



Characterisation of kiwifruit and asparagus enzyme extracts, and their activities toward meat proteins

Minh Ha^{a,b}, Alaa El-Din Bekhit^{b,*}, Alan Carne^a, David L. Hopkins^c

^a Department of Biochemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand

^b Department of Food Science, University of Otago, P.O. Box 56, Dunedin, New Zealand

^c NSW Department of Primary Industries, Centre for Red Meat and Sheep Development, P.O. Box 129, Cowra, NSW 2794, Australia

ARTICLE INFO

Article history:

Received 13 May 2012

Received in revised form 24 July 2012

Accepted 5 September 2012

Available online 16 September 2012

Keywords:

Meat

Proteases

Characterisation

Asparagus

Kiwifruit

ABSTRACT

Two plant enzyme extracts from kiwifruit and asparagus were evaluated for their ability to hydrolyse commercially available substrates and proteins present in both beef connective tissue and topside myofibrillar extracts. The results show significant differences in protease activity depending on the assay used. Protease assays with connective tissue and meat myofibrillar extracts provide a more realistic evaluation of the potential of the enzymes for application in meat tenderization. Overall, the kiwifruit protease extract was found to be more effective at hydrolysing myofibrillar and collagen proteins than the asparagus protease extract. The two protease extracts appeared to target meat myofibrillar and collagen proteins differently, suggesting the potential of a synergistic effect of these proteases in improving the tenderness of specific cuts of meat, based on their intrinsic protein composition.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Tenderness plays an important role in dictating the acceptability of red meat. Once the meat is cooked or served in cooked form, many of the appearance attributes become irrelevant and flavour is or can be manipulated with other ingredients in the meal or added flavours. Several publications have reported the willingness of consumers to pay more for meat that has a guaranteed tenderness (Feldkamp, Schroeder, & Lusk, 2005). Therefore, the production of tender meat is important to the meat industry in order to retain consumer confidence in red meat and to maximise financial returns due to the higher retail value of tender meat.

Meat tenderness is affected by several factors that are spread along the production chain (biological, on-farm, processing, and consumer factors) all of which impact on the biophysical and biochemical properties of the muscle post-mortem. Post-mortem protein degradation caused by endogenous proteases during aging is responsible for meat tenderness (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010). However due to anatomical and biological differences, not all of the muscles reach the same level of tenderization during aging and portions of the carcass would be perceived by consumers as tough even after an extended aging period. Higher meat tenderization rates can be achieved by stimulating the activity of endogenous proteases through exposure to relatively high

post-mortem temperatures and/or addition of Ca²⁺, but these treatments often have a negative impact on other quality attributes (e.g. colour and flavour) (Bekhit et al., 2005; Hopkins & Thompson, 2002). The use of exogenous proteases to improve the tenderness of tough meat cuts and potentially increase the monetary value/return of a carcass has attracted much interest recently (Toohey, Kerr, van de Ven, & Hopkins, 2010). Several plant enzymes (such as papain, bromelain and ficin) have been extensively investigated as meat tenderisers. Papain, bromelain and ficin can hydrolyse meat proteins indiscriminately, with most literature reporting papain to have the highest rate of tenderisation at the same concentration. However, these protease enzymes have broad specificities and indiscriminately break down connective tissue and myofibrillar proteins (Ashie, Sorensen, & Nielsen, 2002). This leads to some undesirable attributes in the tenderized meat (mushy texture, bitterness, off flavour). Recent interest in plant proteases has been directed toward proteases from kiwifruit (actinidin, a cysteine protease) which exhibit mild tenderising effects and have other potential beneficial effects on lipid oxidation and colour stability of lamb meat (Bekhit, Han, Morton, & Sedcole, 2007). Commercial actinidin preparations improved the shear force of beef semimembranosus but not the compression values (Toohey, Kerr, van de Ven, & Hopkins, 2011), indicating effective proteolytic activity on the myofibrillar protein fraction and less effective action on the connective tissues. Fresh kiwifruit juice however was reported to be effective in solubilising connective tissues (Mostafaie, Bidmeshkipour, Shirvani, Mansouri, & Chalabi, 2008) and more

* Corresponding author. Tel.: +64 3 479 4994.

E-mail address: aladin.bekhit@otago.ac.nz (Alaa Bekhit).

information on the activities of fresh kiwifruit juice toward meat proteins is needed. A new cysteine protease from asparagus has been reported (Yamaguchi, Yamashita, Takada, & Kiso, 1982), but no information on the potential of this protease as a meat tenderizer is available. In the current study, the proteolytic activities of crude extracts of kiwifruit and of asparagus were characterised using synthetic substrates and meat proteins with the aim of determining their affinities toward different meat proteins and the generation of useful information toward exploiting these proteases as meat tenderizers.

2. Materials and methods

2.1. General

All chemicals used were of analytical reagent grade unless otherwise stated. Fresh Italian (*Agrilepidio*, Cisterna di Latina, Italy) kiwifruit (*Actinidia deliciosa*) and New Zealand asparagus (*Asparagus officinalis*) were obtained from a local market.

2.2. Crude juice extraction

Preparation of in-house enzyme extracts was carried out according to the method outlined by Baker, Boland, Calder, and Hardman (1980) with some modifications. Kiwifruit and asparagus were homogenised with a Sorvall Omni-mixer 17106 polytron (Du Pont Instruments Corp., Wilmington, DE, USA). Juice was extracted from the ground materials by extruding through a triple layer of cheesecloth. Juice was centrifuged at 16,000 g for 5 min at 4 °C. The clarified supernatant was transferred to capped microfuge tubes and stored frozen at –20 °C.

2.3. Total protein determination

The total protein content of the enzyme extracts was determined using a 2D Quant Kit (GE Healthcare, Auckland, NZ) as per the manufacturer's instruction.

2.4. Protein profile of enzyme extracts

Proteins were displayed by one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) using Invitrogen gradient (4–12%) Bis-Tris gels (Invitrogen, Christchurch, NZ). A 10 µl aliquot of each enzyme extract (containing 20 µg protein) was added to 3 µl of Invitrogen SDS sample buffer and 2 µl of Invitrogen reducing agent. The samples were incubated at 90 °C for 10 min and loaded on a gel. Electrophoresis was performed in pre-chilled NuPAGE[®] MES SDS running buffer (1X) at 160 V for 1 h at room temperature. Protein standards (Novex Sharp Pre-stained Protein Standard, Invitrogen, Christchurch, NZ) were included in the gel as a molecular marker. After electrophoresis, the gels were washed three times in Milli-Q water for 5 min each and stained overnight in SimplyBlue[™] SafeStain (Invitrogen) (20 ml per gel) with gentle shaking. The gels were de-stained with Milli-Q water (100 ml per gel) and images were scanned the following day using a CanoScan LiDE 600F scanner.

2.5. Esterase activity assay

The ester hydrolysing activity of the enzyme extracts was investigated using the substrate CBZ-Lys-*p*-nitrophenyl (CBZ-Lys-ONp) ester (Sigma, Saint Louis, MO, USA) as described previously (Han, Morton, Bekhit, & Sedcole, 2009). Hydrolysis of the substrate CBZ-Lys-ONp ester generates *p*-nitrophenol (pNP) with A_{400} absorbance maxima and was quantified with a standard curve of pNP.

Assays were carried out in triplicate over the 5.0–7.0 pH range and 25–85 °C temperature range using 70 mM K₂HPO₄ buffer (pH was adjusted with KOH or H₃PO₄).

