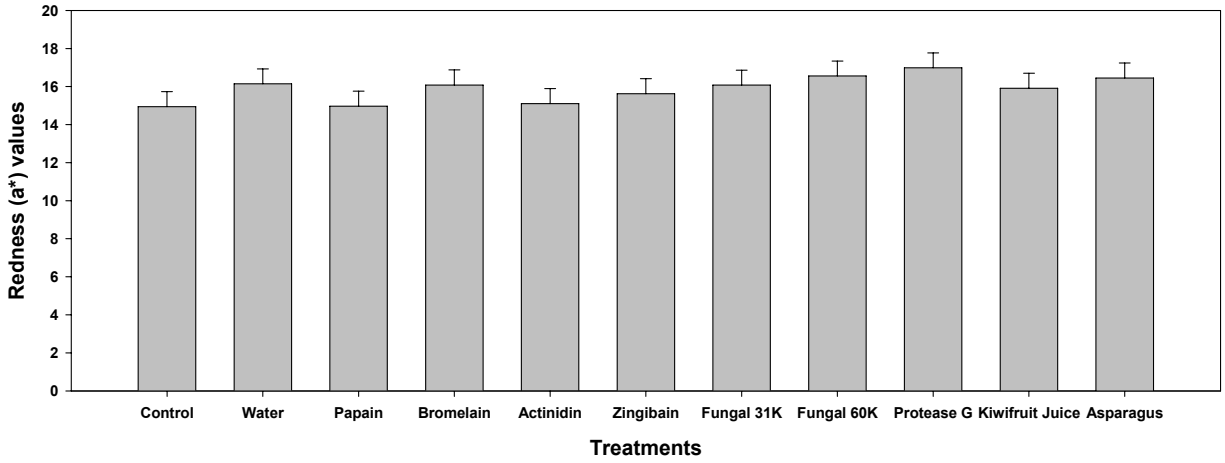
Treatment	Display time (days)								
	1	2	4	6	8	9	10	11	12
Control	37.1^{bc}	37.7^{c}	39.1^{abc}	40.0^{bc}	41.2^{b}	41.7^{bcde}	42.0^{cd}	42.6^{de}	43.4^{cde}
Water	38.8^{abc}	39.9^{abc}	40.9^{ab}	41.8^{ab}	43.9^{a}	42.1^{bcde}	42.2^{bcd}	45.4^{ab}	44.9^{abc}
Papain	38.6^{abc}	38.7^{abc}	39.9^{abc}	41.9^{ab}	42.2^{ab}	43.0^{abc}	42.6^{bcd}	45.0^{abc}	45.6^{abc}
Bromelain	38.5^{abc}	39.1^{abc}	40.5^{abc}	41.6^{ab}	42.1^{ab}	42.7^{abcd}	43.6^{ab}c	45.3^{ab}	46.3^{ab}
Actinidin	38.8^{abc}	40.1^{ab}	40.6^{abc}	42.4^{a}	43.6^{ab}	44.6^{a}	45.0^{a}	46.1^{a}	47.1^{a}
Zingibain	39.8^{a}	39.8^{abc}	40.5^{abc}	41.1^{abc}	42.4^{a}	43.6^{ab}	44.3^{ab}	43.6^{bcde}	44.3^{bcd}
Fungal 31K	39.1^{ab}	39.8^{abc}	39.7^{abc}	41.4^{abc}	42.1^{ab}	43.0^{abc}	43.7^{abc}	43.8^{bcd}	44.7^{bc}
Fungal 60K	38.2^{ab}c	37.9^{bc}	38.9^{bc}	39.3^{c}	41.8^{ab}	40.8^{cde}	41.3^{d}	42.1^{e}	42.4^{d}e
Protease G	38.4^{abc}	38.9^{abc}	39.4^{abc}	40.1^{abc}	41.9^{ab}	41.4^{bcde}	40.8^{d}	42.4^{e}	43.0^{cde}
Kiwifruit Juice	36.5^{c}	38.3^{abc}	38.4^{c}	40.1^{abc}	42.1^{ab}	40.2^{e}	41.0^{d}	42.1^{e}	41.7^{e}
Asparagus	39.1^{ab}	40.3^{a}	41.3^{a}	41.3^{abc}	42.2^{ab}	40.7^{de}	41.1^{d}	42.8^{cde}	44.6^{bcd}

