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Effects of Bromelain Tenderisation on Myofibrillar Proteins, Texture and Flavour of Fish Balls Prepared from Golden Pomfret

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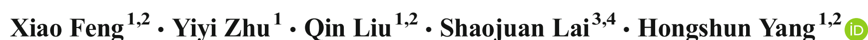

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Abstract An enzymatic method to tenderise golden pomfret (GP, *Trachinotus blochii*) flesh by marinating with bromelain (BML) solution was developed to produce GP fish balls with a texture similar to those of yellowtail fusilier (YF, *Caesio cuning*). Treatment with BML reduced the hardness, chewiness and gel strength significantly but increased the resilience of GP fish balls. As a result, 0.4% (enzyme-substrate ratio, *w*/*w*) BML-treated GP fish balls had the same texture properties as YF fish balls. Meanwhile, changes in myofibrillar proteins in the fish balls were determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and atomic force microscopy. The results indicated that BML degraded myosin light chain and troponin T effectively, without affecting actin. BML treatment generated protein fragments with significantly smaller sizes, thereby improving the tenderness of the flesh. The length, width and height of the myofibrils from 0.4% BML-treated fish ball were 6.42, 1.52 and 1.48 μm, respectively, which were not significantly different from the myofibrils of YF fish balls, indicating that the similar nanostructure determined the comparable texture properties. Gas chromatography-mass spectrometry analysis showed that 0.4% BML decreased the amount of hexanal, hexadecane, 1-octen-3-ol and 2,6,10,14-tetramethylpentadecane but increased the ratio of heptadecane from 18.14 to 38.23% in the treated fish balls, making the flavour of 0.4% BML-treated GP fish balls similar to that of YF balls. Overall, the results suggest that 0.4% BML-tenderised GP could be a promising alternative to YF to produce quality fish balls.

Keywords Myofibril · Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) · Atomic force microscopy (AFM) · Enzyme · Gas chromatography-mass spectrometry (GC-MS) · Seafood · Nanostructure · Nanotechnology

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Introduction

Fish balls are popular in Southeast Asia (Yi et al. 2011), and they are often boiled, steamed or deep-fried, with boiling being the most common cooking method. Apart from their high nutritional values, consuming fish balls also helps to avoid the issue of choking on fish bones, as the bones are removed completely during processing.

In Singapore, fish are typically imported from Malaysia, Thailand, Indonesia, India and Philippines as raw materials for fish ball production; however, many imported species have become increasingly expensive due to limited supply, for example the yellowtail fusilier (YF, *Caesio cuning*), a widespread Indo-Pacific coral food fish that is commonly used to produce premium fish balls. To increase Singapore's self-

sufficiency in fish consumption, the Agri-Food & Veterinary Authority of Singapore (AVA) spawned the golden pomfret (GP, *Trachinotus blochii*) successfully. However, GP has not yet been utilised for commercial fish ball production in Singapore, partly because of the relatively hard texture of its flesh. To utilise GP for fish ball production, appropriate tenderising techniques could be applied to improve the texture of GP flesh.

Techniques to improve meat tenderness have been studied extensively (Bekhit et al. 2014), especially for red meats, such as beef, mutton and pork. Based on their mechanisms, post-mortem interventions for meat tenderisation can be categorised into three types: physical, chemical and enzymatic, among which enzymatic treatment is considered to be the most energy efficient and environmentally friendly approach (Bekhit et al. 2014).

Natural proteases present in plant extracts have long been studied, with papain, bromelain (BML) and ficin extracted from papaya, pineapple and figs being designated as Generally Recognised As Safe (GRAS) (Bekhit et al. 2014). These traditionally used endopeptidases are shown to possess relatively low substrate specificity and demonstrated catalytic ability to hydrolyse a wide range of bonds in proteins (Schwimmer, 1981). As a result, they tend to break down muscle proteins indiscriminately (Ashie et al. 2002; Ha et al. 2012). Among these three types, ficin is less widely used because of its high hydrolytic activity which often renders meat too mushy, and papain has been observed to over-tenderise of meat at times, producing off-flavours (Kim and Taub 1991). By contrast, BML has demonstrated lower activity towards myofibrillar proteins than papain and ficin and has been reported to impart a juicier texture to the tenderised meat, with a higher water holding capacity and better flavours (Kim and Taub 1991).

BML comprises a mixture of cysteine proteases derived from the stem and fruit of the pineapple plant (*Ananas comosus*) (Bekhit et al. 2014). It has a slightly narrower spectrum of enzymatic activity than papain, with its optimum pH and temperature for most substrates falling in the range of pH 6.5–8.0 and 55–60 °C, respectively, and an inactivation temperature of around 75 °C (Arshad et al. 2014). BML has attracted considerable attention of the industry as an inexpensive and yet effective option for meat tenderisation, and many studies have explored the potential application of BML in a variety of meat products (Arshad et al. 2014). For example, Melendo et al. (1996) reported the successful application of BML in toughness reduction of coarse dry pork sausage via limited proteolysis of myofibrillar proteins, and Ketnawa and Rawdkuen (2011) demonstrated that BML extracted from pineapple peels could effectively tenderise beef, chicken and squid. Recently, Eom et al. (2015) also developed a tenderisation method for jumbo squid via injection of BML into the main body, which softened its texture progressively to facilitate consumption by the elderly and individuals with masticatory and dysphagia problems. In addition, BML was utilised by Chuapoehuk and Raksakulthai (1992) to enhance the hydrolysis of minced oyster meat for oyster sauce production, and the product in turn received higher ratings from sensory panellists than commercial oyster sauces. Nevertheless, no systematic report has been published that examines the specific effects of BML on fishery products. Hence, BML was selected as the enzymatic tenderiser for GP flesh in our study.

The aim of this study was to develop fish balls with an acceptable texture through GP enzymatic tenderisation and to study the effects of BML on the protein nanostructure/composition and the flavours of the fish balls. Textures of different fish ball samples were evaluated using Texture Profile Analysis (TPA), fracture gel analysis and sensory evaluation. Myofibrils were extracted from the fish ball samples for protein characterisation to further investigate the effects of BML on GP flesh. Analytical methods applied to the extracted myofibrillar proteins included sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). Atomic force microscopy (AFM) was applied to analyse the nanostructure and morphology of myofibrils. Meanwhile, gas chromatography-mass spectrometry (GC-MS) was applied to analyse the volatile flavour compounds in YF, GP and enzyme-treated GP fish balls to determine the effect of BML on the flavour of the fish balls.