The esterase activity of the enzyme extracts was calculated as µmol of pNP generated per minute per ml of enzyme solution. The total protein amount added of the enzyme extracts to the assays was adjusted during protocol development and validation so that the ester hydrolysis activity of the enzyme extracts at all pHs and temperatures could be clearly presented for comparison. The total protein amounts added were 0.2 and 45.5 µg for the kiwifruit and asparagus enzyme extracts, respectively.

2.6. Caseinolytic activity assay

The assay is based on the ability of proteases to hydrolyse fluorescent-labelled casein (Thompson, Saldana, Cong, & Goll, 2000). The substrate used was a green fluorescent-labelled (BODIPY-FL) casein (Molecular Probes, Portland, OR, USA) and the measurements were carried out using a fluorescence plate reader (POLARstar OPTIMA, BMG labtech, Offenburg, Germany). The change in fluorescence at 520 nm following excitation at 485 nm was recorded every 10 s for 1 min. Validation of the assay resulted in the following protocol. The substrate was dissolved respectively in ten different phosphate buffers with pHs ranging from 4.5 to 9.0. The phosphate buffers were validated to buffer adequately over this pH range. Assays were conducted at 5, 25, 35, 45 and 55 °C. Higher temperatures were not possible due to the effect of heat on the structure of the plates.

The fluorescence readings for the reaction steady state (within the first minute of the assay) were used to generate a progress curve (arbitrary fluorescence units vs. time) from which a line slope value was determined. Enzyme unit was calculated as described previously (Lee, Song, Tannenbaum, & Han, 2008). The slope of the line was taken to be the best approximation to the initial velocity that could be measured in the assay at a particular pH and temperature. The enzyme activity of each enzyme extract was determined as the change in arbitrary fluorescent units per minute per ml of enzyme solution. The total protein amount of the enzyme extracts added to the assays was adjusted during protocol development and validation so that the caseinolytic activity of the enzyme extracts at all pHs and temperatures could be clearly presented for comparison. The total protein amount of the enzyme extracts added were 0.1 and 11.4 µg for kiwifruit and asparagus, respectively.

2.7. Collagenolytic activity

The collagenolytic activity of the two enzyme extracts was determined using collagen impregnated with azo-dye (Azocoll) (Sigma, Saint Louis, MO, USA). Azo-dye labelled peptides released into the assay solution from the insoluble Azocoll matrix (in proportion to peptide bonds hydrolysed) were measured at A_{520} . Validation of the assay resulted in the following protocol. Azocoll (25 mg) was suspended in 12.5 ml of phosphate buffer pH 6.0 and stirred for 2 h at room temperature, followed by decanting of the supernatant containing extracted small azo-dye labelled collagen peptides that can interfere with the assay (Chavira, Burnett, & Hageman, 1984). The washing step was repeated. Aliquots (1.0 ml) of the Azocoll suspension (in 12.5 ml) were stored at –20 °C. After temperature equilibration at 25, 35, 45 or 55 °C, 100 µl of an enzyme extract was added to 1.0 ml of Azocoll suspension and the tubes were tumbled at the incubation temperature in a mini Hybaid oven. The samples were then centrifuged at 16,000 g for 3 min and the absorbance (A_{520}) of the supernatants was recorded after 1, 2, 3, 6 and 24 h of incubation. The total protein amount of the enzyme extracts added to the assays was adjusted during protocol

development and validation so that the collagenolytic activity could be clearly presented for comparison. The total protein amount of the enzyme extracts added to the assays was 2.1 and 227.5 μg for kiwifruit and asparagus extracts, respectively.

2.8. Preparation of myofibrillar protein extract from beef topside

The myofibrillar protein extraction procedure used was described previously with modifications (Goll, Young, & Stromer, 1974). Beef topside (0.5 g) was homogenised with a polytron homogenizer at 11,000 rpm for 0.5 min in 5 ml homogenisation buffer (100 mM KCl, 2 mM MgCl_2 , 2 mM EGTA, 1 mM NaN_3 , 20 mM Na_2HPO_4 and 20 mM NaH_2PO_4 , pH 6.8) and 5 μl freshly made 100 mM PMSF (in isopropanol). The homogenate was centrifuged at 1,000g for 10 min at 4 °C. The supernatant was discarded and the pellet was re-suspended and homogenised again in 5 ml of the above buffer containing 100 mM PMSF (5 μl) and 1% (v/v) Triton X-100 (5 μl), for 0.5 min at 11,000 rpm and then centrifuged at 1,500g for 10 min. The pellet was resuspended in 5 ml 50 mM Tris–HCl, pH 6.8, vortexed and then filtered through Whatman No. 1 filter paper. The retentate was freeze-dried and stored at –20 °C as meat myofibrillar protein extract stock material.

2.9. Hydrolysis assays of myofibrillar protein extract from beef topside

Freeze-dried meat myofibrillar protein extract stock was re-suspended in buffer (0.2 mM formic acid titrated with acetic acid to pH 3.0) to 5 mg ml^{-1} and then adjusted to pH 6.0 with 1 M NaOH. Validation of the assay resulted in the following protocol. Aliquots (200 μl) of the above stock meat myofibrillar extract were equilibrated to the assay temperature of 45 °C for 5 min in a Hybaid mini-oven, followed by the addition of an enzyme extract (40 μl). The tubes were then tumbled in a Hybaid mini-oven. Time course samples of the hydrolysis assay were obtained by retrieving aliquots (10 μl) from the tumbled assay tube after 5, 10, 15, 20 and 30 min for the kiwifruit enzyme extract and after 2, 4, 6 and 24 h for the asparagus enzyme extract. To the hydrolysis time course samples, Invitrogen SDS sample buffer (3 μl) and Invitrogen reducing agent (1 μl) were added, mixed and then immediately frozen in dry ice. Samples were then stored at –20 °C until analysed by 1D-SDS–PAGE. The total protein amount of the enzyme extracts added to the assays was adjusted in order to display a gradual hydrolysis of meat myofibrillar proteins on 1D-SDS gels. The total protein amounts of the enzyme extracts added to the assays were 0.08 and 45.6 μg for kiwifruit and asparagus, respectively.

2.10. Collagen protein extraction from beef tendon

Extraction of collagen protein was carried out as previously described with some modifications (Davis & Mackle, 1981). Beef tendon (2 g), was washed with Milli-Q water, then diced (5 mm cubes) and stirred in 500 ml 0.2 M NaCl, 0.02 M Na_2HPO_4 , pH 7.4, for 16 h. The material was centrifuged for 30 min at 22,000g. The washing step was repeated on the pellet. The pellet was subjected to a similar process with 500 ml 0.5 M NaCl, 0.02 M Na_2HPO_4 , pH 7.4, and then with 500 ml 0.01 M Tris–HCl buffer with 0.167 M EDTA, pH 7.4, followed by two washes with Milli-Q water for 3 h each time. All of the steps were carried out at 4 °C. The tendon residue was then extracted with 100 ml chloroform:methanol (2:1) three times for 16 h each time at room temperature, followed by washing twice with 100 ml acetone and drying with 100 ml diethyl ether. The dried material was then suspended in 100 ml freshly prepared phenol:acetic acid:water (1:1:1, v/v/v) and gently shaken for three days at room temperature. Following this, the material was centrifuged at 1,000g for 30 min. The supernatant was extracted three times with an equal volume of diethyl ether, after which the eth-

real layer was discarded while the aqueous and interface layers were collected. The collagen protein extracted from 2 g wet weight of tendon ended up in a volume of 20 ml and aliquots of the material were stored frozen at –20 °C for assays. The final total protein concentration of the collagen extract was 8 mg/ml.