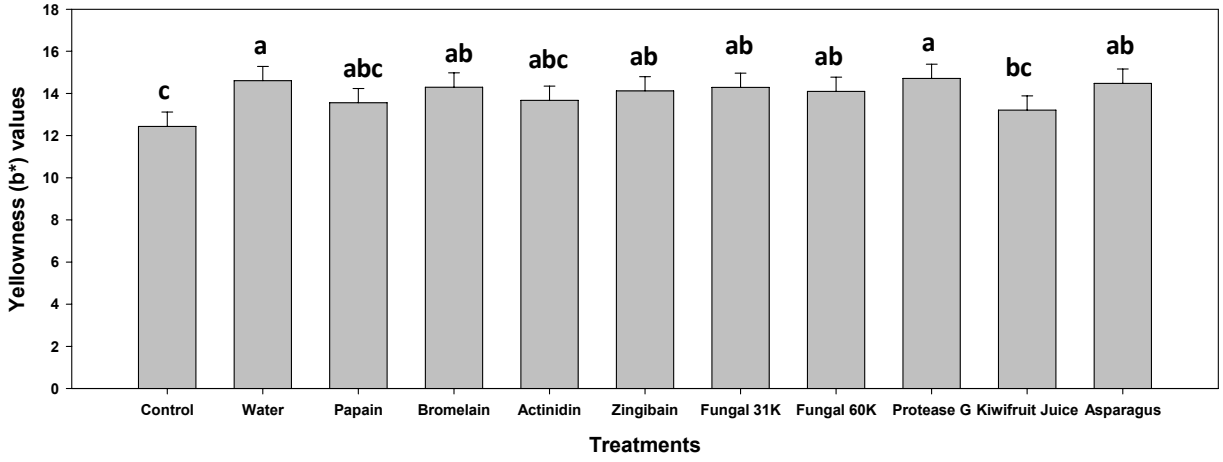
Browning index (630nm/580 nm, SED =0.25)

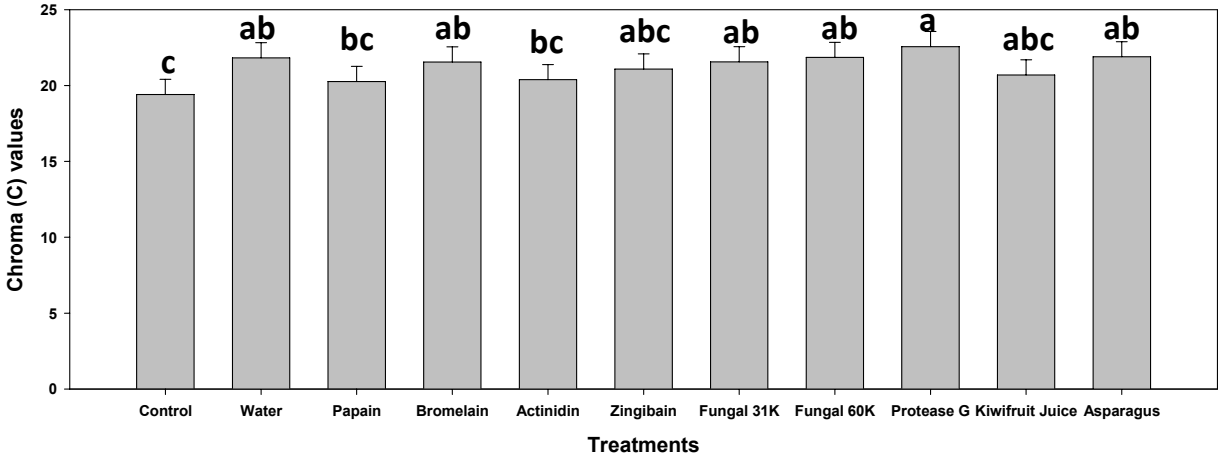
Treatment	Display time (days)								
	1	2	4	6	8	9	10	11	12
Control	4.4^{cd}	4.0^{abc}	3.3	2.9	2.8^{b}	2.4^{b}	2.4^{b}	2.3^{ab}	2.2
Water	5.1^{b}	4.3^{abc}	3.4	3.1	2.9^{ab}	2.6^{ab}	2.4^{b}	2.4^{ab}	2.4
Papain	4.6^{bcd}	3.9^{c}	3.2	2.9	2.6^{b}	2.5^{b}	2.2^{c}	2.3^{ab}	2.1
Bromelain	5.1^{b}	4.4^{abc}	3.6	3.2	2.7^{b}	2.7^{ab}	2.5^{abc}	2.4^{ab}	2.1
Actinidin	4.5^{cd}	4.1^{abc}	3.3	2.9	2.7^{b}	2.6^{b}	2.3^{b}	2.2^{b}	2.2
Zingibain	4.3^{d}	4.2^{abc}	3.3	3.0	2.9^{ab}	2.6^{ab}	2.6^{abc}	2.5^{ab}	2.4
Fungal 31K	4.8^{bc}	4.5^{ab}	3.4	3.1	2.9^{ab}	2.8^{ab}	2.7^{abc}	2.6^{ab}	2.4
Fungal 60K	5.7^{a}	4.3^{abc}	3.3	3.2	3.1^{ab}	2.7^{ab}	2.7^{abc}	2.6^{ab}	2.5
Protease G	5.7^{a}	4.7^{a}	3.7	3.2	2.9^{ab}	2.9^{ab}	2.6^{abc}	2.6^{ab}	2.5
Kiwifruit Juice	4.4^{cd}	4.2^{abc}	3.4	3.3	3.0^{ab}	2.8^{ab}	2.8^{ab}	2.7^{ab}	2.5
Asparagus	4.2^{d}	3.9^{c}	3.3	3.1	3.3^{a}	3.1^{a}	2.9^{a}	2.8^{a}	2.6

