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Characterisation of commercial papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat proteins

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ABSTRACT

Four commercially available plant preparations containing respectively papain, bromelain, actinidin and zingibain were evaluated for their ability to hydrolyse proteins present in both beef connective tissue and topside myofibril extracts. The results show significant differences in protease activity depending on the assay used and that protease assays with connective tissue and meat myofibril extracts provide a realistic evaluation of the potential of the enzymes for application in meat tenderisation. The actinidin protease preparation was found to be most effective at hydrolysing beef myofibril proteins and the zingibain protease preparation most effective at hydrolysing connective tissue proteins. This indicates the potential of these proteases for targeting specific tenderising applications, in contrast to trying to achieve control of tenderisation with the more active papain and bromelain proteases.

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1. Introduction

There is considerable interest in the evaluation and development of methods to produce meat with consistent tenderness and to improve the tenderness of tougher cuts of red meat while still maintaining meat quality (Koohmaraie, 1996). A number of studies have evaluated a variety of physical procedures to improve meat tenderness, including muscle stretching (Sorheim & Hildrum, 2002; Toohey, Hopkins, Lamb, Neilsen, & Gutkze, 2008), electrical stimulation (Hwang & Thompson, 2001) and blade tenderisation (Jeremiah, Gibson, & Cunningham, 1999), which have been demonstrated to be beneficial to some extent. Meat tenderisation during ageing has been extensively studied, including evaluation of the effect of endogenous enzymes of the calpain and cathepsin families (Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Hopkins & Thompson, 2001) and other factors, such as pH, temperature and Ca^{2+} on the tenderisation rate and extent during post-mortem ageing (Huff-Lonergan, Zhang, & Lonergan, 2010). There is still significant debate about the contribution of different endogenous proteases in meat tenderisation (Hopkins & Geesink, 2009; Kemp, Sensky, Bardsley, Buttery, & Parr, 2010; Koohmaraie & Geesink, 2006). Other methods that can be incorporated with meat cooking strategies, such as marination with organic acids (Lind, Griswold, & Bramblett, 1971) and calcium salt (Koohmaraie, Babiker, Schroeder, Merkel, & Dutson, 1988; Wheeler, Crouse, & Koohmaraie, 1992) have also been shown to be beneficial for improving tenderness.

The main components of meat, myofibrils, consist mainly of actin, myosin and various accessory proteins, and connective tissue, which consists of various collagen sub-types and elastin (Bailey & Light, 1989; Lawrie, 1998). The relative proportions of these components are considered to influence meat structure and texture and hence the relative tenderness of a meat cut (Wheeler, Shackelford, & Koohmaraie, 2000). It is reported that disruption of these components in meat structure in various ways, including partial hydrolysis of meat proteins, contributes to tenderness (Koohmaraie, 1994). The potential to use exogenous proteases to tenderise meat has also attracted considerable interest, with a focus on members of the cysteine protease class and particularly plant cysteine proteases, some of which have long been used in home cooking (Naveena, Mendiratta, & Anjaneyulu, 2004; Sullivan & Calkins, 2010). The catalytic properties of purified cysteine proteases of the papain family, such as papain from papaya latex (EC 3.4.22.2) (Glazer & Smith, 1971), bromelain from pineapple fruit (EC 3.4.22.33) and stem (EC 3.4.22.32) (Inagami & Murachi, 1963), and actinidin from kiwifruit (EC 3.4.22.14) (Baker, Boland, Calder, & Hardman, 1980) have been studied for some time. Additionally a protease from ginger rhizome (zingibain, EC 3.4.22.67) (Kim, Hamilton, Guddat, & Overall, 2007) has been characterised. Previous work has focused on protease assay methods using small molecules or chemically labelled proteins as substrates, which has

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provided useful comparative information, but these data do not relate well to applications where proteases are interacting with large polymer substrates assembled in complex higher order structures, such as are present in meat. The ability of papain family cysteine proteases to improve beef meat tenderness has been demonstrated (Ashie, Sorensen, & Nielsen, 2002; Lewis & Luh, 1988), with the effect attributed to an increased degradation of myofibril proteins and disruption of muscle fibre structure (Han, Morton, Bekhit, & Sedcole, 2009; Kim & Taub, 1991; Naveena et al., 2004). However, there are relatively few reports describing the proteolysis of meat proteins using exogenous protease application. The present study characterised four commercially available plant protease preparations (papain, bromelain, actinidin and zingibain) using both traditional (small molecule synthetic substrate and chemically labelled protein) assays, as well as analysis of the hydrolysis of beef tendon collagen protein extract and a beef topside myofibril protein extract.

2. Materials and methods

All chemicals used were of analytical reagent grade unless otherwise stated. Protease preparations were kind gifts from international suppliers. Papain 25,000 MG (a papaya latex powder preparation) and bromelain 110 (a pineapple fruit powder preparation) were from Enzyme Solutions Pty. Ltd. (Croydon South, Victoria, Australia). Actinidin KFPE 2 Fold (a kiwifruit powder preparation) was from Ingredient Resources Pty. Ltd. (Warriewood, New South Wales, Australia). Zingibain protease (a ginger rhizome solution preparation) was from Biohawk Foundation (Archerfield, Queensland, Australia).

2.1. Total protein determination

The total protein content of the commercial enzyme preparations was determined using a 2D Quant Kit (GE Healthcare, #80-6484-51) as per the manufacturer's instructions. Stock solutions (10–100 mg mL^{-1}) of the commercial plant protease preparations supplied as powders were prepared by dissolving in Milli-Q water.

2.2. Protein profile of commercial protease preparations

Proteins were displayed by one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS–PAGE) using Invitrogen gradient (4–12%) Bis-Tris gels (#NP0322BOX). A 10 μL aliquot of each enzyme preparation solution (containing 20 μg protein) was added to 3 μL of Invitrogen SDS sample buffer (#NP0007) and 2 μL of Invitrogen reducing agent (#NP0004). The samples were then incubated at 90 °C for 10 min and then loaded on a gel. Electrophoresis was performed in pre-chilled NuPAGE® MES SDS running buffer (1X) (Invitrogen #NP0002) at 160 V for 1 h at room temperature. Protein standards (Novex Sharp Pre-stained Protein Standard, Invitrogen, #57318) were included on 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 #LC6060) (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 Canon CanoScan LiDE 600F scanner.