Materials and Methods

Optimisation of Enzyme Conditions and Fish Ball Preparation

To optimise the enzymatic tenderisation method for GP flesh, a series of preliminary trials were conducted with commercial powder preparations of BML (50,000 U/g, Hebei Baiwei Biotechnology Co. Ltd., Handan, Hebei, China) using various application methods (direct addition or marinating in solution), different enzyme/substrate (E/S) ratio (0.01–0.8%, *w*/*w*), incubation time (15 min to 3 h) and temperatures (in ice water bath or at room temperature). According to the results of the texture analysis, the marinating approach using 0.4% BML in an ice water bath for 15 min was selected as the optimal treatment for GP in this study. Another sample was treated with 0.8% BML as a comparison to study the effect of a higher enzyme concentration.

Commercial-sized frozen YF and GP (Pasar, NTUC Fairprice Co-Operative, Singapore) were purchased from a local supermarket and transferred to the laboratory within 1 h for processing (Klomklao et al. 2016). Fish skins and

bones were removed completely, and flesh from the dorsal part of the fish was divided into four groups: YF, GP, GP marinated with five volumes of 0.4% (BML-myofibril protein ratio, *w*/*w*) BML solution and GP marinated with five volumes of 0.8% BML solution. The BML solutions were prepared by dissolving commercial BML powder (50,000 U/g, Hebei Baiwei Biotechnology Co. Ltd., China) in deionised water at room temperature. Each group of fish flesh was submerged in the respective solution (deionised water for untreated GP and YF) in an ice water bath for 15 min and then rinsed thoroughly with water to remove any residual solution. The washed flesh was blended with 5% (*w*/*w*) potato starch and 1.25% (*w*/*w*) table salt purchased from a local supermarket using a hand blender (300 W, Braun GmbH, Germany) for 90 s until thoroughly mixed. The blended paste was hand-rolled into balls (20 g each) using a spoon and boiled in deionised water for 10 min.

Texture Analysis

Each freshly prepared fish ball sample was cut from the centre into a cylinder of 15 mm in height and 15 mm in diameter for TPA using a TA.XT2i Texture Analyser (Stable Micro Systems Co. Ltd., UK) equipped with a flat-ended cylindrical probe (SMSP/20, 20 mm diameter). The test speed was 1 mm/s, and the distance of compression was 6 mm (40% of the sample height) (Mohtar et al. 2013; Sow and Yang 2015). Textural parameters, including hardness, cohesiveness, springiness, chewiness and resilience, were determined for each sample using the computer software, Exponent (Stable Micro Systems Co. Ltd., UK), derived from the respective force-time curve obtained.

Fracture gel analysis, as described by Poowakanjana et al. (2015), was performed using the same texture analyser and software equipped with a spherical probe (SMSP/5S, 5 mm diameter). Each fish ball sample was cut from the centre into a cylinder of 20 mm in height and 15 mm in diameter and punctured at a test speed of 1 mm/s. Fracture gel properties, including breaking force and penetration distance, were measured for each sample (Zhang et al. 2015).

Myofibrillar Protein Extraction

Myofibrillar proteins were extracted from each group of fish ball samples both before and after boiling, using the method described by Martone and others (1986), with modifications. Solution A [20 mM Tris-HCl buffer containing 0.10 M KCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN_3, pH adjusted to 7.5 using an HCl solution] and solution B [20 mM Tris-maleate buffer containing 0.45 M KCl, 5 mM β-mercaptoethanol (β-MCE), 1 mM ethylene glycol-bis (β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.2 M $Mg(CH_3COO)_2$, pH adjusted to 6.8 using a maleic acid solution] were prepared freshly before the extraction. Each sample (5 g) was minced and stirred with 10 volumes of solution A (50 mL) for 1 h in an ice water bath. The mixture was centrifuged at 16,000×*g*, 4 °C for 10 min before the pellet was collected, followed by a second wash under the same conditions with five volumes of solution B (30 mL) with the addition of 10 mM adenosine 5′-triphosphate (ATP). The mixture was centrifuged at 20,379×*g*, 4 °C for 25 min, and the supernatant was collected as the myofibrillar protein extract (Pazos et al. 2015).

SDS-PAGE and MALDI-TOF-MS Analyses

The soluble protein concentration in each myofibrillar protein extract was determined according to Bio-Rad Protein Assay Protocol (Bulletin 9004, Bio-Rad Laboratories, Inc., USA). Protein (20 μg) from each extract was loaded into the gel for analysis (4–20% gradient gel). The SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 1 L) and sample loading buffer (250 mM Tris-HCl buffer pH 6.8, 4% SDS, 40% glycerol, 0.02% bromophenol blue, 15% β-mercaptoethanol, 1/3 sample volume per well) were prepared, and protein standard (10–250 kDa) was used as the protein marker. Electrophoresis was performed on a Bio-Rad Mini-PROTEAN Tetra Cell at 200 V for approximately 50 min (Tian et al. 2015). The subsequent gel staining and distaining processes used Coomassie Brilliant Blue R-250, methanol and acetic acid (Chen et al. 2016; Deng et al. 2014). The experiment was repeated five times for the protein band intensity quantification. The gel image was captured using the gel imaging system G:BOX EF2 (Syngene, Synoptics Ltd., UK) and analysed using the computer software CLIQS 1D Pro (TotalLab Ltd., UK).

Myofibrillar protein extract (1 mL) was first dialysed in a precut dialysis tube in deionised water overnight with stirring in an ice water bath. The matrix for MALDI-TOF-MS was prepared by dissolving sinapic acid powder (matrix substance for MALDI-MS grade, ≥99.0%, Sigma-Aldrich Co. LLC., St. Louis, MO, USA) in acetonitrile at room temperature until saturated. Each dialysed sample of 2 μL was mixed with 2 μL of matrix before being pipetted onto a MALDI target plate (Bruker Daltonics, Bruker Corporation, USA) and air-dried at room temperature before analysis using a Bruker autoflex™ MALDI-TOF/TOF Mass Spectrometer with a smartbeam™ laser (Bruker Daltonics, Bruker Corporation, USA) (Feng et al. 2017a). Mass spectra obtained for each sample were analysed using the computer software flexAnalysis Version 3.0 (Bruker Daltonics, Bruker Corporation, USA).