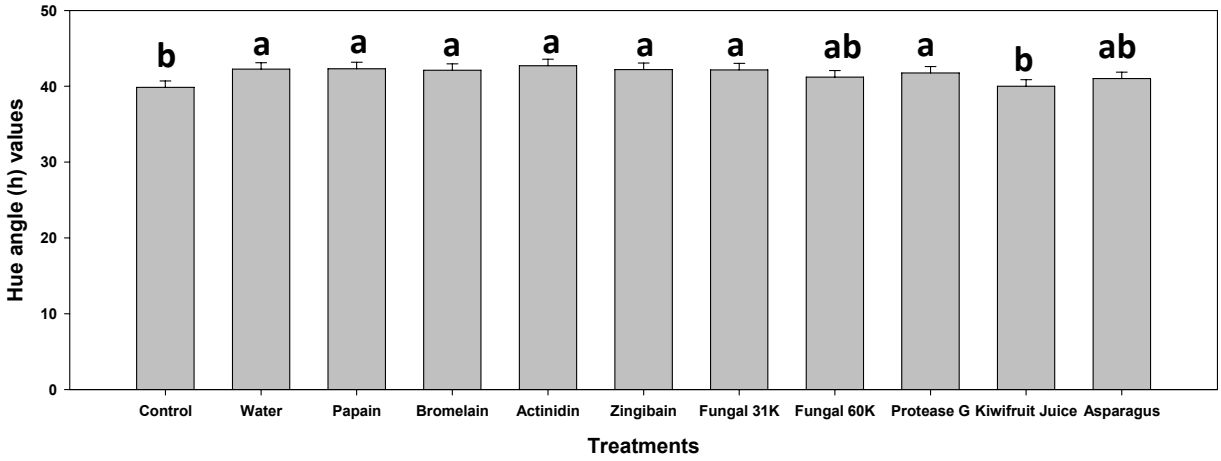
2) Main Effects of treatments of hot boned beef topsides with enzyme tenderizers on colour parameters (L*, a*, b*, C and h) at 1 day post-mortem.








Chroma (C) values
25
20
15
10
5
0
c
ab
bc
ab
bc
abc
ab
ab
a
abc
ab
Control
Water
Papain
Bromelain
Actinidin
Zingibain
Fungal 31K
Fungal 60K
Protease G
Kiwifruit Juice
Asparagus
Treatments


Hue angle (h) values
50
40
30
20
10
0
b
a
a
a
a
a
a
ab
a
b
ab
Control
Water
Papain
Bromelain
Actinidin
Zingibain
Fungal 31K
Fungal 60K
Protease G
Kiwifruit Juice
Asparagus
Treatments

Appendix E.

1) Effects of treatment of hot boned beef topsides with enzyme tenderizers on colour parameters (L*, a*, b*, C and h) at 3 weeks post-mortem.

Lightness = L* (SED =3.5)

Treatment	Display time (days)					
	1	2	3	4	5	6
Control	36.2	34.5	35.0	35.0	34.5	34.4
Water	40.0	38.6	38.3	39.2	39.0	38.6
Papain	41.4	39.8	40.5	40.8	40.4	39.6
Bromelain	39.1	38.5	38.3	38.6	38.3	38.9
Actinidin	40.5	38.9	38.7	39.1	39.1	39.0
Zingibain	41.2	39.4	39.2	39.5	40.3	39.6
Fungal 31K	39.9	39.4	38.1	37.9	37.7	37.9
Fungal 60K	40.1	39.9	39.0	39.3	38.6	39.4
Protease G	39.8	38.9	39.3	39.5	38.7	38.5
Kiwifruit Juice	37.2	36.3	35.3	36.0	35.3	34.9
Asparagus	40.1	40.5	40.0	39.6	40.1	39.7

Redness = a* (SED =0.83)