2.3. Characterisation of esterase, caseinolytic and collagenolytic activities of commercial proteases

2.3.1. Esterase activity assay

The ester hydrolysing activity of the plant protease preparations was investigated using the substrate CBZ-Lys-*p*-nitrophenyl (CBZ-Lys-ONp) ester (Sigma, #A4341) as described previously (Bowers, McComb, Christensen, & Schaffer, 1980; Han et al., 2009). Hydrolysis of the substrate CBZ-Lys-ONp ester generates *p*-nitrophenol (*p*NP) with A_{400} absorbance maxima and was quantitated with a standard curve of *p*NP. Assays were carried out in triplicate over the 5.0–7.0 pH range and 25–85 °C temperature range.

Validation of the fixed time assay resulted in the following protocol: a fixed-time assay set of four capped microfuge tubes containing phosphate buffer (450 μL) was first equilibrated for 3 min at the assay temperature. Then 10 μL of 20 mM CBZ-Lys-ONp ester in acetonitrile were added to all four tubes. Subsequently, one of the commercial enzyme preparation solutions (40 μL) was added into three of the four tubes while the same volume of Milli-Q water was added to the fourth tube as control. Substrate hydrolysis was allowed to occur for 30 s. After the incubation, 500 μL of 1 M sodium carbonate solution were added to each tube to quench the enzyme reaction. The samples were then centrifuged at 16,000*g* for 5 min and the supernatant was measured at A_{400}. There was negligible A_{400} change due to the addition of the commercial protease preparation solution. The esterase activity of the commercial protease preparations was calculated as μmol of *p*NP generated per minute per mL of enzyme solution. The total protein amount added of the commercial protease preparations to the assays was adjusted during protocol development and validation so that the ester hydrolysis activity of the commercial protease preparations at all pHs and temperatures could be clearly presented for comparison. The total protein amounts added were 4.6, 0.2, 2.0 and 17.9 μg for papain, bromelain, actinidin and zingibain, respectively.

2.3.2. Caseinolytic activity assay

The assay is based on the ability of proteases to hydrolyse fluorescent-labelled casein as described by Thompson, Saldana, Cong, and Goll (2000) .The substrate used was a green fluorescent-labelled (BODIPY-FL) casein (Molecular Probes, #E6638) and the measurements were carried out using a fluorescence plate reader (POLARstar OPTIMA, BMG labtech). 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.

One hundred microlitres of BODIPY-FL casein stock solution (10 μg mL^{-1}) was added to each well of a microtitre plate and then 90 μL of one of the phosphate buffers were added to each of triplicate wells. The plate was temperature equilibrated in an oven at the particular assay temperature for 3 min. The plate was then placed in a POLARstar plate reader at the assay temperature. Then 10 μL of a commercial enzyme preparation solution were added to each microtitre plate well using a multichannel pipette and the fluorescence readings in all wells were measured immediately after the plate was gently shaken for 3 s. The rate of hydrolysis in each assay was recorded at 15 s intervals over 3 min. This assay was repeated three times for each pH (4.5–9.0) and temperature (5, 25, 35, 45 and 55 °C) combination. The temperature control of the POLARstar OPTIMA fluorescence microtitre plate reader was limited to the range 25–45 °C. The assays at 5 and 55 °C were performed with the buffer and substrate components pre-equilibrated to assay temperature, and readings were recorded with the POLARstar OPTIMA set at the nearest temperature (25 or 45 °C, respectively).

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 by Lee, Song, Tannenbaum, and Han (2008). The line

slope value 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 commercial protease preparation was determined as the change in arbitrary fluorescent units per minute per mL of enzyme solution. The total protein amount of the commercial protease preparations added to the assays was adjusted during protocol development and validation so that the caseinolytic activity of the commercial protease preparations at all pHs and temperatures could be clearly presented for comparison. The total protein amount of the commercial protease preparations added to the assays was 4.6, 0.2, 0.5 and 9.0 μg for papain, bromelain, actinidin and zingibain, respectively.

2.3.3. Collagenolytic activity

The collagenolytic activity of the commercial protease preparations was determined using collagen impregnated with azo-dye (Azocoll) (Sigma, #A4341) according to Chavira, Burnett, and Hageman (1984). 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 et al., 1984). The settled substrate was re-suspended in 12.5 mL of the same buffer and 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 a protease preparation were 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. An additional 100 μL aliquot of the commercial protease preparation was then added into the assay tubes and the A_{520} was again recorded after a further 1 h incubation, to provide an indication as to whether or not there had been a significant loss of enzyme activity during the extended incubation. The total protein amount added of the commercial protease preparations to the assays was adjusted during protocol development and validation so that the collagenolytic activity of the commercial protease preparations could be clearly presented for comparison. The total protein amount of the commercial protease preparations added to the assays was 185.6, 8.2, 20 and 179 μg for papain, bromelain, actinidin and zingibain, respectively.

2.4. The efficacy of commercial proteases toward meat proteins

2.4.1. Collagen protein extraction from beef tendon

Extraction of collagen protein was carried out according to the procedure of Davis and Mackle (1981) with some modifications. 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 22,000*g*. 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 of 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 1000*g* for 30 min. The supernatant was extracted three times with an equal volume of diethyl ether, after which the ethereal layer was discarded while the aqueous and interface layers were collected. The collagen protein extracted from 2 g of tendon ended up in a volume of 20 mL and aliquots of the material were stored frozen at −20 °C for assays.

2.4.2. Collagen protein extract 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. Commercial protease preparation stock solution (40 μL) was added to the tubes which were then tumbled in the 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 commercial zingibain, papain and bromelain preparations and after 0.5, 1, 2, 3 and 24 h for the commercial actinidin preparation. The assay samples were frozen on dry ice immediately after addition of Invitrogen SDS sample buffer (3 μL) (#NP0007), Invitrogen reducing agent (1 μL) (#NP0004) and Milli-Q water (6 μL). The samples were then stored at −20 °C until analysis by SDS–PAGE. The total protein amount of the commercial protease preparations added to the assays was 0.07, 0.5, 2.0 and 17.9 μg for papain, bromelain, actinidin and zingibain, respectively.