AFM Analysis

Each dialysed myofibrillar protein extract (20 μL) was pipetted onto a freshly cleaved mica sheet attached to a magnetic

specimen disc. Each specimen was air-dried overnight in a petri dish at room temperature before being scanned by a TT-AFM atomic force microscope (AFM Workshop, USA) equipped with a Sensaprobe TM190-A-15 tip (Applied Nanostructures Inc., USA) at resonance frequencies of 145–230 kHz, force constants of 25–95 N/m and a Z scanner range of 0.2–0.4 Hz (Sow and Yang 2015; Xin et al. 2010; Yang 2014). The vibrating mode was selected with 128–512 scan lines in a maximum scan size of 15 μm × 15 μm. Images obtained from each specimen were analysed using the computer software Gwyddion (Nečas & Klapetek, Czech Republic).

Volatile Flavour Compounds Analysis

Volatile compounds in fish ball samples were measured using headspace solid phase microextraction gas chromatography-mass spectrometry (HS/SPME/GC/MS). Fish ball samples (2 g) were cut and weighed in an empty 10-mL fat-bottomed headspace vial and then capped. Analysis was carried out using a Shimadzu GC-MS-QP2010 Ultra Gas Chromatography-Mass Spectrometer coupled with a Shimadzu AOC-5000 Autosampler (Shimadzu Corporation, Kyoto, Japan). The chromatographic conditions used were as follows: column oven temperature was initially held at 35 °C for 3 min, followed by an increase to 200 °C at 3 °C min^{-1}. The temperature was then held at 200 °C for 3 min, and finally the temperature was increased to 250 at 20 °C min^{-1} and held for 10 min. The injection temperature was set at 220 °C using the splitless injection mode. The Electron Impact (EI) ionisation MS conditions were as follows: interface temperature, 265 °C; ionisation voltage, 70 eV; mass range m/z, 35–350; and scan speed, 1666 amu s^{-1}. The National Institute of Standards and Technology (NIST) mass spectral database (NIST07) was used to identify the volatile compounds. Semi-quantitative analysis was carried out using normalised total ion count (TIC) peak area counts by multiplying the peak area counts by 10^{-5} (Feng et al. 2017b).

Sensory Evaluation

Each fish ball sample was cut into eight bite-size pieces (2.5 g each), and each piece was labelled with a random three-digit code as one serving for use in sensory evaluation. After preparation, the samples were refrigerated at 4 ± 1 °C overnight and reheated in boiling water for 1 min till warm before being served.

Thirty-two untrained panellists (11 male, 21 female, aged between 22 and 57) from the National University of Singapore participated in the evaluation session, which was conducted in a designated sensory evaluation laboratory with individual booths and normal lighting. Each panellist was provided with four servings of samples from the four treatment groups, respectively, which were served in a random order along with water and plain crackers for mouth rinsing between sample tasting. The panellists were tasked to observe, smell, taste and score each sample on a standard nine-point hedonic scale in terms of its appearance, odour, taste, texture and overall quality (Lim 2011; Pang et al. 2017).

Statistical Analysis

All statistical analyses of the experimental data were performed at least in triplicate, and the results were reported in the form of the mean value ± standard deviation. The significance of the differences among the results was determined using one-way Analysis of Variance (ANOVA), accompanied by Duncan's post hoc multiple comparison test with a significance level of 0.05, using the computer software IBM SPSS Statistics Version 22 (International Business Machines Co. Armonk, NY, USA).

Results and Discussion

Texture Analysis

Five textural parameters including hardness (N), chewiness (N), springiness, cohesiveness and resilience of fish ball samples prepared using YF, GP, GP tenderised with 0.4% BML ("GP + 0.4% BML") and 0.8% BML ("GP + 0.8% BML") are also shown in Fig. 1. Fracture gel properties including breaking force (N) and penetration distance (cm) of fish ball samples from the four treatment groups are also presented in Fig. 1.

The hardness and chewiness of the GP fish balls were 12.91 and 9.09 N, respectively, which were significantly higher than those of YF. After 0.4% BML treatment, the hardness and chewiness decreased to 8.65 and 6.06 N, respectively, which were not significantly different from the values of YF fish balls. BML is plant thiol protease, which can break peptide and disulphide bonds, affect myosin structure in myofibrillar proteins and decrease the hardness and chewiness of fish balls (Shin et al. 2008). It was also observed that the GP fish balls had lower resilience than the YF fish balls, and the resilience of the fish balls increased to 0.4 after 0.4% BML treatment, which was not significantly different compared with the YF fish balls. Meanwhile, there were no significant differences in springiness and cohesiveness between YF and GP fish balls, indicating that these two textural parameters were not affected by any intrinsic differences between the flesh of YF and GP. The texture results indicated that 0.4% BML-tenderised GP flesh successfully and made fish balls with identical texture properties to those of YF fish balls. However, the 0.8% BML treatment decreased the hardness