Treatment	Display time (days)					
	1	2	3	4	5	6
Control	21.8	19.2	17.2	15.7	15.1	14.0
Water	21.0	17.7	15.6	14.4	13.4	12.7
Papain	21.1	18.7	16.6	15.5	14.5	13.6
Bromelain	20.9	18.5	16.2	14.7	13.9	12.9
Actinidin	19.5	17.0	15.0	13.8	12.9	12.1
Zingibain	20.8	18.5	17.1	15.7	14.9	14.4
Fungal 31K	21.6	18.6	17.5	16.2	15.3	14.5
Fungal 60K	22.3	19.0	17.5	15.9	15.2	14.5
Protease G	23.2	20.2	17.9	16.5	16.2	15.3
Kiwifruit Juice	21.4	18.9	17.6	16.0	15.1	14.6
Asparagus	20.6	17.8	16.9	16.1	15.6	15.0

Yellowness = b* (SED =1.1)

Treatment	Display time (days)					
	1	2	3	4	5	6
Control	17.1	16.1	15.0	14.5	14.3	13.7
Water	17.7	16.5	15.4	14.8	14.5	14.4
Papain	17.2	16.5	15.6	14.9	15.0	14.3
Bromelain	18.3	17.8	16.3	15.7	15.2	14.8
Actinidin	17.0	16.4	15.2	15.0	14.5	14.1
Zingibain	17.2	16.4	15.7	15.2	14.5	14.3
Fungal 31K	18.0	16.5	16.7	16.0	15.5	15.4
Fungal 60K	18.7	16.8	16.4	15.3	14.7	14.8
Protease G	19.0	17.5	16.3	15.4	16.0	15.5
Kiwifruit Juice	17.0	16.2	15.9	14.8	14.5	14.3
Asparagus	18.0	16.5	16.2	15.9	15.7	15.4

Chroma (SED =1.18)

Treatment	Display time (days)					
	1	2	3	4	5	6
Control	27.6[ab]	25.0	22.7	21.3[ab]	20.7[ab]	19.6[b]
Water	28.0[ab]	25.2	22.9	21.6[ab]	20.7[ab]	20.2[ab]
Papain	27.4[ab]	25.2	23.0	21.6[ab]	21.0[ab]	20.0[ab]
Bromelain	28.0[ab]	25.4	22.9	21.3[ab]	20.4[b]	19.4[b]
Actinidin	26.6[b]	24.3	22.1	21.0[b]	20.1[b]	19.3[b]
Zingibain	26.6[b]	24.4	22.9	21.5[ab]	20.4[b]	20.0[ab]
Fungal 31K	27.7[ab]	24.4	23.8	22.4[ab]	21.4[ab]	20.8[ab]
Fungal 60K	28.3[ab]	24.6	23.2	21.3[ab]	20.3[b]	20.0[ab]
Protease G	29.1[a]	25.9	23.4	21.7[ab]	21.9[ab]	21.0[ab]
Kiwifruit Juice	26.9[ab]	24.5	23.3	21.4[ab]	20.6[ab]	20.1[ab]
Asparagus	28.3[ab]	25.2	24.3	23.6[a]	23.0[a]	22.4[a]

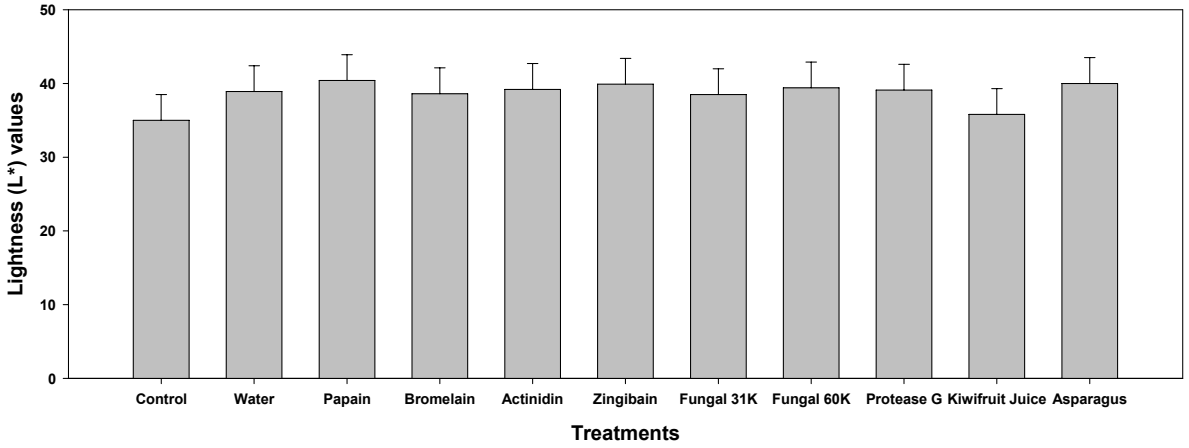
Hue (SED =1.18)