2.4.3. Beef topside myofibril protein extract preparation

The myofibril protein extraction procedure used was that described by Goll, Young, and Stromer (1974). Beef topside (0.5 g) was homogenised with a polytron homogeniser at 11,000 rpm for 30 s 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 phenylmethylsulfonyl fluoride (PMSF) in isopropanol. The homogenate was centrifuged at 1000*g* 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 30 s at 11,000 rpm and then centrifuged at 1500*g* 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 filtrate was freeze-dried and stored at −20 °C as meat myofibril protein extract stock material.

2.4.4. Beef topside myofibril protein extract hydrolysis assays

Freeze-dried meat myofibril 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 myofibril extract were equilibrated to the assay temperature of 55 °C for 5 min in a Hybaid mini-oven, followed by the addition of a commercial protease preparation stock solution (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 commercial zingibain, papain and bromelain preparations and after 2, 4, 6 and 24 h for the commercial actinidin preparation. To the hydrolysis time course samples, Invitrogen SDS sample buffer (3 μL) (#NP0007) and Invitrogen reducing agent (1 μL) (#NP0004) 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 added of the commercial protease preparations to the assays was adjusted in order to display a gradual hydrolysis of meat myofibril proteins on 1D-SDS gels. The total protein added was

0.07, 0.5, 4.0 and 17.9 μg for papain, bromelain, actinidin and zingibain, respectively.

2.5. 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 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. A MALDI-TOF MS target plate was calibrated with trypsin in a 4800 MALDI TOF/TOF analyser (Applied Biosystems MDX SCIEX) prior to application of peptide extracts with α-cyano-4-hydroxycinnamic acid (10 mg mL^{-1} in 65% acetonitrile). Analysis was conducted in Reflector Positive Ion-Mode and parameters set by MS Reflector Positive (acquisition mode) and MS Sigma Positive (processing mode). The peptide mass range was set at 900–4000 Da with a focus mass of 2100 Da. The laser intensity was set at 4300, with a total of 800 pulses fired on each band. The 15–20 strongest precursor ions of each sample band were subjected to MS/MS collision-induced dissociation (CID) analysis. Air at a pressure of 1×10^{-6} Torr was used as the collision gas. The laser intensity was increased to 5400, with a total of 4000 pulses fired on each spot for MS/MS. The modes MS–MS 2KVPositive and MSMS_1533_pos were used for the acquisition and processing methods respectively. An interpretation method was also required for multi-dimensional MS, for which the "General1" mode was used.

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 NCBInr 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 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.

3. Results and discussion

3.1. Protein profile of the commercial enzyme preparations

The commercial plant protease preparations were initially subjected to 1D-SDS–PAGE analysis (Fig. 1) to provide information on the proteins present in the preparations. These preparations displayed a complex protein profile on SDS–PAGE, indicating that they were either crude or partially purified preparations. A band can be observed in each lane in Fig. 1 corresponding to the mass reported for actinidin (23.5 kDa) (Carne & Moore, 1978), papain (23.4 kDa) (Kamphuis, Kalk, Swarte, & Drenth, 1984), bromelain (25–26 kDa) (Rowan, Buttle, & Barrett, 1988; Rowan, Buttle, & Barrett, 1990), and zingibain protease (29 kDa) (Ohtsuki, Taguchi, Sato, & Kawabata, 1995). Immunological and chromatographic studies on crude extracts of pineapple stem and fruit have identified at least five distinct cysteine proteases of similar molecular mass, namely fruit bromelain, stem bromelain, ananain, comosain and pinguinain (Napper et al., 1994; Rowan et al., 1990). Similar studies with kiwifruit extract also identified several proteases and multiple actinidin isoforms (Nieuwenhuizen et al., 2007; Sugiyama, Ohtsuki, Sato, & Kawabata, 1996; Tamburrini et al., 2005). A study on ginger rhizome, using chromatography and isoelectric focusing, separated three different cysteine proteases with pI values of 4.5, 4.6 and 4.8 (Ohtsuki et al., 1995). This is consistent with the detection of multiple bands within columns (Fig. 1).

3.2. Ester hydrolysis activity of the commercial protease preparations

Ester hydrolysis assays are often used to provide an initial indication of the catalytic activity of various proteases. In this study, the ester hydrolysis activity of the four commercial enzyme preparations was investigated over the pH 5–7 range and temperature 5–85 °C range using the commercially available substrate CBZ-Lys-ONp (Sigma–Aldrich) (Table 1).

The commercial actinidin protease preparation exhibited very low ester hydrolysis activity. It is understood that the actinidin preparation is formulated with added dextran as a bulking agent and other reducing agents. The commercial bromelain, zingibain and papain protease preparations exhibited maximal esterase activity at 55, 65 and 75 °C respectively. The present study is the first study to have examined comparatively the ester-hydrolysing activity of these proteases under a wide range of pH and temperature. Although ester compounds have been used extensively in protease activity analyses and are traditionally used as substrates in kinetic studies (Baker et al., 1980; Boland & Hardman, 1973), these compounds do not necessarily represent the true kinetic behaviour of enzymes' ability to hydrolyse amide bonds in protein polymers. Hence, BODIPY-FL casein was employed to assess the

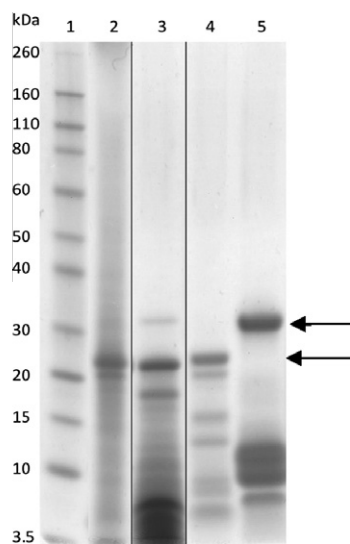

Fig. 1. Commercial plant protease preparation protein profiles displayed by 1D-SDS–PAGE. Aliquots of the enzyme preparations (containing 20 μg protein each) were loaded onto a 4–12% gradient 12 well Bis–Tris NuPAGE mini-gel (Invitrogen) and stained with Simply Blue (Invitrogen). Lane 1 = molecular marker, lane 2 = commercial actinidin; lane 3 = commercial papain, lane 4 = commercial bromelain and lane 5 = commercial zingibain.