2.11. Collagen protein extract for hydrolysis assays

Collagen protein extract was diluted with Milli-Q water (1:1) and the pH was adjusted to 6.0 using 4 M NaOH. Validation of the assay resulted in the following protocol. Aliquots (200 μl) of the stock connective tissue extract were equilibrated to the assay temperature in a Hybaid oven. Enzyme extract (40 μl) was added to the tubes which were then tumbled in a Hybaid oven at 55 °C. Time course hydrolysis assay samples were obtained by removing 4 μl aliquots of the tumbled mix after 5, 10, 15, 20 and 30 min for the kiwifruit preparation and after 0.5, 1, 2, 3 and 24 h for the asparagus preparation. The assay samples were frozen on dry ice immediately after addition of Invitrogen SDS sample buffer (3 μl), Invitrogen reducing agent (1 μl) and Milli-Q water (6 μl). The samples were then stored at –20 °C until analysis by SDS–PAGE. The total protein amounts added of the enzyme extracts to the assays were 0.08 and 45.6 μg for kiwifruit and asparagus, respectively.

2.12. Protein identification by matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS)

Stained protein bands were excised from SDS–PAGE gels and cut into 1 mm cubes. The gel bands were destained with 80% acetonitrile for 10 min and washed with ammonium bicarbonate (ABC) buffer 2X (100 mM, pH 8.0) for 20 min. The gel pieces were dehydrated by two consecutive incubations with 80% acetonitrile and reduced with 10 mM DTT in ABC buffer 1X. Gel bands were then incubated in 20 mM iodoacetic acid in ABC buffer 1X in the dark for 15 min, and washed with 80% acetonitrile four times for

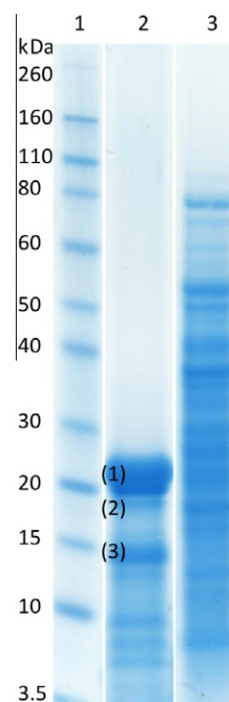


Fig. 1. Enzyme extract protein profiles displayed by 1D-SDS–PAGE. Indicated on the gel are the protein molecular marker sizes, the band reference numbers (in brackets) for mass spectrometry analysis. Lane 1 = molecular marker, lane 2 = kiwifruit extract; lane 3 = asparagus extract.

Table 1
 Mascot search results against NCBI nr and SwissProt databases for the hydrolysed protein fragment analysis of the meat myofibril and meat connective tissue by kiwifruit and asparagus enzyme extracts.

Gel band	References	Protein identification	Intact mass (Da)	Apparent mass on gel (Da)	Species	Total ion score	Accession No.	Database
1		Actinidin	23,543	23,500	Actinidia chinensis	567	157829826	NCBI nr
2		Thaumatin-like protein	24,205	20,000	Actinidia deliciosa	556	TLP_ACTDE	SwissProt
3		Kiwellin	19,955	23,000	Actinidia deliciosa	116	KIWEL_ACTDE	SwissProt
		Kirola	17,393	15,000	Actinidia deliciosa	105	KIRO_ACTDE	SwissProt
4		Myosin heavy chain-1	222,851	220,000	Bos Taurus	889	MYH1_BOVIN	SwissProt
		Myosin heavy chain-2	223,180	220,000	Bos Taurus	764	MYH2_BOVIN	SwissProt
5		Myosin heavy chain-7	223,091	130,000	Bos Taurus	662	MYH7_BOVIN	SwissProt
6		Myosin binding protein C	128,214	100,000	Bos Taurus	115	160425243	NCBI
7		α -actinin-2	103,713	95,000	Bos Taurus	457	ACTN2_BOVIN	SwissProt
		α -actinin-3	103,086	95,000	Bos Taurus	266	ACTN3_BOVIN	SwissProt
8		ATPase-1	109,220	82,000	Bos Taurus	207	AT2A1_BOVIN	SwissProt
9		Myosin heavy chain-7	223,091	65,000	Bos Taurus	197	MYH7_BOVIN	SwissProt
10		Calsequestrin-1	45,619	43,000	Bos Taurus	152	118150866	NCBI
11		Actin	42,024	40,000	Bos Taurus	638	ACTS_BOVIN	SwissProt
12		Tropomyosin β chain	32,817	37,500	Bos Taurus	284	TPM2_BOVIN	SwissProt
13		Tropomyosin α chain	32,675	33,000	Bos Taurus	230	TPM1_BOVIN	SwissProt
14		Myosin light chain	20,919	21,000	Bos Taurus	257	MYL1_BOVIN	SwissProt
		Troponin I	20,828	21,000	Bos taurus	87	300797481	NCBI
15		Myosin light chain-2	19,000	20,000	Bos taurus	181	MLRS_BOVIN	SwissProt
		Troponin C	18,098	20,000	Mus musculus	113	TNNC2_MOUSE	SwissProt
16		Collagen type I α -1	138,854	280,000	Bos taurus	110	CO1A1_BOVIN	SwissProt
17		Collagen type I chain A	95,928	250,000	Bos taurus	73	109156929	NCBI
18		Collagen type I α -1	138,854	150,000	Bos taurus	94	CO1A1_BOVIN	SwissProt
19		Collagen type I chain B	93,272	130,000	Bos taurus	79	109156930	NCBI
20		Myosin heavy chain-7	223,091	140,000	Bos taurus	375	MYH7_BOVIN	SwissProt
		Myosin heavy chain-1	222,851	140,000	Bos taurus	363	MYH1_BOVIN	SwissProt
		Myosin heavy chain-2	223,180	140,000	Bos taurus	361	MYH2_BOVIN	SwissProt
21		Myosin heavy chain-1	222,851	85,000	Bos taurus	381	MYH1_BOVIN	SwissProt
22		Myosin heavy chain-1	222,851	70,000	Bos taurus	574	MYH1_BOVIN	SwissProt
		Myosin heavy chain-2	223,180	70,000	Bos taurus	573	MYH2_BOVIN	SwissProt
23		Myosin heavy chain-7	223,091	67,000	Bos taurus	363	MYH7_BOVIN	SwissProt
		Myosin heavy chain-1	222,851	67,000	Bos taurus	224	MYH1_BOVIN	SwissProt
		Myosin heavy chain-2	223,180	67,000	Bos taurus	222	MYH2_BOVIN	SwissProt
24		Myosin heavy chain-1	222,851	65,000	Bos taurus	404	MYH1_BOVIN	SwissProt
		Myosin heavy chain-7	223,091	65,000	Bos taurus	93	MYH7_BOVIN	SwissProt
25		Myosin heavy chain-1	222,851	57,000	Bos taurus	468	MYH1_BOVIN	SwissProt
		Myosin heavy chain-2	223,180	57,000	Bos taurus	415	MYH2_BOVIN	SwissProt
26		Actinidin	42,145	22,000	Actinidia chinensis	198	ACTN_ACTCH	SwissProt
		Kiwellin	19,955	22,000	Actinidia deliciosa	75	KIWEL_ACTDE	SwissProt
27		Thaumatin-like protein	24,205	20,000	Actinidia deliciosa	250	TLP_ACTDE	SwissProt
28		Actin	41,982	15,000	Bos taurus	98	ACTA_BOVIN	SwissProt
		Myosin-2	223,180	15,000	Bos taurus	71	MYH2_BOVIN	SwissProt
29		Myosin heavy chain-7	223,091	220,000	Bos taurus	316	MYH7_BOVIN	SwissProt
		Myosin heavy chain-1	222,851	220,000	Bos taurus	239	MYH1_BOVIN	SwissProt
30		Myosin heavy chain-1	222,851	160,000	Bos taurus	525	MYH1_BOVIN	SwissProt
		Myosin heavy chain-2	223,180	160,000	Bos taurus	447	MYH2_BOVIN	SwissProt
31		Myosin heavy chain-1	222,851	150,000	Bos taurus	542	MYH1_BOVIN	SwissProt
		Myosin heavy chain-2	223,180	150,000	Bos taurus	541	MYH2_BOVIN	SwissProt
32		Myosin heavy chain-1	222,851	140,000	Bos taurus	572	MYH1_BOVIN	SwissProt
		Myosin heavy chain-2	223,180	140,000	Bos taurus	570	MYH2_BOVIN	SwissProt
33		α -actinin-3	103,086	95,000	Bos taurus	232	ACTN3_BOVIN	SwissProt
		α -actinin-2	103,713	95,000	Bos taurus	181	ACTN2_BOVIN	SwissProt
		Myosin heavy chain-1	222,851	95,000	Bos taurus	49	MYH1_BOVIN	SwissProt
34		Collagen type I α -1	138,854	140,000	Bos taurus	1377	75775290	NCBI nr
		Collagen type I chain A	95,928	140,000	Bos taurus	115	109156929	NCBI nr
35		Collagen type I α -1	138,854	130,000	Bos taurus	937	75775290	NCBI nr
		Collagen type I chain A	95,928	130,000	Bos taurus	226	109156929	NCBI nr
36		Collagen type I α -1	138,854	120,000	Bos taurus	1180	75775290	NCBI nr
		Collagen type I chain A	95,928	120,000	Bos taurus	234	109156929	NCBI nr
37		Collagen type I α -1	138,854	110,000	Bos taurus	1,380	75775290	NCBI nr
		Collagen type I chain A	95,928	110,000	Bos taurus	170	109156929	NCBI nr
38		Collagen type I α -1	138,854	90,000	Bos taurus	1,078	75775290	NCBI nr
		Collagen type I chain A	95,928	90,000	Bos taurus	295	109156929	NCBI nr
39		Collagen type I α -1	138,854	75,000	Bos taurus	978	75775290	NCBI nr
		Collagen type I chain A	95,928	75,000	Bos taurus	255	109156929	NCBI nr
40		Collagen type I α -1	138,854	70,000	Bos taurus	940	75775290	NCBI nr
		Collagen type I chain A	95,928	70,000	Bos taurus	143	109156929	NCBI nr
41		Collagen type I α -1	138,854	57,500	Bos taurus	263	75775290	NCBI nr
		Fibromodulin	42,906	57,500	Bos taurus	102	27806623	NCBI nr
		Collagen type I chain A	95,928	57,500	Bos taurus	77	109156929	NCBI nr
		Prolargin	43,655	57,500	Bos taurus	56	PRELP_BOVIN	SwissProt