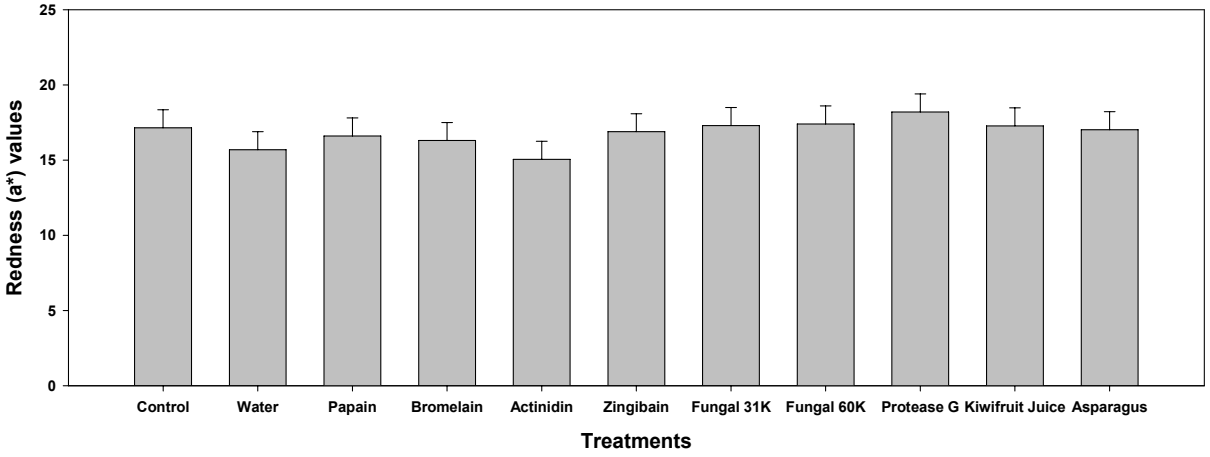
Treatment	Display time (days)					
	1	2	3	4	5	6
Control	38.2	40.3	41.4	42.9	43.8	44.6
Water	40.1	43.2	44.8	45.9	47.4	48.8
Papain	39.1	41.4	43.4	43.7	46.1	46.6
Bromelain	41.4	44.1	45.6	47.2	48.1	49.4
Actinidin	41.3	44.1	45.4	47.6	48.4	49.4
Zingibain	39.6	41.6	42.7	44.2	44.2	45.1
Fungal 31K	40.0	41.7	44.0	44.9	45.7	47.0
Fungal 60K	40.0	41.5	43.1	44.0	44.2	45.7
Protease G	39.4	41.0	42.5	43.1	44.9	45.7
Kiwifruit Juice	38.4	40.5	42.1	42.8	43.8	44.6
Asparagus	41.0	42.5	43.6	44.4	45.0	45.5

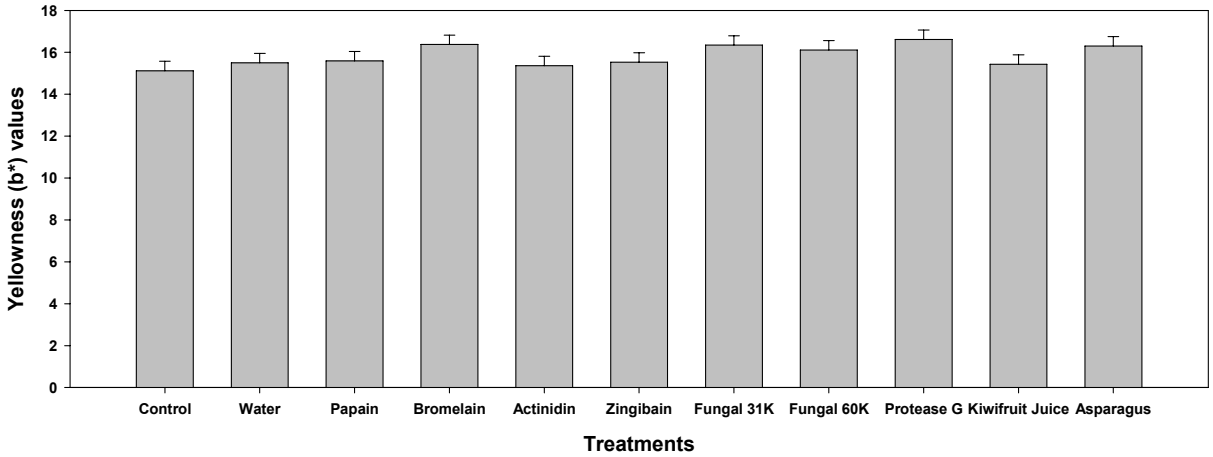
Browning index (SED =0.55)

