Food Chemistry 172 (2015) 197–206

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characterisation of novel fungal and bacterial protease preparations and evaluation of their ability to hydrolyse meat myofibrillar and connective tissue proteins

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ARTICLE INFO

Article history:
Received 8 June 2014
Received in revised form 31 July 2014
Accepted 13 September 2014
Available online 22 September 2014

Keywords:
Meat
Myofibrillar
Connective tissue
Fungal proteases
Tenderisation

ABSTRACT

The catalytic capability of four commercially available food-grade fungal and bacterial protease preparations (AFP, FPII, F60K and HT) was evaluated over a range of pH, temperature and substrate conditions using esterase and caseinolytic activity assays and time course hydrolysis over 120 and 60 min of myofibrillar and connective tissue proteins, respectively. The protease preparations displayed similar casein hydrolysis kinetics and were active in hydrolysing BODIPY-FL casein to varying extents at postmortem aging meat pH (5.0–6.0). All of the four proteases exhibited selective hydrolytic activity towards meat myofibrillar proteins including myosin and actin. Significant hydrolysis of two meat tenderisation protein markers troponin T and desmin by the four proteases was detected by western blot. The results obtained indicate that the new fungal protease preparations AFP and FPII, bacterial protease preparation HT and the new source of fungal protease preparation F60K have potential for use in meat tenderising applications.

1. Introduction

Tenderness is considered to be a major meat eating quality and consumers are willing to pay a premium for meat with guaranteed tenderness (Lusk, Fox, Schroeder, Mintert, & Koohmaraie, 2001). Meat with a consistent and acceptable level of tenderness can result in increased monetary returns for suppliers (Polkinghorne, Philpott, Gee, Doljanin, & Innes, 2008). This has led to a strong interest in the development of reliable methods to produce cuts of meat that are consistently tender, whilst still retaining other desirable meat attributes such as texture and flavour (Koohmaraie, 1996).

Aging of meat carcasses, as well as the use of various physical treatments, has been the traditional method to increase meat tenderness (Bekhit, Carne, Ha, & Franks, 2014). Aging of meat has utilised the ability of endogenous proteases in meat to degrade structural proteins, however this method has been found to be not so effective with tougher cuts of meat such as topside (Koohmaraie, 1994). Proteolysis of connective tissue proteins such as collagen, as well as meat myofibrillar proteins is regarded to be important for achieving meat tenderisation (Bailey & Light, 1989) and lowering the shear force value of meat (Han, Morton, Bekhit, & Sedcole, 2009). Previous studies have investigated the use of exogenous proteases from various sources to aid in the tenderisation of meat (Ha, Bekhit, Carne, & Hopkins, 2013a) with the aim of providing opportunities to produce consistent meat tenderness and increase the value of cuts of meat that are typically less tender as obtained direct from the animal.

The effectiveness of plant derived proteases, including papain from papaya, bromelain from pineapple and actinidin from kiwifruit, in hydrolysing meat proteins has been well documented (Ha, Bekhit, Carne, & Hopkins, 2012; Payne, 2009) and preparations based on these proteases are now commercially available (Toohey, Karr, van de Ven, & Hopkins, 2011). Disadvantages of the use of some plant derived proteases, such as papain and bromelain, include their lack of substrate specificity towards meat proteins, leading to extensive hydrolysis of both the myofibrillar and connective tissue proteins (Ashie, Sorensen, & Nielsen, 2002; Miller, Strange, & Whiting, 1989). This leads to meat with unwanted attributes such as mushy texture and a bitter taste. More recently, there has been a move towards the evaluation of microbial proteases which have greater substrate specificity, in an attempt to overcome these problems (Ha et al., 2013a).

Microbial proteases from bacterial and fungal sources that are non-pathogenic have been found to be useful in applications including meat tenderisation as they have better substrate

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http://dx.doi.org/10.1016/j.foodchem.2014.09.061
0308-8146/

specificity (Rao, Tanksale, Ghatge, & Deshpande, 1998). A common source of commercially available fungal proteases is *Aspergillus oryzae*. This fungus has been used for more than 2000 years in the production of oriental foodstuffs such as soy sauce, thus *A. oryzae* is regarded as being a safe microorganism and a source of proteases (Barbesgaard, Heldt-Hansen, & Diderichsen, 1992). Various proteases derived from *A. oryzae* have been shown to hydrolyse collagen and elastin and, to a limited extent, myofibrillar proteins (Ashie et al., 2002; Payne, 2009). Aspartic proteases from *A. oryzae* in particular have demonstrated maximal activity at pH 2.5–6.0, making them particularly suitable for applications in meat tenderising as table meat cuts typically have a pH in the range of 5.4–5.8 (Ashie et al., 2002; Silva, Patarata, & Martins, 1999).

Commercial proteases from bacterial sources are generally derived from strains of the *Bacillus* genus, such as *Bacillus amyloliquefaciens* and *Bacillus subtilis*. They have been demonstrated to have a low thermotolerance which is advantageous for application in food hydrolysis as their catalytic activity is easier to control (Beckhorn, Labbee, & Underkofler, 1965; Rao et al., 1998).

Two myofibrillar proteins, troponin and desmin, are of particular interest in meat tenderisation. Desmin polymerises to form intermediate filaments, 10 nm in diameter. This provides a framework for connecting individual actomyosin myofibrils by bridging the gap between neighbouring Z-disks. It has been reported that the breakdown of these inter-myofibrillar linkages plays an important role in effecting muscle tenderisation (Takahashi, 1996; Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). Troponin is comprised of three regulatory proteins, troponin C, troponin I and troponin T, and is involved in the regulation of muscle contraction by preventing the binding of myosin to actin. Troponin T is of particular interest as it forms a complex with tropomyosin and its degradation has been used as a marker of myofibrillar protein degradation in aged beef (Sun et al., 2014).