Table 1 (continued)

Gel band References	Protein identification	Intact mass (Da)	Apparent mass on gel (Da)	Species	Total ion score	Accession No.	Database
42	Collagen type I α -1	138,854	52,000	Bos taurus	393	75775290	NCBIInr
	Collagen type I α -2	128,985	52,000	Bos taurus	293	2967448	NCBIInr
	Fibromodulin	42,906	52,000	Bos taurus	236	27806623	NCBIInr
43	Collagen type I α -1	138,854	37,500	Bos taurus	652	75775290	NCBIInr
	Collagen type I chain A	95,928	37,500	Bos taurus	66	27806623	NCBIInr
	Collagen type I α -2	128,985	37,500	Bos taurus	46	2967448	NCBIInr
44	Collagen type I α -1	138,854	36,000	Bos taurus	545	75775290	NCBIInr
45	Collagen type I α -2	128,985	32,000	Bos taurus	584	27806623	NCBIInr
	Collagen type I chain B	93,272	32,000	Bos taurus	124	109156930	NCBIInr
	Collagen type I α -1	138,854	32,000	Bos taurus	62	75775290	NCBIInr
46	Collagen type I α -1	138,854	78,000	Bos taurus	102	CO1A1_BOVIN	SwissProt
47	Collagen type I α -1	138,854	69,000	Bos taurus	105	CO1A1_BOVIN	SwissProt
48	Fibromodulin	43,010	54,000	Bos taurus	127	FMOD_BOVIN	SwissProt
	Collagen type I α -1	138,854	54,000	Bos taurus	106	CO1A1_BOVIN	SwissProt
49	Collagen type I α -1	138,854	51,500	Bos taurus	140	CO1A1_BOVIN	SwissProt
50	Collagen type I α -1	138,854	45,000	Bos taurus	77	CO1A1_BOVIN	SwissProt
51	Collagen type I α -1	138,854	37,500	Bos taurus	121	CO1A1_BOVIN	SwissProt