Table 1
Proteolytic activities (ester hydrolysis activity of commercial protease preparations assayed with the substrate CBZ-Lys-ONp and Caseinolytic activity using BODIPY-FL casein) of commercial protease preparations.[a] The zingibain preparation stock was supplied as a solution.

Commercial enzyme preparation	Ester hydrolysis activity using the substrate CBZ-Lys-ONp					Caseinolytic activity using BODIPY-FL casein				
	Enzyme powder[b] per assay (μg)	Total protein per assay (μg)	Specific activity (μmol min^{-1} mg^{-1} protein)	Optimal pH	Optimal temperature (°C)	Enzyme powder per assay (μg)	Total protein amount per assay (μg)	Specific activity (Δfluorescence min^{-1} mg^{-1} protein)	V_{max} (Δfluorescence min^{-1})	K_M (μM)
Papain	50	4.6	19.6	6.0	75	0.2	0.02	2.3×10^5	352.5	0.20
Bromelain	50	0.2	25.0	6.0	55	2.0	0.01	4.6×10^6	11.0	0.01
Actinidin	2000	2.0	ND	–	–	1000	1.00	1.4×10^4	53.0	0.80
Zingibain	[a]	17.9	2.2	6.0	65	[a]	0.20	1.3×10^5	364.5	0.50

ND = No activity detected.
[a] Enzyme preparation stock available as a solution.
[b] Protease supplied as powders were prepared as 100 mg mL^{-1} stock solutions.

proteolytic activity (amide bond hydrolysis) of the commercial enzyme preparations.

3.3. Caseinolytic activity of the commercial protease preparations

The casein hydrolysis activity of the protease preparations was examined over the pH range 4.5–9.0, using the commercially available substrate BODIPY FL-casein (Fig. 2). The casein hydrolysis activity of papain, bromelain and zingibain protease preparations appeared to be maximal at relatively high temperature (>55 °C) under the assay conditions, in comparison to the actinidin protease preparation, which exhibited maximal activity at 45 °C. The protease preparations appeared to be active over a wide range of assay pH. Maximum activities were at 7.0–8.0 (papain), 5.0–6.0 (bromelain), 4.5–5.5 (commercial actinidin) and 6.5–7.0 (zingibain). Significant casein hydrolysis occurred with most of the commercial protease preparations in the acidic pH range typically found in meat (pH 5.5–6.0), indicating the potential application of these commercial protease preparations in meat tenderisation. The maximal casein hydrolysis specific activities of papain, bromelain, actinidin and zingibain proteases were 2.3×10^5, 4.6×10^6, 1.4×10^4 and 1.3×10^5 Δfluorescence min^{-1} mg^{-1} protein, respectively

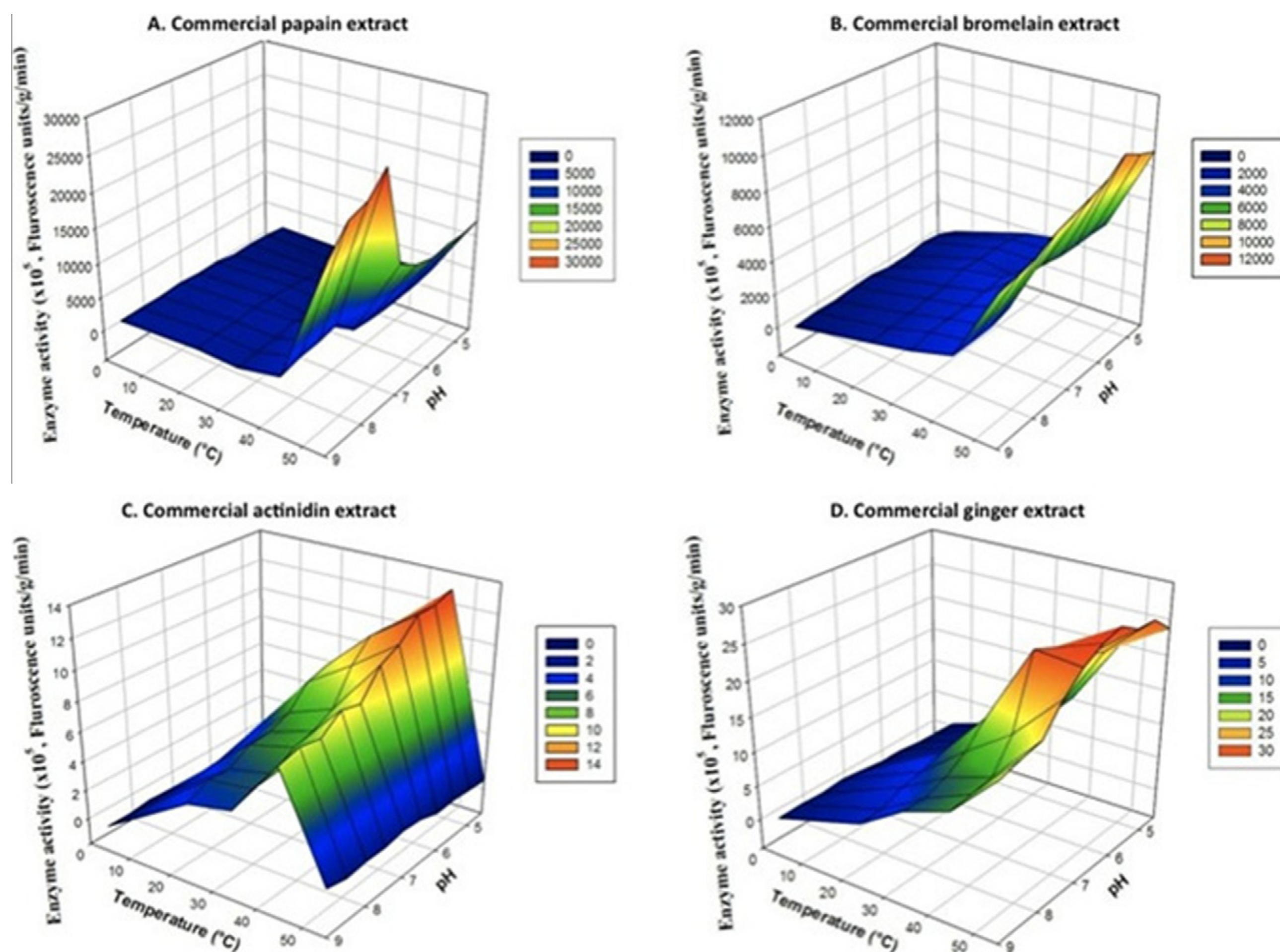

Fig. 2. Casein hydrolysis activity profiles of the commercial protease preparations. Assays at each pH and temperature were carried out in triplicate over 3 min.