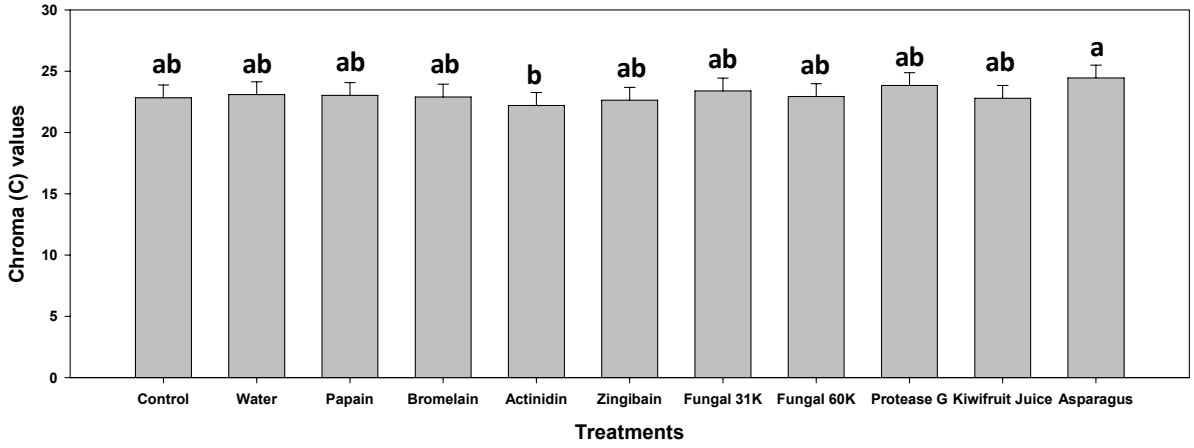
Treatment	Display time (days)					
	1	2	3	4	5	6
Control	5.1	4.3	3.5	3.1	2.9	2.6
Water	4.6	3.6	3.0	2.6	2.4	2.2
Papain	4.6	3.8	3.2	3.0	2.6	2.4
Bromelain	4.8	4.0	3.3	2.8	2.6	2.4
Actinidin	4.2	3.4	2.9	2.5	2.3	2.1
Zingibain	4.5	3.9	3.4	3.0	2.8	2.7
Fungal 31K	4.9	3.9	3.6	3.2	3.0	2.7
Fungal 60K	5.0	3.9	3.5	3.0	2.8	2.6
Protease G	5.3	4.3	3.5	3.1	3.1	2.8
Kiwifruit Juice	4.8	4.0	3.6	3.2	2.9	2.8
Asparagus	4.5	3.6	3.3	3.1	3.0	2.8

2) Main effects of treatments of hot boned beef topsides with enzyme tenderizers on colour parameters (L*, a*, b*, C and h) at 3 weeks post-mortem.








Chroma (C) values
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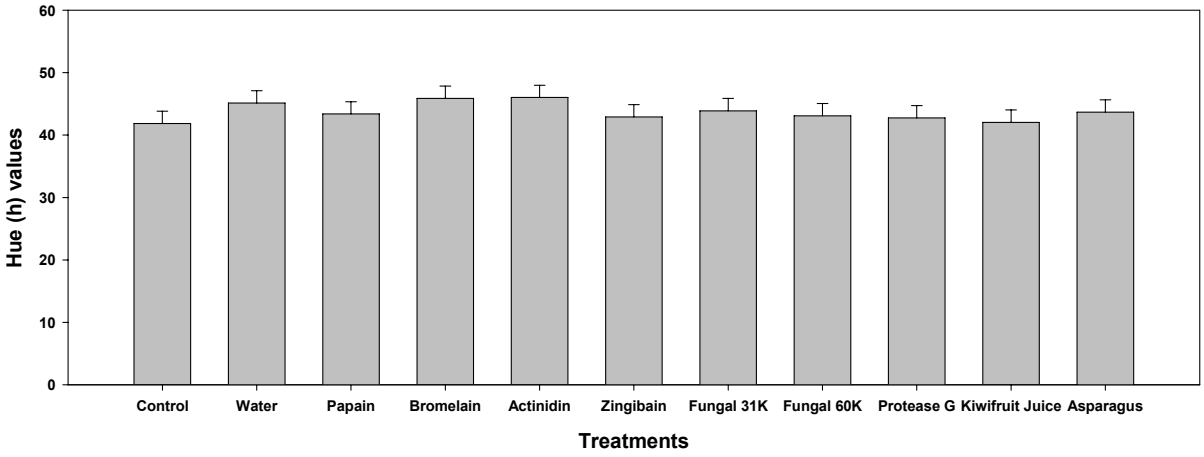
25
20
15
10
5
0
ab
ab
ab
ab
b
ab
ab
ab
ab
ab
a
Control
Water
Papain
Bromelain
Actinidin
Zingibain
Fungal 31K
Fungal 60K
Protease G
Kiwifruit Juice
Asparagus
Treatments