To evaluate the potential use of an exogenous protease in a meat tenderising application, it is of interest to characterise a new protease as it is known that different proteases can exhibit a range of different hydrolytic capabilities towards meat proteins (Ha et al., 2012, 2013a). Recently, two new fungal protease preparations, AFP and fungal protease II (FPII), and a new bacterial protease preparation, HT, have become available, along with a new sourced formulation of a previously studied fungal protease 60,000 (F60K) (Ha et al., 2013a). This study aimed to investigate the hydrolytic capabilities of these four fungal and bacterial proteases. A fluorescent labelled casein substrate, BODIPY-FL-casein, was used to determine and compare the caseinolytic and kinetic properties of the proteases. Additionally, the hydrolysis of collagen proteins extracted from bovine *Achilles* tendon and myofibrillar proteins in meat myofibrils extracted from *M. semimembranosus* were investigated to determine the relative affinity of each protease preparation towards each of these meat components. In addition, the time course hydrolysis of desmin and troponin was analysed by western blot to compare the rate of hydrolysis of these proteins compared to, in particular, that of myosin and actin in meat myofibrillar extracts.

2. Materials and methods

2.1. Materials

All chemicals used were of analytical reagent grade or higher. Protease preparations Acidic Fungal Protease (AFP), Fungal Protease II (FPII), Fungal 60,000 (F60K) and HT-proteolytic (HT) were supplied by Enzyme Solutions Pty. Ltd. (Croydon South, Victoria, Australia 3136). All four protease preparations were supplied in powder form and stock solutions were prepared in Milli-Q water. The total protein present was 47, 44, 13 and 55 mg mL^{-1} for AFP, FPII, F60K and HT, respectively in a 100 mg mL^{-1} protease powder stock solution.

2.2. Total protein determination

The total protein content of each of the commercial protease preparations was determined with a 2D Quant Kit (GE Healthcare, #80-6484-51) according to the manufacturers' instructions.

2.3. Analysis of proteins in the fungal and bacterial protease preparations

Proteins present in the fungal and bacterial protease preparations were displayed by one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D-SDS–PAGE) using Bolt gradient (4–12%) Bis–Tris gels (Life Technologies, #BG04122BOX). An aliquot of a stock solution of each protease preparation (100 mg mL^{-1}) was added to an appropriate volume of Bolt LDS sample buffer (Life Technologies, #B0007) and Bolt sample reducing agent (Life Technologies, #B0004). The samples were incubated at 90 °C for 5 min prior to being loaded on to the gel. Protein standards (Novex Sharp Pre-stained Protein Standard, Life Technologies, #LC5800) were included on the gel as a molecular marker. The electrophoresis was conducted in 1× Bolt MES SDS running buffer (Life Technologies, #B0002) at 164 V for 34 min. Following electrophoresis, the gel was washed for 5 min in Milli-Q water three times and stained in SimplyBlue™ SafeStain solution (Invitrogen #LC6060). The gel was then de-stained with Milli-Q water and an image was captured with a Canon CanoScan LiDE 600F scanner.

Proteins in the fungal and bacterial protease preparations were also displayed on large format 2D-PAGE. Aliquots (150 μL) of stock solutions of the protease preparations (100 mg mL^{-1}) were processed using a 2-D Clean-Up Kit (GE Healthcare, #80-6484-51). Then each protein sample was prepared in 360 μL of a rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT and 4 mM TCEP) that was used to rehydrate 18 cm pH 3–10 NL Immobiline DryStrip IEF strips (GE Healthcare #17-1235-01) for the first dimension electrophoresis. IEF was conducted over 24 h in an IPGphor isoelectric focusing instrument (Pharmacia) at a maximum voltage of 8000 V for a total of 96.4 kVh. After electrophoresis the IEF strips were incubated in a reduction solution (6 M urea, 130 mM DTT, 375 mM Tris–HCl, pH 8.8) for 10 min, followed by incubation in an alkylation solution (6 M urea, 135 mM iodoacetamide, 375 mM Tris–HCl, pH 8.8) for 10 min, at 20 °C. The IEF strips were then transferred immediately to large format 12.5% SDS–PAGE gels with an acrylamide to bis-acrylamide ratio of 30:0.8 and prepared in an ETTAN-DALT6 electrophoresis system (GE Healthcare). Second dimension electrophoresis was conducted for 6 h at 15 °C at 2 W per gel for the first hour followed by 15 W per gel for the rest of the electrophoresis. After electrophoresis the 2D-PAGE gels were soaked in 10% methanol, 7% acetic acid, for two changes of 30 min each, before staining with in-house prepared colloidal Coomassie (Candiano et al., 2004). 2D-PAGE gels were destained in 5% phosphoric acid, 10% ethanol and then equilibrated in Milli-Q water, prior to imaging.

The major protein components in each fungal and bacterial protease preparation were identified by a proteomic method involving excising protein spots from each 2D-PAGE and subjecting them to an in-gel digest procedure prior to MALDI-TOF-TOF mass spectrometry, as previously reported (Ha et al., 2012).

2.4. Determination of esterase activity

Esterase activity of each protease preparation was evaluated using the substrate CBZ-Lys-*p*-nitrophenyl (CBZ-Lys-ONp) ester