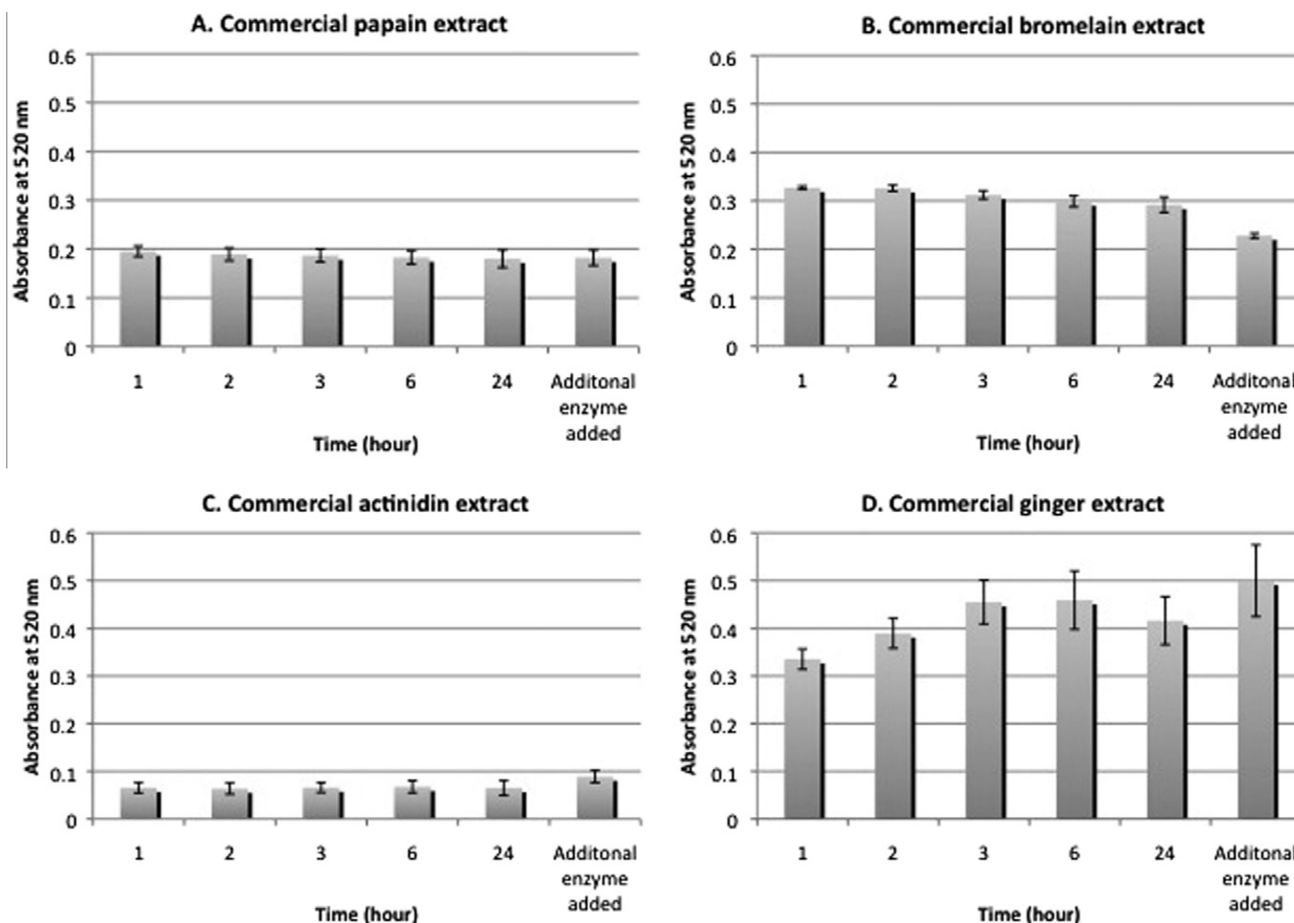

Fig. 3. Azocoll hydrolysis activity of the protease preparations. All of the assays were carried out at pH 6.0 and 55 °C in triplicate and the error bars represent the standard deviation. The A520 values at each time point are the total accumulated absorbance up to that time in the assay.

(Table 1). The hydrolytic activities of papain-family proteases have been examined with various substrates as reported in the literature (Baker et al., 1980; Inagami & Murachi, 1963; Whitaker & Bender, 1965). However, enzyme activity of pure proteases in these studies was limited to a particular pH and temperature. The present study provides a more comprehensive picture of the hydrolytic activity of commercial proteases under a wide range of pH and temperature.

The K_M values obtained for the commercial protease preparations using the BODIPY-FL casein substrate varied significantly with the greatest difference being over eighty-fold between the bromelain (0.01 μM) and actinidin (0.8 μM) protease preparations. The significant difference in K_M values of the protease preparations indicates significant differences in substrate binding affinity which is influenced by differences in the amino acid sequences of the proteases as suggested by Baker et al. (1980). Various ester and amide bond-containing substrates have been used to obtain kinetic parameters of papain-family proteases (Baker et al., 1980; Inagami & Murachi, 1963). A study by Homaei, Sajedi, Sariri, Seyfzadeh, and Stevanato (2010), using casein as a substrate, determined the K_M value of papain to be 0.62 μM, which is more than threefold higher than that found in the present study (0.2 μM), indicating that the papain preparation in the present study has a higher specificity for casein.