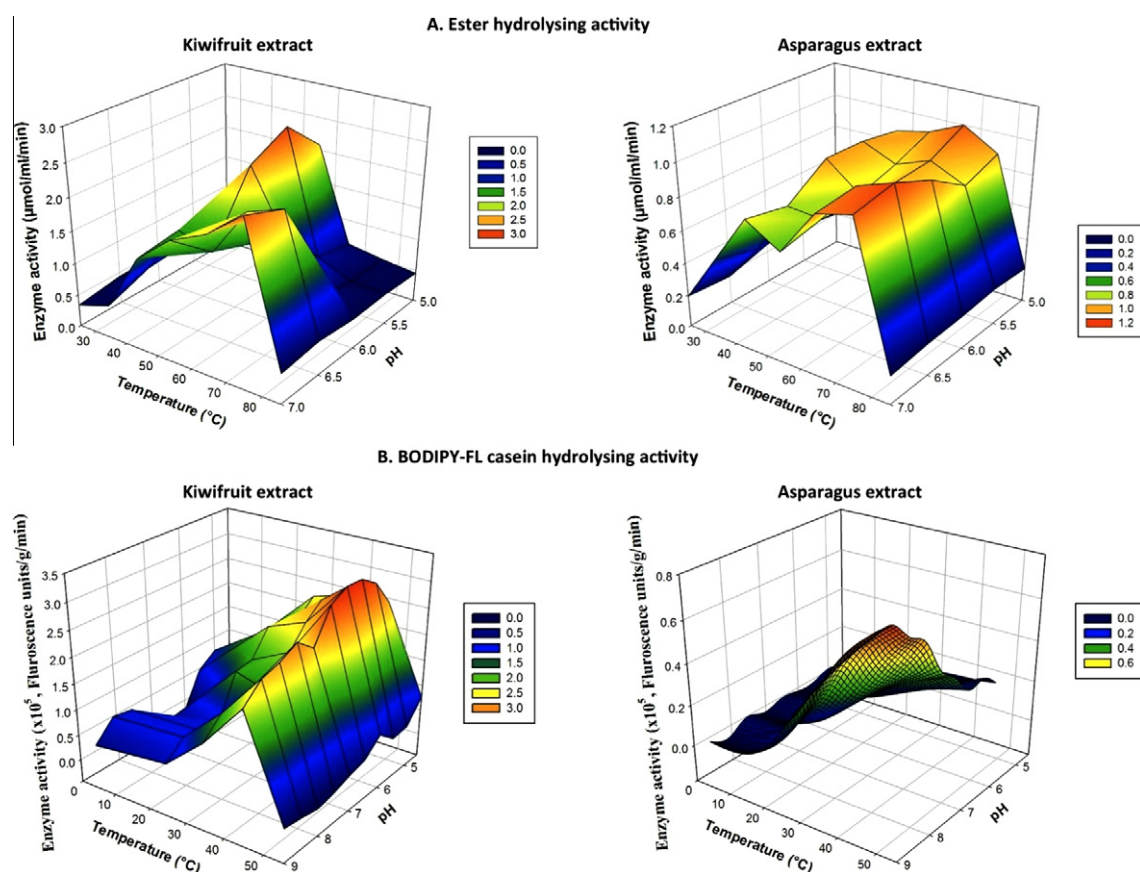


Fig. 2. Enzyme activity profiles of in-house prepared kiwifruit and asparagus enzyme extracts. (A) Ester hydrolysing activity. (B) BODIPY-FL casein hydrolysing activity.

10 min each, and in ABC buffer 1X for 20 min. The gel bands were then vacuum dried. Subsequently, the gel pieces were rehydrated and incubated with trypsin in ABC buffer 1X (trypsin/protein ratio of 1:2) at 37 °C overnight. The gel bands were then incubated for 30 min with 80% acetonitrile in 0.1% trifluoroacetic acid (Shevchenko et al., 2001). The supernatants containing digested peptides were retained for matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS was carried out in the Centre for Protein Research, Department

of Biochemistry, University of Otago. The spectra produced by MS/MS were exported to GPS Explorer™ Software for data analysis. The Mascot search engine (<http://www.matrixscience.com>) was used to search the mass spectrometry peak data against both the SwissProt and NCBIInr amino acid sequence databases. A general template was used to define the parameters of the database searches, which included the allowance of the variable protein modifications carboxyamidomethyl cysteine and oxidised methionine. The parameters which were altered were; a precursor

Table 2
Hydrolytic activities of Kiwifruit and asparagus enzyme extracts.

Enzyme extracts	Esterolytic activity using CBZ-Lys-ONp		Caseinolytic activity using BODIPY-FL casein			Collagenolytic activity using Azocoll		
	Total protein per assay (μg)	Specific activity (μmol min ⁻¹ mg ⁻¹ protein)	Total protein per assay (μg)	Specific activity (Δfluorescence min ⁻¹ mg ⁻¹ protein)	V _{max} (Δfluorescence min ⁻¹)	K _M (μM)	Total protein per assay (μg)	Specific activity (ΔA ₂₂₀ min ⁻¹ mg ⁻¹ protein)
Kiwifruit	0.2	249.7 ± 26.7 ^a	0.1	1.1 × 10 ⁶ ± 0.19 × 10 ^{6a}	196.1 ± 12.8 ^a	1.4 ± 0.2 ^a	2.1	1.2 ± 0.2 ^a
Asparagus	45.5	1.0 ± 0.3 ^b	11.4	2.8 × 10 ³ ± 0.49 × 10 ^{3b}	23.8 ± 3.1 ^b	0.2 ± 0.4 × 10 ^{-1b}	227.5	3.6 × 10 ⁻³ ± 0.6 × 10 ^{-3b}

^{a,b} Within each column, means with different superscripts are different at $P < 0.05$.

tolerance of 75 ppm, a fragment tolerance of 0.4 Da, peptide charges of +1 and a maximum of three missed cleavages. Protein identity was only reported when the ion score threshold was at least 45, which indicates identity or extensive homology ($p < 0.05$).

2.13. Statistical analysis

Statistical analysis was performed with Minitab® Software (Version 16.0, Minitab Inc., Pennsylvania, USA). The results from the protease assays were from three independent experiments in which the measurements were carried out in triplicates. The statistical differences between the means were determined by *T* test and the results are presented as means ± SD.

3. Results and discussion

3.1. Protein profile of the enzyme extracts

The enzyme extracts were initially subjected to 1D-SDS-PAGE analysis (Fig. 1) to provide information on the proteins present in the preparations. The enzyme extracts displayed a complex protein profile on SDS-PAGE, indicating that many protein species were present in the crude juice extracts. A dominant band observed in lane 2 corresponding to a mass of 23.5 kDa was confirmed by mass spectrometry as actinidin (Table 1). The protein display shows that actinidin is only a portion of the total protein complement, in agreement with previous studies in the literature, which have identified several proteases and multiple actinidin isoforms in kiwifruit extract using chromatographic approaches. By means of anion exchange high resolution liquid chromatography, four actinidin isoforms with the same molecular weight were isolated (Tello-Solis, Valle-Guadarrama, & Hernandez-Arana, 1995). A later study found that actinidin contained up to ten different isoforms with pI ranging from 3.9–9.3 (Nieuwenhuizen et al., 2007). The acidic isoforms appeared to have different substrate specificities and pH optima (Sugiyama, Ohtsuki, Sato, & Kawabata, 1996). Furthermore, mass spectrometry results (Table 1) revealed the presence of other proteins namely thaumatin-like protein (24 kDa), kiwellin (20 kDa) and kirola (17.4 kDa), in agreement with other studies (D'Avino et al., 2011; Tamburrini et al., 2005). The asparagus extract contained proteins with molecular weights ranging from 5–75 kDa with a cysteine protease expected to be approximately 28 kDa (Yonezawa, Kaneda, & Uchikoba, 1998). Mass spectrometry of the asparagus protein bands generated inconclusive results (data not shown), suggesting a need of further purification in order to obtain a more pure cysteine enzyme extract for mass spectrometry analysis.

3.2. Esterolytic, caseinolytic and collagenolytic activity of the enzyme extracts

Ester bond containing substrates are often used as a preliminary indication of the catalytic activity of various proteases. The ester hydrolysis activity of the kiwifruit and asparagus enzyme extracts were investigated over the pH 5–7 range and temperature 25–85 °C range using the commercially available substrate CBZ-Lys-ONp (Sigma-Aldrich) (Fig. 2A). While the asparagus enzyme extract was catalytically active over the entire assay pH range and most of the assay temperature range (45–75 °C), the kiwifruit extract hydrolysed the ester substrate over a narrower range of pH and temperature. The activity profile of the kiwifruit extract, which exhibited two hydrolysis maxima (at pH 5.5, 45 °C and at pH 7.0, 75 °C), was likely due to the presence of various proteases, and actinidin isoforms as discussed earlier.