Hue (h) values
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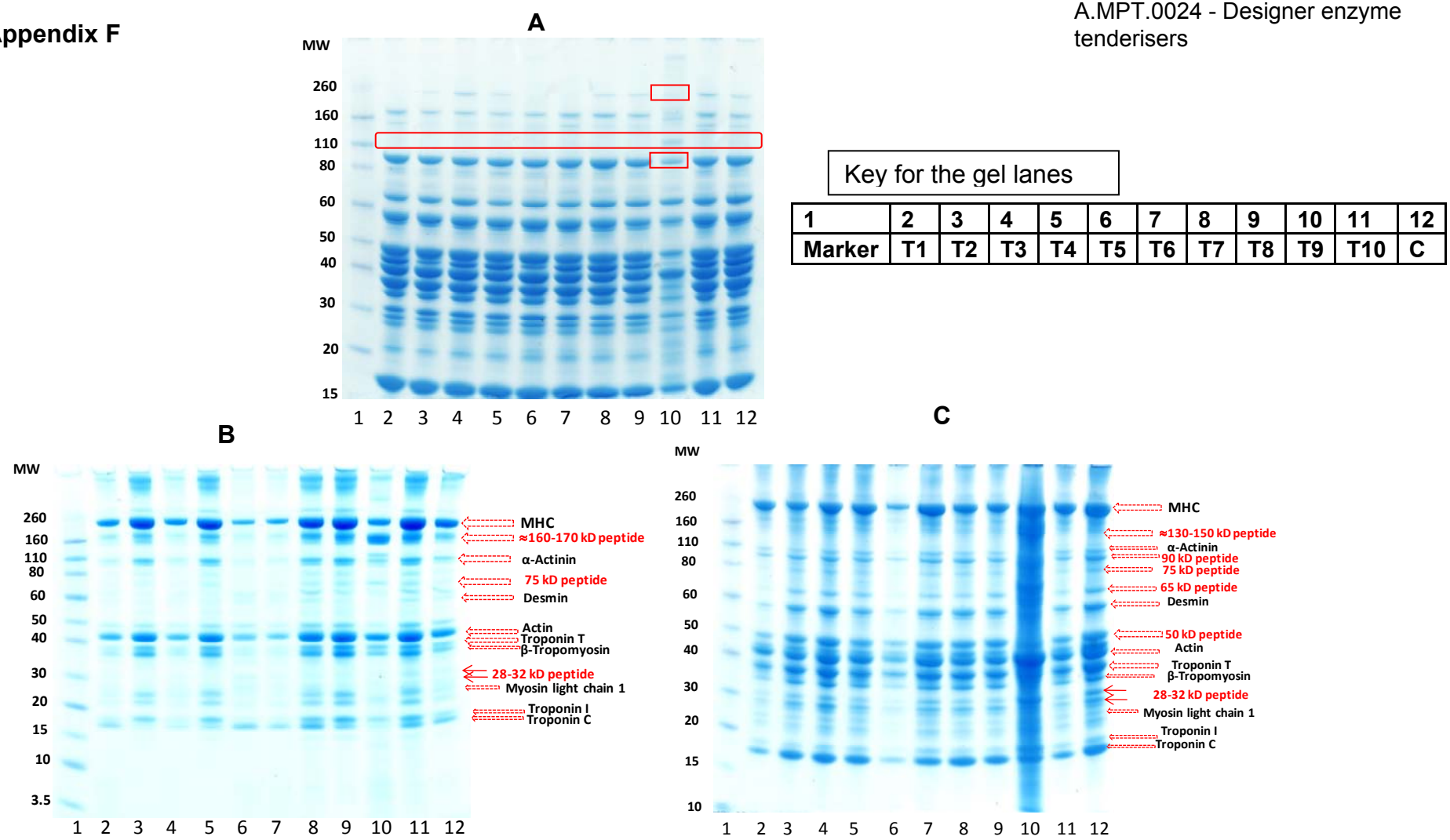
50
40
30
20
10
0
Control
Water
Papain
Bromelain
Actinidin
Zingibain
Fungal 31K
Fungal 60K
Protease G
Kiwifruit Juice
Asparagus
Treatments

Appendix F



Key for the gel lanes

1	2	3	4	5	6	7	8	9	10	11	12
Marker	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	C

Figure 1. SDS-PAGE profile of meat sarcoplasmic proteins from one day post mortem uncooked meat (A); myofibrillar proteins from one day post mortem uncooked meat (B) and myofibrillar proteins extracted from one day post mortem broiled meat (C) from beef topsides injected with several plant and microbial protease preparations as outlined in table3.