3.4. Collagenolytic activity of the protease preparations

Collagen hydrolysis capability of the protease preparations was tested with Azocoll, a commercially available azo dye-labelled collagen (Sigma–Aldrich), and a commonly used substrate for collagen hydrolysis assays that has been used in enzyme kinetic studies (Rowe & Brown, 1988). It was found that extensive prewashing of Azocoll stock suspensions reduced non-specific release of azo dye, as reported by Chavira et al. (1984). The Azocoll was kept suspended by agitation during the assay time course, as this step has been shown to have a dramatic effect on Azocoll hydrolysis (Chavira et al., 1984).

Azocoll hydrolysis activity of the four commercial protease preparations differed significantly, with the collagen hydrolysis capability as follows: zingibain > bromelain > papain > commercial actinidin (Fig. 3). Most of the activity occurred in the first hour of assay incubation, although the commercial zingibain preparation showed a gradual increase over the first three hours. The addition of an additional aliquot of commercial protease preparation to each assay after 24 h of incubation did not result in an increase in absorbance, i.e., no further Azocoll hydrolysis occurred. This indicates that the lack of Azocoll hydrolysis after the first hour of incubation was not a result of protease instability. A decrease in absorbance observed after the additional aliquot of bromelain was added to the assay and tendency of decreased absorbance at 24 h of incubation in ginger protease (Fig 3D) suggested interactions between the azo-dye labelled peptides and the assay mixture upon prolonged incubation (i.e., 24 h), which may have resulted in absorbance interference. The specific activity of the four protease preparations, expressed as A_{520} min^{-1} mg^{-1} Azocoll, was 0.02, 0.7, 0.05 and 0.03 for papain, bromelain, actinidin and zingibain enzyme preparations, respectively, showing that the bromelain protease preparation displayed the highest specific activity towards the Azocoll

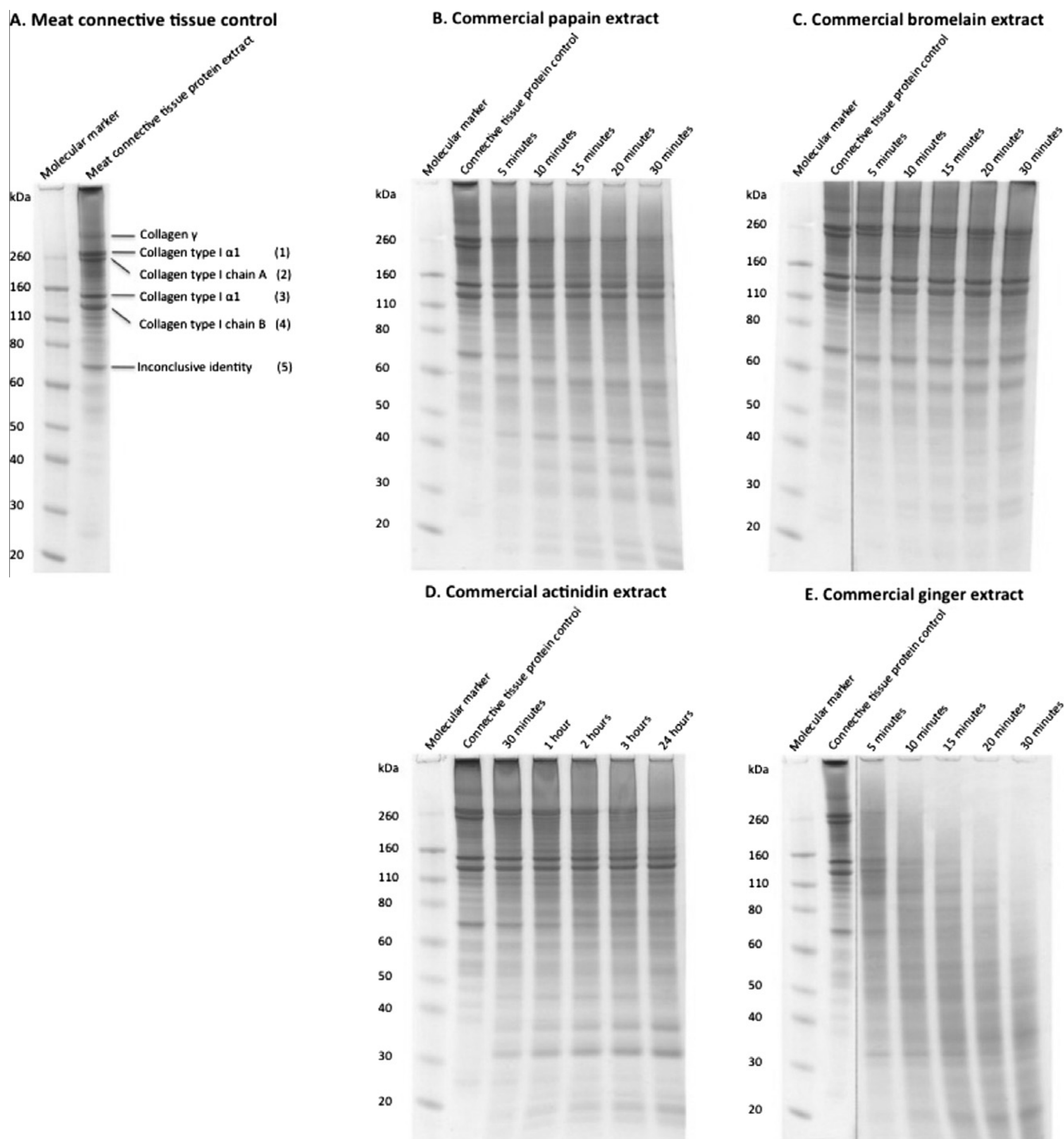

Fig. 4. The ability of commercial protease preparations to hydrolyse meat connective tissue proteins with analysis by 1D-SDS–PAGE. A 20 μg collagen protein extracted from beef tendon (A) and time course hydrolysis assays of extracted meat connective tissue collagen proteins using papain (B), bromelain (C), actinidin (D) and zingibain (E) commercial protease preparations. The amount of each commercial protease used was adjusted so as to generate an informative protein hydrolysis display, focused on the early protein partial hydrolysis occurring.

substrate. Using Azocoll, Foegeding and Larick (1986) demonstrated higher collagenolytic activity with bromelain compared to papain, which is in agreement with our findings, with minimal activity by actinidin (Fig. 3).