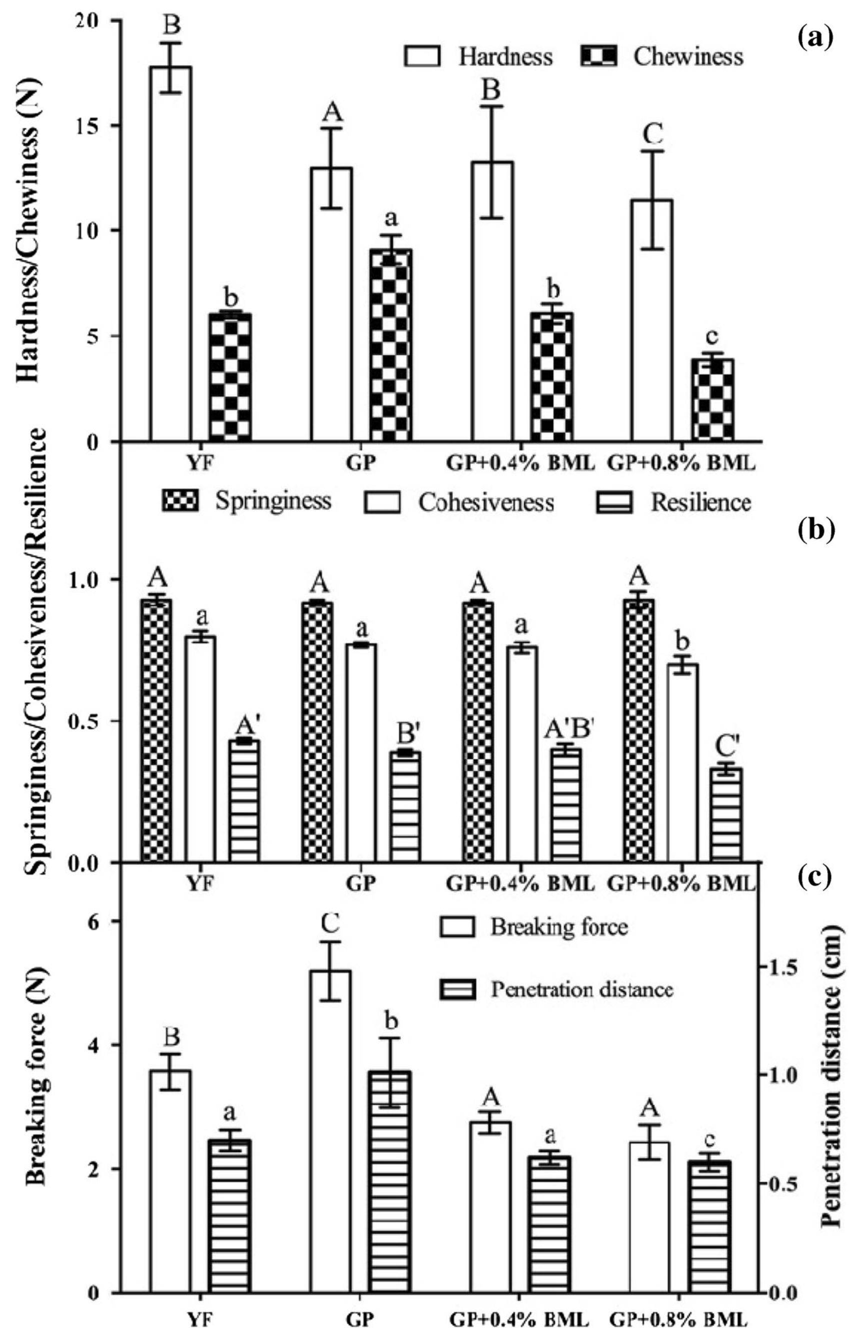

Fig. 1 Hardness and chewiness (**a**). Springiness, cohesiveness and resilience (**b**). Breaking force and penetration distance (**c**) of fish balls from four different groups. Means of the same parameter with *different letters* are significantly different ($n = 3$, $P < 0.05$)

and chewiness to 5.96 and 3.87 N, respectively, and its cohesiveness and resilience became significantly lower than both GP and YF fish balls, indicating the undesirable textures and an over-tenderisation effect. Figure 1 showed a significant decrease in both breaking force and penetration distance of fish balls prepared from BML-treated GP, indicating BML's tenderising effects on GP flesh. The breaking force of beef also showed a significant reduction after being treated by BML. Meanwhile, an increase of the BML concentration led to further reductions in the breaking force, which was consistent with our results (Zhao et al. 2012).

SDS-PAGE and MALDI-TOF-MS

SDS-PAGE

To further examine the specific effects of BML on the GP flesh, protein characterisation by SDS-PAGE and MALDI-TOF-MS was performed on myofibrillar proteins extracted from fish ball samples both before and after boiling. A representative gel image obtained from SDS-PAGE is shown in Fig. S1. M_W (kDa), band intensities and functions of common myofibrillar proteins are presented in Table 1. The protein

bands were defined according to a previous study (Alberts et al. 2002).

Based on Fig. S1 and Table 1, myosin heavy chain (MHC, 220 kDa) was not detected in the boiled samples, which was probably caused by myosin cross-linking bond formation after thermal processing (Li et al. 2016). It has also been reported that endogenous transglutaminase can catalyse the cross-linking between myofibrillar proteins (Takahashi et al. 2016). The myosin cross-linking caused the increased texture properties of the fish balls formed after boiling (Kim and Taub 1991). Meanwhile, it could be observed that actin remained in each BML-treated sample with a similar band intensity, suggesting that actin was not affected significantly by heating or BML degradation. It was reported that BML did not have a significant degradation effect on actin unless prolonged digestion and thermal treatment was applied (e.g. >1 h). For example, the band intensity of actin decreased significantly after heating at 60 °C for 120 min (Kim and Taub 1991; Takahashi et al. 2016).

Table 1 shows that the band intensity of myosin light chain (MLC, 16–25 kDa) was 5.40% in the GP samples before boiling, whereas 0.4 and 0.8% BML treatment decreased the band intensity of MLC to around 3.3%, which was slightly higher than the band intensity of MLC in the YF samples (2.12%). The degradation effect of BML on MLC was also demonstrated in a previous study (Lopez et al. 2015). The degradation of MLC tenderised the GP flesh, making its protein composition and texture more similar to that of the YF. Meanwhile, 0.8% BML treatment decreased the band intensity of troponin T (29 kDa) significantly from 1.55 to 1.00%, leading to over-tenderisation of the GP flesh, while 0.4% BML treatment did not demonstrate a hydrolytic effect on troponin T. Troponin T regulates contraction in the muscle, and it is a meat tenderisation protein marker (Ryder et al. 2015). Moreover, several new bands with M_W ranging from 60 to 150 kDa were detected in the BML-treated samples, which were likely to be the proteolytic degradation products of MHC, as reported by Gerelt and others (2000). However, BML had no hydrolytic effect on tropomyosin (35 kDa), which was consistent with a previous report (Ha et al. 2012). Meanwhile, GP had significantly higher levels of troponin C (15 kDa) compared with that in YF. Heat treatment digested the troponin C completely, while BML had no hydrolytic effect on it. In the YF sample, a band of 50 kDa was detected, which was not present in GP and BML-treated GP samples. Another protein of 12 kDa was detected in both GP and YF, and 0.8% BML degraded it to 0.15%, while the heat treatment did not further digest this protein in YF and GP fish ball samples. Neither of these proteins has been reported so far; protein sequencing could be performed in future studies to identify the unknown proteins.