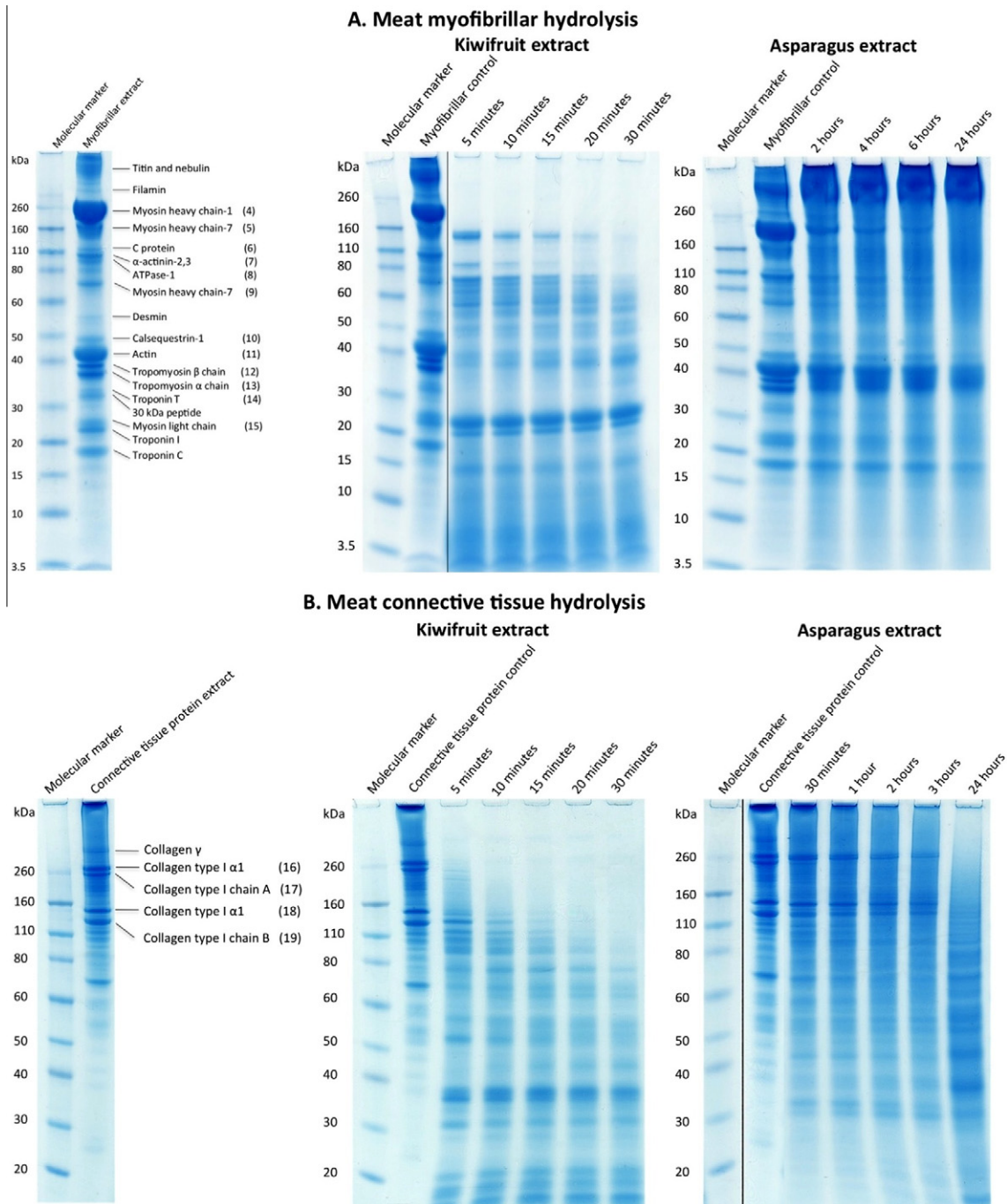


Fig. 3. Meat protein hydrolysing activity of the kiwifruit and asparagus enzyme extracts analysed with 1D-SDS-PAGE. (A) Meat myofibrillar protein hydrolysis time course assays. (B) Connective tissue protein hydrolysis time course assays.

In order to assess the amide bond hydrolysing activity of the two enzyme extracts, a commercially available BODIPY-FL casein was employed (Fig. 2B). The caseinolytic activity of the enzyme extracts were examined over the pH range of 4.5–8.5 and temperature range of 5–55 °C. Both of the enzyme extracts were catalytically active over the entire assay pH range, however, temperature appeared to affect the enzyme activity differently in the two enzyme extracts. While the caseinolytic activity of the kiwifruit extract was detectable over most of the assay temperature range and peaked at 45 °C, the asparagus enzyme extract was only active at temperatures greater than 25 °C.

Similar to the esterolytic and caseinolytic activities, the collagenolytic activity of the asparagus enzyme extract was limited com-

pared to that of the kiwifruit enzyme extract (Table 2). The collagenolytic specific activity of the asparagus enzyme extract was 300-fold less than that of the kiwifruit enzyme extract. However, because these enzyme extracts were crude protease preparations, this low specific activity was most likely caused by a significantly higher total protein (not necessarily protease) content per assay of the asparagus enzyme extract (227.5 μ g) compared to 2.1 μ g of the kiwifruit extract.

The differences in hydrolytic activities of the two enzyme extracts presented in this study are potentially beneficial in that more than one exogenous tenderizer can be used to target different meat proteins, in order to achieve desirable tenderness level in a meat tenderization application. The present study is the first study

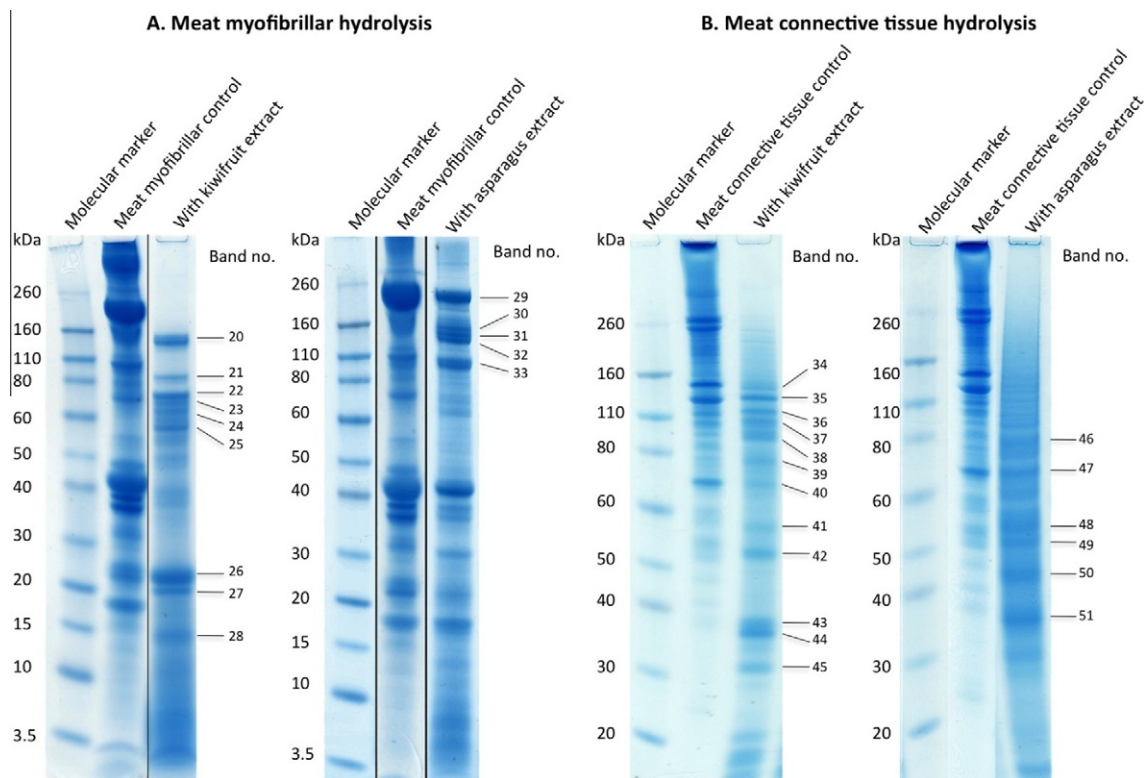


Fig. 4. Meat protein hydrolysis time course assay with kiwifruit and asparagus enzyme extracts and analysed by 1D-SDS-PAGE. Indicated on the gels are the molecular protein marker sizes, the band reference numbers (in brackets) for mass spectrometry analysis. (A) Control and hydrolysed meat myofibril and (B) Control and hydrolysed meat connective tissue.

to have examined comparatively the catalytic activity of these enzyme extracts under a wide range of pH and temperature, and thus provides a more comprehensive picture of the hydrolytic activity of the two enzyme extracts. This study also contributes to an understanding of asparagus protease hydrolytic activity that has not attracted much investigation previously.

