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

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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.

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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 Ca^{2+}, 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 carcase 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

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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 prechilled 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 (*p*NP) with A_{400} absorbance maxima and was quantified 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 using 70 mM K_2HPO_4 buffer (pH was adjusted with KOH or H_3PO_4).

The esterase activity of the enzyme extracts was calculated as μmol of *p*NP 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

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