MALDI-TOF-MS

To complement the results of SDS-PAGE, MALDI-TOF-MS was employed to determine the M_W of myofibrillar proteins present in each sample with higher accuracy. The mass spectra obtained through MALDI-TOF-MS are shown in Fig. S2, where the peak *m/z* values represent the M_W (Da) of certain myofibrillar proteins detected from the untreated GP sample before boiling. The results for the eight treatment groups are shown in Table 2.

Table 2 shows that in general, more proteins were identified in samples before boiling than in those after boiling, which confirmed the observation obtained through SDS-PAGE. Meanwhile, more myofibrillar proteins and peptides ranging from 2 to 36 kDa were identified by MALDI-TOF-MS in the YF flesh compared with the GP flesh. Consistent with the SDS-PAGE results, MLC, as well as other minor proteins such as troponin T, were largely degraded in the presence of BML, and almost all the proteins that were detected in YF and GP with a M_W higher than 15 kDa disappeared upon treatment with BML, which was consistent with previous findings (Lopez et al. 2015). Meanwhile, a number of newly formed myofibrillar protein fragments were identified in the BML-treated samples as the hydrolysis products, such as 2451-, 3052-, 7136- and 12,045-Da protein fragments.

Table 2 and Fig. S2 show that peptides of 2451 and 3052 Da were generated after 0.4 and 0.8% BML treatment, and a peptide of 7136 Da was produced after 0.8% BML hydrolysis. In addition, both YF and GP samples had molecules of 9109, 10,022 and 11,487 Da, which were digested by 0.4 and 0.8% BML hydrolysis. A peptide of 12,944 Da was produced after 0.8% BML treatment, which was not present in the other groups. However, the proteins from which the new peptide fragments were produced could not be determined from the current results.

The results obtained through MALDI-TOF-MS were relatively straightforward and indicated clear trends of compositional changes in myofibrillar proteins among the different treatment groups, which were in line with the results obtained from SDS-PAGE. Nevertheless, the main drawback of this myofibrillar protein characterisation method was that the *m/z* signals of larger protein molecules became weakened and only those with a M_W lower than 40 kDa were detected in the mass spectra obtained (Feng et al. 2016). Meanwhile, MALDI-TOF-MS only provided the molecular weight information. MALDI-TOF/TOF-MS/MS and HPLC-ESI-Q-TOF-MS could be performed in a future study to identify the unknown proteins and peptides and to demonstrate the BML cleavage sites and tenderising mechanism (Nessen et al. 2016).

Table 1 Effects of bromelain treatment and boiling on the band intensity (≥0.005%) of myofibrillar proteins from SDS-PAGE results

M_W (kDa)	Band intensity/%									
	Before boiling				After boiling				Protein	Function (Alberts et al. 2002)
	YF	GP	GP + 0.4% BML	GP + 0.8% BML	YF	GP	GP + 0.4% BML	GP + 0.8% BML		
210–220	0.13a	0.08a	0.13a	0.87b	–	–	–	–	Myosin heavy chain	Slides actin filaments
150	–	–	0.08a	0.38b	–	–	–	–	M3	Myosin breakdown product
130	–	–	0.02	–	–	–	–	–	C protein	Myosin breakdown product
90	0.18a	–	–	0.75b	–	–	–	–	Gelsolin	Fragments actin filaments
80	–	–	0.14a	0.12a	–	–	–	–	–	Myofibril degradation product
68	0.39b	–	–	0.09a	–	–	–	0.33b	Fimbrin	Bundles actin filaments
50	1.57	–	–	–	–	–	–	–	–	–
42	6.21b	6.47b	6.70b	6.29b	5.92b	6.35b	5.75b	4.33a	Actin	Forms filaments
35	0.63ab	0.68b	0.85b	0.77b	–	–	0.46a	1.3c	Tropomyosin	Strengthens actin filaments
29	1.59c	1.55c	1.57c	1.00b	0.16a	0.25a	0.09a	0.40ab	Troponin T	Regulates contraction
16–25	2.12b	5.40d	3.23c	3.38c	0.85a	3.92c	3.46c	3.68c	Myosin light chains	Slide actin filaments
15	0.50a	0.71b	0.97b	0.98b	–	–	–	–	Troponin C	Regulates contraction
12	1.10b	0.81b	1.02b	0.15a	1.06b	0.98b	0.95b	–	–	–

Band intensity is the percentage of the band area in the total band area on the gel image. Means of the band intensity with different lowercase letters are significantly different ($n = 5$, $P < 0.05$)

YF yellowtail fusilier, *GP* golden pomfret, *BML* bromelain, – the protein is not detected

AFM Analysis

AFM has been applied successfully to image the nanostructures of biomolecules, such as fish gelatin and polysaccharides (Chong et al. 2015; Sow and Yang 2015). In this study, AFM analysis was used to examine the nanostructural changes of myofibrillar proteins as a result of BML's tenderising effects. Examples of images obtained through AFM are shown in Fig. 2. The dimensions including length, width and height of the myofibrillar proteins are shown in Table 3.

Myofibrillar proteins in the GP samples before boiling appeared as relatively long rod-like structures (Fig. 2b), which was consistent with previous findings (Chaurasiya et al. 2015; Feng et al. 2016). After treatment with BML, the fibres dissociated into isolated circular structures (Fig. 2c, d), which resembled the nanostructure of myofibrils from YF (Fig. 2a). Zhao et al. (2012) reported a similar dissociation of myofibrillar proteins in BML-treated beef using scanning electron microscopy (SEM). The circular structure of myofibrils in YF was also found in the myofibrils from European pearlfish (*Rutilus frisii meidingeri*) (Stoiber et al. 1998).