3.3. Kinetic parameters the enzyme extracts

BODIPY-FL casein was employed to obtain kinetic parameters for the two enzyme extracts shown in Table 2. While maximum velocity ($V_{\max}$) of the kiwifruit extract is higher than that of the asparagus extract, the K_M value (the substrate concentration at which the reaction rate is half of $V_{\max}$) of the asparagus extract is significantly lower, indicating that asparagus protease had a higher specificity for casein compared to protease(s) present in the kiwifruit extract. This difference in substrate binding affinity is influenced by differences in the amino acid sequences of the proteases as suggested previously (Baker et al., 1980). Using various ester and amide bond-containing substrates to obtain kinetic parameters of papain-family proteases, previous studies have shown that the K_M value of an enzyme varied widely with a change in substrate (Baker et al., 1980). Our kinetic investigation on well-studied members of the papain-family proteases in conjunction with the current study found that, when using casein as substrate, the asparagus enzyme extract had the same K_M value as that of papain (Ha, Bekhit, Carne, & Hopkins, 2012). These results illustrate that although the asparagus enzyme extract exhibited relatively low ester hydrolysing and caseinolytic activity, it can have a high substrate binding affinity and thus be potentially useful in various applications. For example, the low catalytic activity of the asparagus enzyme extract can be advantageous to avoid over-tenderiza-

tion resulting in “mushy meat”, as encountered with papain in a meat tenderization application (Ashie et al., 2002).

3.4. The efficacy of enzyme extracts in hydrolysing beef myofibrillar and tendon collagen protein extract

Hydrolysis of meat myofibrillar and collagen proteins has been regarded as the principle basis of meat tenderization (Bailey & Light, 1989). Degradation of these meat proteins was shown to associate with a lower shear force value (Han et al., 2009) and can be used as an indication for meat tenderness level. In this study, extracted meat myofibrillar and collagen proteins were subjected to time-course hydrolysis by the two enzyme extracts and displayed on SDS-PAGE (Fig. 3). The major bands from the hydrolysed samples were excised (Fig. 4) and subjected to an in-gel proteolysis digestion/peptide extraction/mass spectrometry workflow, followed by informatic analysis using international databases, of partial amino acid sequences generated by mass spectrometry, to obtain protein homologue identity.

The major protein bands identified in the meat myofibrillar extract by mass spectrometry (Table 1) were myosin heavy chain (223 kDa), C protein (128 kDa), α -actinin (103 kDa), actin (42 kDa), α - and β -tropomyosin (33 kDa), troponin T (30.5 kDa), 30 kDa protein species, myosin light chain (25 kDa), troponin I (22 kDa) and troponin C (17.8 kDa). In addition, other meat myofibrillar protein bands were present on the SDS-PAGE and could be tentatively identified as: titin and nebulin (650–3200 kDa), filamin (280 kDa) and desmin (53 kDa), based on apparent molecular weights reported in the literature (Claeys, Uytterhaegen, Buts, & Demeyer, 1995).

The two enzyme extracts appeared to hydrolyse meat myofibrillar proteins at different rates and target different proteins. When compared to other well-studied members of papain family proteases, such as papain and bromelain, the kiwifruit enzyme extract

exhibited comparable meat protein hydrolytic activities. Our previous study has found that under similar catalytic conditions, papain and bromelain were able to hydrolyse actomyosin, titin and nebulin (Ha et al., 2012). Findings in this study demonstrated that the kiwifruit extract hydrolysed the aforementioned proteins, as well as filamin, actinin and desmin, as shown by 1D SDS–PAGE (Fig. 3A) and confirmed with mass spectrometry (Table 1), in agreement with findings in previous studies (Christensen et al., 2009; Han et al., 2009). Furthermore, the kiwifruit extract also appeared to have a similar meat myofibril hydrolytic activity to that of a commercially prepared actinidin preparation as presented in another study reported from our group (Ha et al., 2012). Nonetheless, this in-house juice extract exhibited a much higher catalytic rate, suggesting enzyme activity interference of added compounds, such as dextran, in the commercial protease preparation. The asparagus enzyme extract hydrolysed myofibrillar proteins at a much slower rate, which is consistent with the caseinolytic enzyme activity result presented earlier. The asparagus extract targeted mainly the myosin heavy chain, however, an extended incubation of twenty-four hours of meat myofibrillar proteins with the asparagus extract resulted in degradation of all myofibrillar proteins except troponin C.

The extracted collagen proteins displayed by SDS–PAGE (Fig. 3B) and the results obtained from the mass spectrometry analysis (Table 1) indicate the presence of several collagen subtypes previously reported to be associated with meat connective tissue. Type I collagen is reported to exist as a heterotrimer which consists of two identical α -1 chains and an α -2 chain (Vuorio & de Crombrughe, 1990). Type I collagen is found in most connective tissues (van der Rest & Garrone, 1991). In this current study, three types of tropocollagen, α -1, chain A and chain B with the apparent mass of 270, 250 and 135 kDa respectively were identified. It has been shown that the electrophoretic mobility of collagen α chains is lower than that of globular proteins with similar molecular weights (140 kDa) and that the collagen α -2 chain had a higher electrophoretic mobility than collagen α -1 chain (van der Rest & Garrone, 1991) which was ascribed to a small difference (6 kDa) in the molecular weight of the two chains (Sykes & Bailey, 1971).

The collagenolytic activity of the two enzyme extracts was similar to their myofibril hydrolysing and collagenolytic (with Azocoll) activities, in that the kiwifruit extract was able to hydrolyse most proteins in the collagen extract after a five minute incubation, whereas the asparagus extract required an incubation of up to twenty-four hours for a complete hydrolysis of all collagen proteins. The presence of collagen type I α -1 in most of the excised bands confirmed that this protein was the most abundant collagen protein and was more extensively hydrolysed by the kiwifruit extract than by the asparagus extract.

The current study, using SDS–PAGE and mass spectrometry, identifies specific hydrolysis of various meat proteins by the two enzyme extracts. This study is also only one of a few reports that use SDS–PAGE to display meat protein hydrolysis by exogenous enzymes and the first to use mass spectrometry to identify hydrolysed peptides by these enzyme extracts.

4. Conclusions

The catalytic activity of the asparagus enzyme extract was examined under a wide range of pH and temperature conditions and its meat protein hydrolysing activity was investigated comparatively with well-studied members of the cysteine proteases such as actinidin. This has extended our understanding of the potential of an asparagus enzyme extract in a meat tenderizing application as well as identified the hydrolytic activity of proteases from kiwifruit toward myofibrillar and connective

tissue proteins. The current study has shown the difference in meat myofibrillar and connective tissue protein hydrolytic activities between the kiwifruit and asparagus enzyme extracts, and as such it is conceivable to develop preparations of these enzyme extracts that could be used to improve the tenderness of specific cuts of meat, based on their intrinsic protein composition.