3.5. The efficacy of commercial protease preparations in hydrolysing beef tendon collagen protein extract

Meat connective tissue protein hydrolysis is considered to be a key factor in determining meat tenderness. Hydrolysis of these proteins has been shown to disrupt connective tissue structure, with an associated decrease in shear force and an improvement in meat tenderness (Bailey & Light, 1989). Collagen protein was extracted from beef tendon and used as a substrate for the analysis of the ability of the four commercial protease preparations to hydrolyse collagen protein extract. Initially the proteins present in the meat connective tissue extract were displayed on SDS–PAGE (Fig. 4A) and the major bands were excised and subjected to an in-gel proteolysis digestion/peptide extraction/mass spectrometry workflow, followed by informatic analysis using international dat-

Table 2
Summary of the data generated by MALDI-TOF–TOF tandem mass spectrometry and informatics analysis of the meat connective tissue protein extract (Fig. 4A) and meat myofibril protein extract (Fig. 5A) displayed by SDS–PAGE.

Gel band ref.	Protein Identification	Mass (Da)	Species	Total ion score	Accession No.	Database
01	Collagen type I α-1	138,854	*Bos taurus*	110	CO1A1_BOVIN	SwissProt
02	Collagen type I chain A	95,928	*Bos taurus*	73	109156929	NCBInr
03	Collagen type I α-1	138,854	*Bos taurus*	94	CO1A1_BOVIN	SwissProt
04	Collagen type I chain B	93,272	*Bos taurus*	79	109156930	NCBInr
05	Inconclusive identity					
06	Myosin heavy chain-1	222,851	*Bos Taurus*	889	MYH1_BOVIN	SwissProt
	Myosin heavy chain-2	223,180	*Bos Taurus*	764	MYH2_BOVIN	SwissProt
07	Myosin heavy chain-7	223,091	*Bos Taurus*	662	MYH7_BOVIN	SwissProt
08	Myosin binding protein C	128,214	*Bos Taurus*	115	160425243	NCBInr
09	α-Actinin-2	103,713	*Bos Taurus*	457	ACTN2_BOVIN	SwissProt
	α-Actinin-3	103,086	*Bos Taurus*	266	ACTN3_BOVIN	SwissProt
10	ATPase-1	109,220	*Bos Taurus*	207	AT2A1_BOVIN	SwissProt
11	Myosin heavy chain-7	223,091	*Bos Taurus*	197	MYH7_BOVIN	SwissProt
12	Calsequestrin-1	45,619	*Bos Taurus*	152	118150866	NCBInr
13	Actin	42,024	*Bos Taurus*	638	ACTS_BOVIN	SwissProt
14	Tropomyosin β chain	32,817	*Bos Taurus*	284	TPM2_BOVIN	SwissProt
15	Tropomyosin α chain	32,675	*Bos Taurus*	230	TPM1_BOVIN	SwissProt
16	Myosin light chain	20,919	*Bos Taurus*	257	MYL1_BOVIN	SwissProt
	Troponin I	20,828	*Bos taurus*	87	300797481	NCBInr
17	Myosin light chain-2	19,000	*Bos taurus*	181	MLRS_BOVIN	SwissProt
	Troponin C	18,098	*Mus musculus*	113	TNNC2_MOUSE	SwissProt

abases of partial amino acid sequences generated by mass spectrometry to obtain protein homologue identity (Table 2).

The extracted collagen proteins displayed by SDS–PAGE (Fig. 4A) and the results obtained from the mass spectrometry analysis (Table 2) 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 Crombrugghe, 1990). Type I collagen is found in most connective tissues (Van Der Rest & Garrone, 1991). Three types of tropocollagen, α-1, chain A and chain B, were also 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 (Furthmayr & Timpl, 1971) which was ascribed to a small difference (6 kDa) in the molecular weight of the two chains (Sykes & Bailey, 1971).

Aliquots of collagen protein extract were subjected to hydrolysis with the commercial plant protease preparations and the extent of protein hydrolysis analysed by time course sampling followed by display on 1D-SDS–PAGE (Fig. 4B–E). The amount of each commercial protease used was adjusted so as to generate an informative protein hydrolysis display, focused on the initial protein partial hydrolysis. All four protease preparations were able to hydrolyse the collagen protein extract, but targeted different protein species in the collagen extract. The papain, bromelain and actinidin preparations were able to hydrolyse collagen type I chain A, but not collagen type I chain B under the assay conditions. Collagen type I α1 was partially hydrolysed by the papain and actinidin preparations over longer incubation time (30 min and 24 h, respectively). In a separate experiment, higher concentrations of the papain and bromelain preparations were able to hydrolyse the meat connective tissue proteins in a non-specific manner (data not shown) and generated a SDS–PAGE time course protein fragment profile similar to that of the zingibain protease preparation shown in Fig. 4E.

3.6. The ability of the commercial protease preparations to hydrolyse meat myofibril proteins

Meat myofibril protein hydrolysis is considered to be a key factor in determining meat tenderness. Hydrolysis of these proteins has been shown to disrupt muscle fibre structure with an associated decrease in shear force and an improvement in meat tenderness (Kemp et al., 2010). The proteins in the meat myofibril protein extract displayed on SDS–PAGE and analysed by mass spectrometry (Fig. 5A) are consistent with the proteins reported in the literature to be present in meat myofibril (Claeys, Uytterhaegen, Buts, & Demeyer, 1995). The major protein bands identified here by mass spectrometry (Table 2) 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 myofibril 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 et al., 1995; Huff-Lonergan, Parrish, & Robson, 1995). Aliquots of meat myofibril extract proteins were subjected to hydrolysis with the commercial plant protease preparations and the extent of protein hydrolysis analysed by time course sampling and display on 1D-SDS–PAGE (Fig. 5B–E). The amount of each commercial protease used was adjusted so as to generate an informative protein hydrolysis display, focused on the initial protein partial hydrolysis.

The commercial actinidin preparation hydrolysed a wide range of myofibril proteins, including actomyosin (myosin and actin) (223 and 42 kDa), and Z-disk associated proteins such as nebulin and titin (650–3200 kDa), filamin (280 kDa), actinin (100 kDa) and desmin (53 kDa), in agreement with findings in previous studies (Christensen et al., 2009; Han et al., 2009; Kaur, Rutherfurd, Moughan, Drummond, & Boland, 2010). Although able to hydrolyse meat proteins in a non-selective manner, the actinidin enzyme preparation exhibited a much slower hydrolysing rate than the other enzyme preparations. This result is consistent with the caseinolytic enzyme activity assays presented earlier.