Table 3 shows that the lengths of myofibrils from YF, GP and GP + 0.4% BML before boiling were longer than 15 μm, while the length decreased to 10.95 μm for GP + 0.8% BML, which confirmed the over-tenderisation indicated by the texture results. The myofibrils were 1.76 μm wide for the GP group, which was significantly wider than the myofibrils from the YF samples. After 0.4% BML treatment, the width decreased to the same level as that of YF and further decreased to 1.06 μm after 0.8% BML treatment, which was significantly lower than that of the YF and GP + 0.4% BML groups, indicating the over-tenderisation effect of 0.8% BML treatment. The AFM result was consistent with the texture result and indicated an over-tenderisation effect of 0.8% BML treatment. However, a further decrease in myofibril height was not observed for GP + 0.8% BML group before boiling.

After boiling, granulation of myofibrillar proteins was observed (Fig. 2e–h), similar to the proteins from sardine after boiling (Vate and Benjakul 2016). Thermal treatment may partially unfold the myofibrils and aggregate them through both covalent and non-covalent bonds to form the sphere-shaped microstructures (Vate and Benjakul 2016). It was also observed that the myofibrils from boiled GP fish balls remained rod-shaped, with some dissociation, indicating the degradation effect of thermal treatment via heat-activated endogenous proteases (Takahashi et al. 2016; Vate and Benjakul

Table 2 Effects of bromelain treatment and boiling on the myofibrillar proteins from fish balls, detected by MALDI-TOF-MS (indicated as "✓")

M_W (kDa)	Before boiling				After boiling			
	YF	GP	GP + 0.4% BML	GP + 0.8% BML	YF	GP	GP + 0.4% BML	GP + 0.8% BML
2.0	✓				✓			
2.5			✓	✓				
3.0			✓	✓				
4.0	✓	✓	✓	✓		✓	✓	✓
4.5					✓	✓		
5.0	✓	✓	✓	✓			✓	
5.5	✓							
6.0		✓	✓	✓	✓	✓	✓	✓
7.0				✓				
7.5							✓	
8.5	✓						✓	✓
9.0	✓	✓				✓		
10.0	✓	✓			✓			✓
10.5						✓		
11.5	✓	✓			✓	✓		
12.0		✓	✓		✓		✓	✓
12.5	✓							
13.0				✓	✓			✓
13.5							✓	
14.0	✓						✓	
14.5							✓	
15.5	✓						✓	
16.5	✓							
17.5						✓		
18.0	✓	✓	✓		✓	✓		
19.0	✓							
19.5	✓							
21.5	✓							
22.0	✓							
23.0		✓				✓		
23.5	✓							
26.5	✓							
28.0	✓							
29.5	✓	✓						
33.5	✓							
36.0		✓						

MALDI-TOF-MS matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry, *YF* yellowtail fusilier, *GP* golden pomfret, *BML* bromelain

2016). There was a noticeably larger degree of myofibrillar protein disintegration and fragmentation in the BML-treated samples after boiling (Fig. 2g–h) compared with untreated GP, indicating a higher level of degradation of myofibrillar proteins induced by BML (Christensen et al. 2009).

The myofibrils from GP fish balls were significantly longer than those of the other three groups (Table 3), which explained their integrity and harder texture. It was also noticeable that the lengths of myofibrils from YF and GP + 0.4% BML were 6.83 and 6.42 μm, respectively, and there were no significant differences in the lengths, widths and heights, indicating that the similar dimensions, morphology and nanostructure of myofibrils determined the similar textures between YF and GP + 0.4% BML fish balls. Interestingly, the length of the