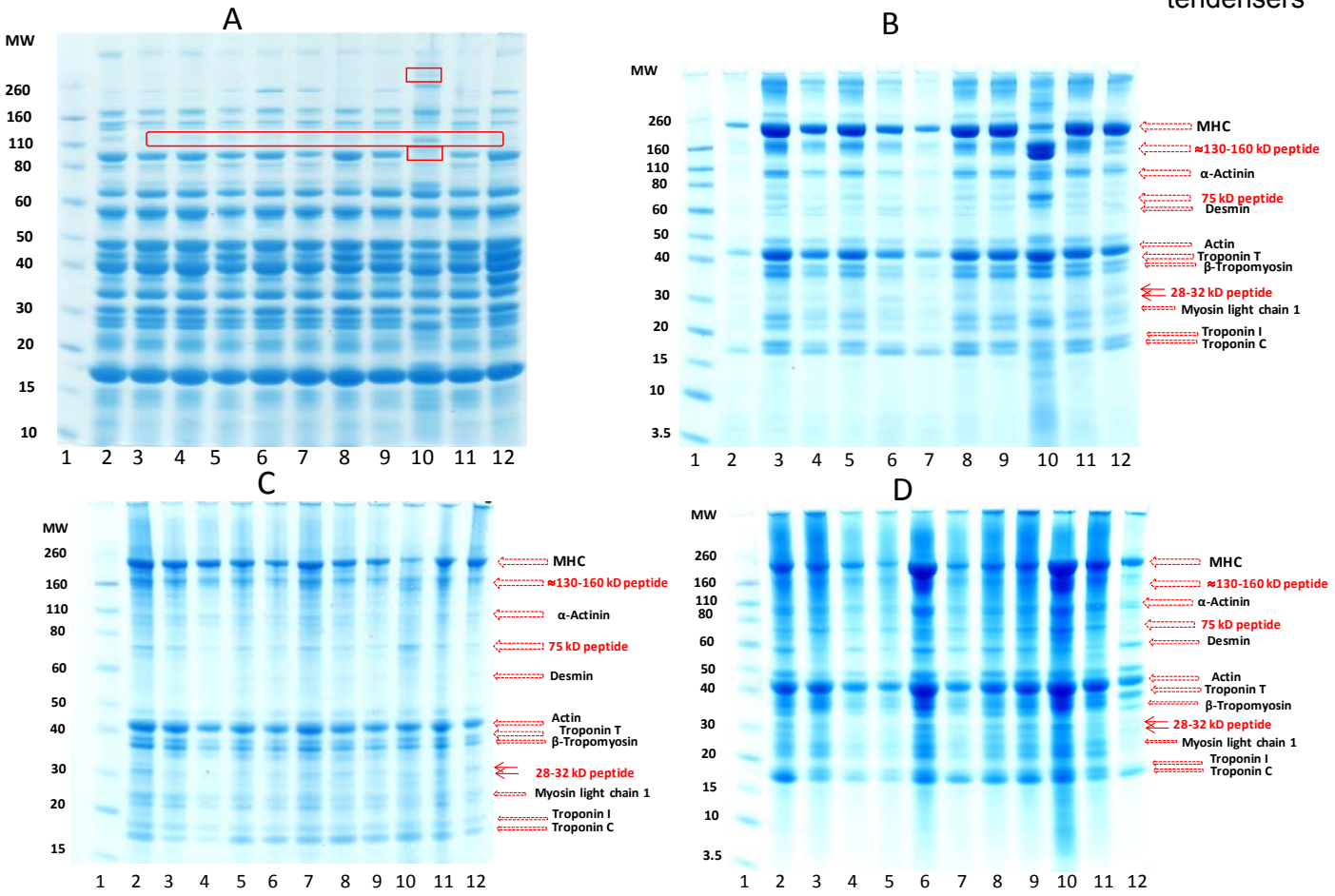


Figure 2. SDS-PAGE profile of meat sarcoplasmic proteins from 3 weeks post mortem uncooked meat (A); myofibrillar proteins from 3 weeks post mortem uncooked meat (B); myofibrillar proteins extracted from 3 weeks post mortem broiled meat (C) and myofibrillar proteins extracted from 3 weeks post mortem roasted meat (D) from beef topsides injected with several plant and microbial protease preparations as outlined in table3.

Acknowledgements

The comments and advice from Dr David Hopkins during the course of this project is gratefully acknowledged. Dr Alan Carne is gratefully thanked for his help and advice on the Biochemical assays. The help of Gary Maclennan, Sonja Lindsay and the support from the management and staff of Alliance Group Ltd (Pukeuri Plant, Oamaru) is greatly acknowledged. The technical assistance of Minh Ha, Clara Bah and Jordan Taylor is gratefully acknowledged.