The papain, bromelain and zingibain preparations appeared to have a similar hydrolysis profile. The preparations were efficient in hydrolysing a few proteins, such as actomyosin, titin and nebulin, but not other myofibril proteins. The predominant heavy myosin (223 kDa) and actin (42 kDa) protein bands were hydrolysed at a significant rate while the bands of C protein (140 kDa), α-actinin (90 kDa), tropomyosins (35 kDa) and troponins (22 and 17.8 kDa) remained unchanged throughout the entire assay

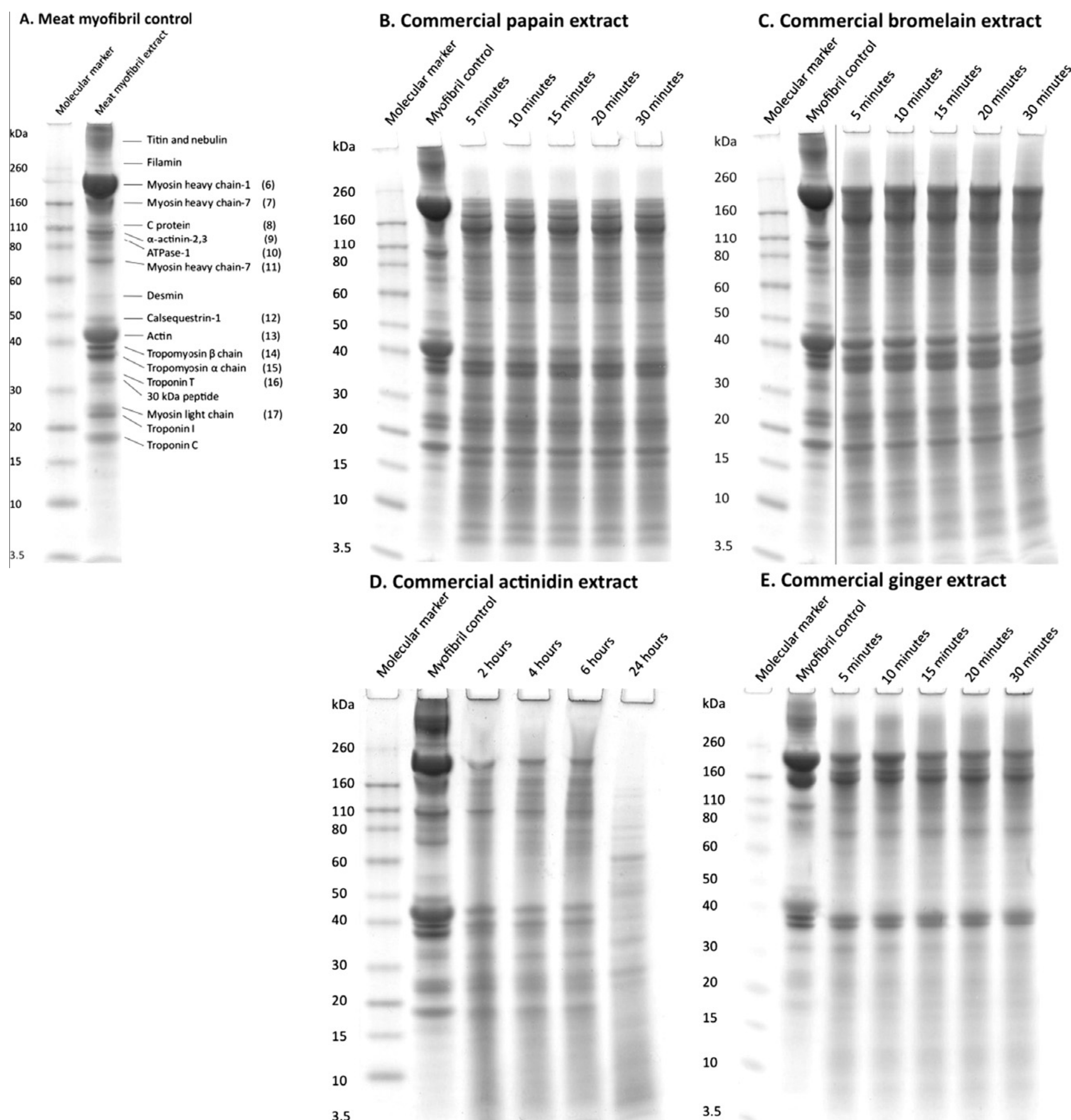

Fig. 5. Comparison of the ability of commercial protease preparations to hydrolyse meat myofibril proteins with analysis by 1D-SDS–PAGE. 20 μg meat myofibril proteins were displayed by SDS–PAGE and time course hydrolysis assays of meat myofibril proteins using papain (B), bromelain (C), actinidin (D) and zingibain (E) protease. The amount of each commercial protease used was adjusted so as to generate an informative protein hydrolysis display, focused on the early protein partial hydrolysis.

duration. The similarity in catalytic activity of these three enzyme preparations can be explained by the homologous amino acid sequences, especially in the vicinity of the active site, which results in a similar substrate specificity of zingibain protease, papain and bromelain (Choi & Laursen, 2000).

4. Conclusion

The four commercial plant protease preparations (papain, bromelain, actinidin and zingibain) were each found by SDS–PAGE to contain several proteins and were therefore either crude preparations or only partially purified. These commercial protease preparations demonstrated differing activities using synthetic substrates. While these substrates have been used traditionally in assays to obtain enzyme activity information on proteases, they do not relate well to the degradation of meat proteins, where a complex higher order structure of biopolymers exists. The use of meat collagen protein extract and a meat myofibril protein extract as substrates can be a more effective method to screen for protease activities. The results presented here indicate that the zingibain protease preparation appears to be most specifically effective at hydrolysing meat connective tissue proteins and the actinidin protease most specifically effective at hydrolysing meat myofibril

proteins, suggesting that these two enzymes may have potential for targeting specific meat tenderising applications.

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