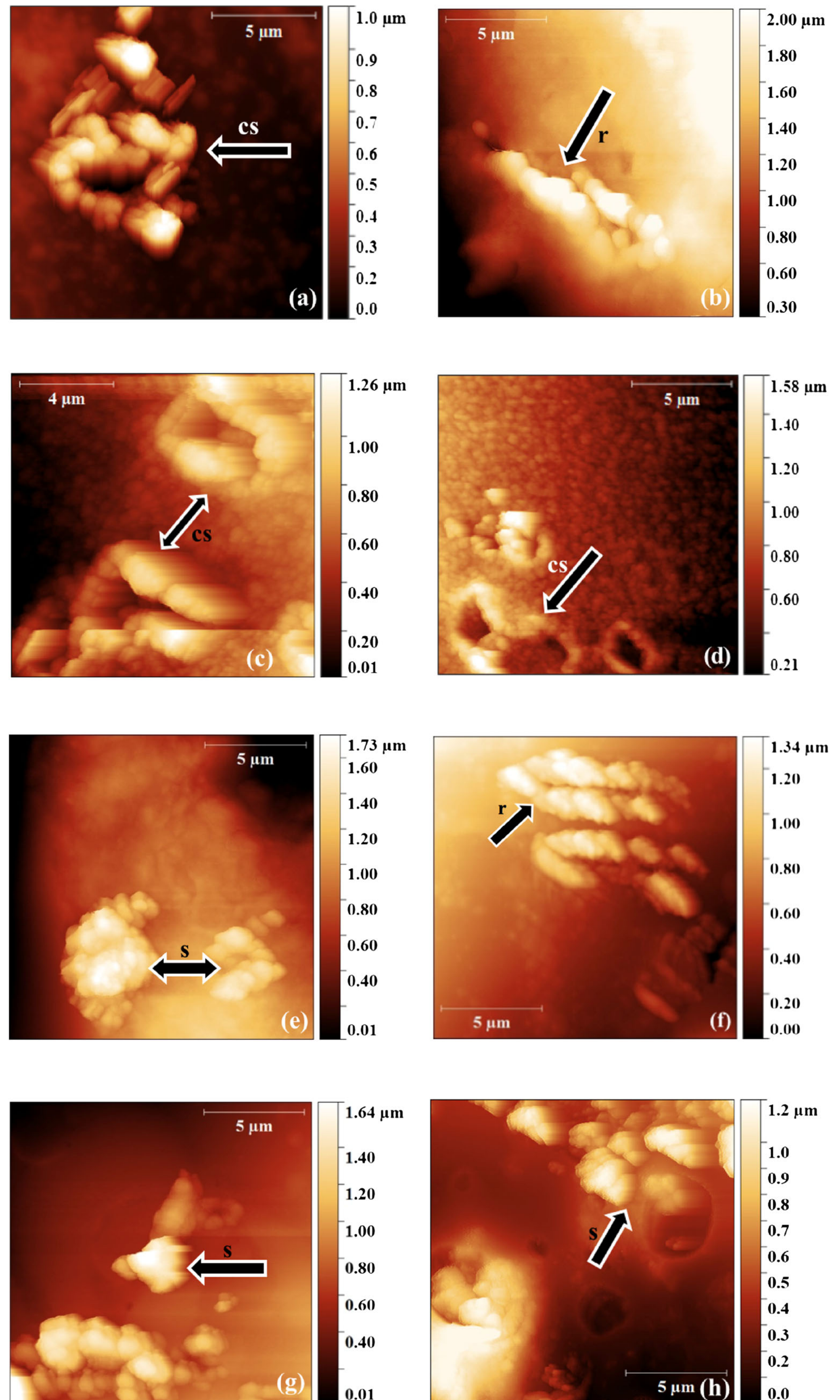

Fig. 2 Atomic force microscopy (AFM) images of myofibrils from different groups: **a** YF, **b** GP, **c** GP + 0.4% BML and **d** GP + 0.8% BML before boiling and **e** YF, **f** GP, **g** GP + 0.4% BML and **h** GP + 0.8% BML after boiling. *cs* circular structure, *r* rod-like structure, *s* sphere-shaped structure, *YF* yellowtail fusilier, *GP* golden pomfret, *BML* bromelain

Table 3 Effects of bromelain treatment and boiling on the dimensions of myofibrillar proteins imaged by AFM

Processing	Dimension/μm	YF	GP	GP + 0.4% BML	GP + 0.8% BML
Before boiling	Length	>15[a]a	>15a	>15a	10.95 ± 0.29b
	Width	1.44 ± 0.21b	1.76 ± 0.08a	1.61 ± 0.03ab	1.06 ± 0.07c
	Height	1.21 ± 0.14b	1.66 ± 0.26a	1.24 ± 0.09b	1.33 ± 0.06b
After boiling	Length	6.83 ± 0.76B	9.03 ± 0.62A	6.42 ± 0.52BC	4.50 ± 1.45C
	Width	1.42 ± 0.17B	2.06 ± 0.13A	1.52 ± 0.13B	1.37 ± 0.09B
	Height	1.40 ± 0.28A	1.66 ± 0.30A	1.48 ± 0.15A	1.33 ± 0.39A

Means of the same dimension with different letters (a–c for samples before boiling; A–C for samples after boiling) are significantly different ($n = 15$, $P < 0.05$)

AFM atomic force microscopy, *YF* yellowtail fusilier, *GP* golden pomfret, *BML* bromelain

[a] Larger than the maximum scan size of 15 μm × 15 μm

myofibrils from GP + 0.8% BML was 4.5 μm, which was significantly lower than that of YF and GP + 0.4% BML after boiling, indicating over-tenderisation after 0.8% BML treatment.

The results obtained from quantitative analysis of myofibrillar protein dimensions were consistent with the proteolytic activity of BML (Gravelle et al. 2016). Moreover, the decrease in protein length and width became more significant as the concentration of BML used increased, which agreed with the finding by Christensen and others (2009) that the particle sizes of myofibrillar proteins in pork tended to decrease with the increasing concentration of actinidin used for tenderisation. Furthermore, the decrease in myofibril length and width correlated with the decreased hardness and chewiness of the fish balls, which agreed well with previous findings that the detachment among fibres correlated with the decreased hardness and chewiness of fish fillets (Ayala et al. 2011).

The results of AFM analysis implied that BML modified the morphology of myofibrils effectively by transforming them into circle-shaped structures and fragments, which was also reflected by the change in M_W of proteins and peptides in different samples, as revealed by SDS-PAGE and MALDI-TOF-MS. Therefore, a clear illustration of BML's tenderising effects on GP was obtained by characterising fish myofibrillar proteins in terms of protein composition and nanostructure.

Volatile Flavour Compound Analysis

GC-MS was applied to compare the flavour compounds of fish balls made from GP and YF, as well as to evaluate the effects of BML treatment on the flavour changes of the fish balls. There were more volatile flavour compounds identified in the YF fish balls compared with those in the GP fish balls, and the common volatiles were present in different ratios. Table 4 shows the quantification of the different volatile flavour compositions, and compounds generated from the column during heating are not presented. BML treatment modified the volatile composition of the fish balls. For example, the hexanal level in the GP fish balls was 6.68%, which was significantly higher than the 2.07% of the YF fish balls. However, after 0.4 and 0.8% BML treatment, the hexanal levels decreased to 2.11 and 2.52%, respectively, which were not significantly different compared with the levels in YF fish balls. Hexanal was found to have a warmed-over flavour in meat (Shahidi and Botta 2012), which was not desirable. Decreased hexanal in the GP + 0.4% BML fish balls would make their flavours more acceptable and pleasant, which agreed with previous finding that BML-tenderised meat had better flavours (Kim and Taub 1991).

BML treatment also helped to decrease the hexadecane percentage in GP fish balls, a flavour compound of cooked shrimp (Shahidi and Botta 2012), from 29.83% to around 25%, which was slightly closer to the 18.02% found in YF fish balls. BML also decreased the ratio of 1-octen-3-ol, a flavour compound found in cooked crayfish, oyster, prawn and shrimp (Shahidi and Botta 2012). However, BML increased peptadecane from 18.1 to 38.2%. The GP and enzyme-treated fish balls had significantly higher amounts of heptadecane compared with that in the YF fish balls, in which the heptadecane percentage was only 8.37%. Furthermore, BML decreased the 2,6,10,14-tetramethylpentadecane content from 17.58% in GP fish balls to 16.07% in GP + 0.4% BML fish balls, which was closer to that of YF fish balls (14.4%). It was reported that this volatile compound was found in cooked crayfish and shrimp (Shihadi and Botta 2012). The decreased hexanal, hexadecane and 2,6,10,14-tetramethylpentadecane levels after BML treatment made the flavour composition of GP + 0.4% BML fish balls similar to that of YF fish balls. Meanwhile, the YF fish balls had more volatile flavour compounds that were not detected in GP and in enzyme-treated GP fish balls, such as 2,6,10,15-tetramethylheptadecane and 2-undecanone. 2,6,10,15-Tetramethylheptadecane contributes to the flavours of shrimp (Zhang et al. 2011), and 2-undecanone is a flavour and aroma compound discovered in miso products (Giri et al. 2010). These natural flavour compounds could be further added to GP + 0.4% BML fish balls to increase their resemblance to YF fish balls.