Acknowledgements

We acknowledge funding support from Meat and Livestock Australia (Project No: A.MPT.0024) and assistance from the Centre for Protein Research, Department of Biochemistry, University of Otago, with mass spectrometry.

References

- Ashie, I. N. A., Sorensen, T. L., & Nielsen, P. M. (2002). Effects of papain and a microbial enzyme on meat proteins and beef tenderness. *Journal of Food Science*, 67, 2138–2142.
- Bailey, A. J., & Light, N. D. (1989). *Connective tissue in meat and meat products*. Essex, UK: Elsevier Science Publishers, Ltd.
- Baker, E. N., Boland, M. J., Calder, P. C., & Hardman, M. J. (1980). The specificity of actinidin and its relationship to the structure of the enzyme. *Biochimica et Biophysica Acta*, 616, 30–34.
- Bekhit, A. E. D., Han, J., Morton, J., & Sedcole, R. (2007). Effect of kiwifruit juice and water pre-rigor infusion on lamb quality. In *Proceedings of the 53rd International Conference of Meat Science and Technology* (pp. 377–378). Beijing, China.
- Bekhit, A. E. D., Ilian, M. A., Morton, J. D., Vanhanan, L., Sedcole, J. R., & Bickerstaffe, R. (2005). Effect of calcium chloride, zinc chloride, and water infusion on metmyoglobin reducing activity and fresh lamb color. *Journal of Animal Science*, 83, 2189–2204.
- Chavira, R., Jr., Burnett, T. J., & Hageman, J. H. (1984). Assaying proteinases with azocoll. *Analytical Biochemistry*, 136, 446–450.
- Christensen, M., Torngren, M. A., Gunvig, A., Rozlosnik, N., Lametsch, R., Karlsson, A. H., et al. (2009). Injection of marinade with actinidin increases tenderness of porcine M. biceps femoris and affects myofibrils and connective tissue. *Journal of the Science of Food and Agriculture*, 89, 1607–1614.
- Claeys, E., Uytterhaegen, L., Buts, B., & Demeyer, D. (1995). Quantification of beef myofibrillar proteins by SDS–PAGE. *Meat Science*, 39, 177–193.
- D'Avino, R., Bernardi, M. L., Wallner, M., Palazzo, P., Camardella, L., Tuppo, L., et al. (2011). Kiwifruit Act d 11 is the first member of the ripening-related protein family identified as an allergen. *Allergy*, 66, 870–877.
- Davis, P. F., & Mackle, Z. M. (1981). A simple procedure for the separation of insoluble collagen and elastin. *Analytical Biochemistry*, 115, 11–17.
- Feldkamp, T. J., Schroeder, T. C., & Lusk, J. L. (2005). Determining consumer valuation of differentiated beef steak quality attributes. *Journal of Muscle Foods*, 16, 1–15.
- Goll, D. E., Young, R. B., & Stromer, M. H. (1974). Separation of subcellular organelles by differential and density gradient centrifugation. In *Proceedings of 27th Reciprocal Meat Conference* (pp. 250–297). Chicago, IL, USA.
- Ha, M., Bekhit, A. E. D., Carne, A., & Hopkins, D. L. (2012). Characterization of commercial papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat proteins. *Food Chemistry*, 134, 95–105.
- Han, J., Morton, J. D., Bekhit, A. E. D., & Sedcole, J. R. (2009). Pre-rigor infusion with kiwifruit juice improves lamb tenderness. *Meat Science*, 82, 324–330.
- Hopkins, D. L., & Thompson, J. M. (2002). Factors contributing to proteolysis and disruption of myofibrillar proteins and the impact on tenderisation in beef and sheep meat. *Australian Journal of Agricultural Research*, 53, 149–166.
- Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., & Parr, T. (2010). Tenderness – An enzymatic view. *Meat Science*, 84, 248–256.
- Lee, J. H., Song, Y. A., Tannenbaum, S. R., & Han, J. (2008). Increase of reaction rate and sensitivity of low-abundance enzyme assay using micro/nanofluidic preconcentration chip. *Analytical Chemistry*, 80, 3198–3204.
- Mostafaie, A., Bidmeshkipour, A., Shirvani, Z., Mansouri, K., & Chalabi, M. (2008). Kiwifruit actinidin: a proper new collagenase for isolation of cells from different tissues. *Applied Biochemistry and Biotechnology*, 144, 123–131.
- Nieuwenhuizen, N. J., Beuning, L. L., Sutherland, P. W., Sharma, N. N., Cooney, J. M., Bielecki, L. R. F., et al. (2007). Identification and characterisation of acidic and novel basic forms of actinidin, the highly abundant cysteine protease from kiwifruit. *Functional Plant Biology*, 34, 946–961.
- Shevchenko, A., Sunyaev, S., Loboda, A., Bork, P., Ens, W., & Standing, K. G. (2001). Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. *Analytical Chemistry*, 73, 1917–1926.
- Sugiyama, S., Ohtsuki, K., Sato, K., & Kawabata, M. (1996). Purification and characterization of six kiwifruit [Actinidia chinensis] proteases isolated with two ion-exchange resins, toyopearl-superQ and bakerbond WP-PEI. *Bioscience, Biotechnology, and Biochemistry*, 60, 1994–2000.
- Sykes, B. C., & Bailey, A. J. (1971). Molecular weight heterogeneity of the alpha-chain sub-units of collagen. *Biochemical and Biophysical Research Communications*, 43, 340–345.

- Tamburrini, M., Cerasuolo, I., Carratore, V., Stanziola, A. A., Zofra, S., Romano, L., et al. (2005). Kiwellin, a novel protein from kiwi fruit. Purification, biochemical characterization and identification as an allergen. *Protein Journal*, 24, 423–429.
- Tello-Solis, S. R., Valle-Guadarrama, M. E., & Hernandez-Arana, A. (1995). Purification and circular dichroism studies of multiple forms of actinidin from *Actinidia chinensis* (kiwifruit). *Plant Science*, 106, 227–232.
- Thompson, V. F., Saldana, S., Cong, J., & Goll, D. E. (2000). A BODIPY fluorescent microplate assay for measuring activity of calpains and other proteases. *Analytical Biochemistry*, 279, 170–178.
- Toohy, E. S., Kerr, M. J., van de Ven, R., & Hopkins, D. L. (2010). The effect of “kiwi fruit solution” on meat traits in beef topside. In *56th International Conference of Meat Science and Technology (ICoMST)*. Jeju, South Korea.
- Toohy, E. S., Kerr, M. J., van de Ven, R., & Hopkins, D. L. (2011). The effect of a kiwi fruit based solution on meat traits in beef m. semimembranosus (topside). *Meat Science*, 88, 468–471.
- van der Rest, M., & Garrone, R. (1991). Collagen family of proteins. *FASEB Journal*, 5, 2814–2823.
- Vuorio, E., & de Crombrughe, B. (1990). The family of collagen genes. *Annual Review of Biochemistry*, 59, 837–872.
- Yamaguchi, T., Yamashita, Y., Takeda, I., & Kiso, H. (1982). Proteolytic enzymes in green asparagus, kiwi fruit and miut: occurrence and partial characterization. *Agricultural and Biological Chemistry*, 46, 1983–1986.
- Yonezawa, H., Kaneda, M., & Uchikoba, T. (1998). A cysteine protease from young stems of asparagus: Isolation, properties, and substrate specificity. *Bioscience, Biotechnology, and Biochemistry*, 62, 28–33.