Table 4 Identification and semi-quantification of volatile flavour compounds identified in fish balls after boiling

Volatile compound	Group				Flavour description
	YT	GP	GP + 0.4% BML	GP + 0.8% BML	
Hexanal	2.07a	6.68b	2.11a	2.52a	Warmed-over meat (Shahidi and Botta 2012)
Hexadecane	18.02a	29.83c	25.97b	23.68b	Cooked shrimp (Shahidi and Botta 2012)
1-Octen-3-ol	3.07c	2.97b	1.59a	1.43a	Cooked crayfish, oyster, prawn, shrimp (Shahidi and Botta 2012)
Heptadecane	8.37a	18.14b	38.23c	38.21c	Cooked cray fish (Shahidi and Botta 2012)
3,5-Octadien-2-one	ND	3.38b	3.36b	1.38a	Fatty, fruity (Saxby 2012)
2,6,10,14-Tetramethyl pentadecane	14.4a	17.58c	16.07b	15.43ab	Cooked crayfish and shrimp (Shihadi and Botta 2012)
Butyl hexanoate	1.23	ND	ND	ND	Fatty, sweaty, cheesy (Rowan et al. 1999)
2,6,10,15-Tetramethyl heptadecane	4.37	ND	ND	ND	Shrimp (Zhang et al. 2011)
2-Undecanone	4.88	ND	ND	ND	Miso product (Giri et al. 2010)
1-Nonanol	1.62	ND	ND	ND	Cooked rice, fruits and vegetables (Song et al. 2016)
2-Propyl-1-pentanol	1.86	ND	ND	ND	Processed cheese (Sunesen et al. 2002)

Compounds were identified by comparison with reference substances from the MS NIST library and were present in three replicates. Semi-quantification was expressed in arbitrary unit area (absolute area multiplied by 10^{-5}) and indicated as the mean. Means within the same storage time with different lowercase letters are significantly different ($n = 3$, $P < 0.05$)

YF yellowtail fusilier, *GP* golden pomfret, *BML* bromelain, *ND* not detected

Sensory Evaluation

To evaluate consumer acceptability, sensory attributes including appearance, odour, taste, texture and overall quality of the fish ball samples from the four treatment groups were examined. Using the scores of YF as a reference, a relative score for each sensory attribute was obtained by subtracting the respective YF scores (Gerelt et al. 2000), and the average relative scores of the four treatment groups are shown in Fig. 3.

No significant differences were observed among the four treatment groups in terms of all sensory attributes except appearance, because the intrinsically whiter colour of YF flesh was highly preferred. The use of BML did not cause any apparent changes in the appearance of the GP fish balls, while the YF fish balls were rated with a significantly higher score for appearance regardless of treatment for the GP fish balls. In terms of odour and flavour, the GP and BML-treated fish balls showed no significant differences compared with the YF fish balls, indicating there was no off-flavour generated by BML treatment. This is supported by other studies which demonstrate that BML treatment would not cause off-flavours during meat processing (Chuapoehuk and Raksakulthai 1992; Sullivan and Calkins 2010).

The results suggested that the other four sensory attributes of fish balls, i.e. odour, taste, texture and overall quality, were not affected significantly by the intrinsic differences between

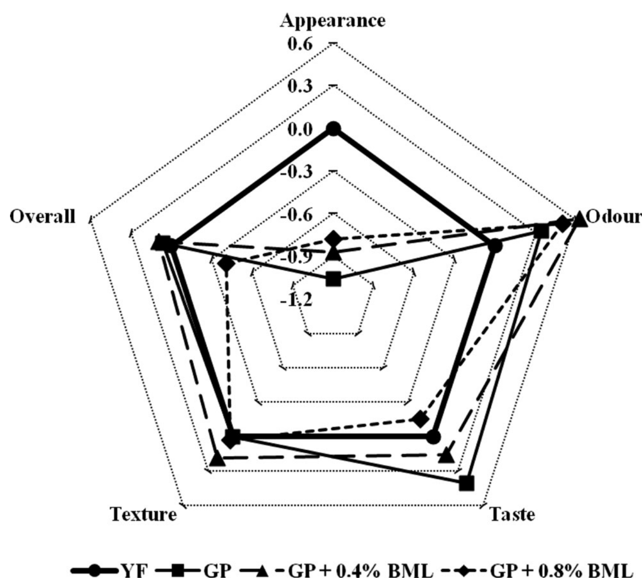

Fig. 3 Appearance, odour, taste, texture and overall quality of fish balls from four different groups

YF and GP or by the addition of BML, which indicated that the differences of flavour and texture detected by GC-MS and TPA were not sufficient to cause the differences in the sensory results. Well-trained panellists could be invited to conduct the sensory evaluation, or the number of untrained panellists could be increased to 150 to obtain more reliable results (Tee and Siow 2014). The sensory results indicated that the optimal treatment with 0.4% BML was unlikely to have any negative impact on consumers' acceptability of the tenderised GP fish balls, which would be a prerequisite for the adoption of this tenderisation method by the food industry.

Conclusions

In this study, an enzymatic tenderisation method, which comprises the marinating of GP with a 0.4% BML solution, was developed to produce fish balls with similar texture and flavour to YF fish balls. The tenderisation method reduced the hardness and chewiness significantly to 8.65 and 6.06 N, respectively, while it increased the resilience of fish balls. No significant differences in texture properties were found between GP + 0.4% BML and YF fish balls. BML degraded myosin light chain and troponin T effectively without affecting actin, and generated myofibrillar protein fragments, thereby improving the tenderness of GP. Circle-shaped myofibrils were observed after BML treatment, which resembled the myofibril nanostructure in YF before boiling. Meanwhile, the similar dimensions of the sphere-shaped myofibrils between the GP + 0.4% BML and YF fish balls explained their equivalent texture properties. The GC-MS results showed that YF fish balls had more volatile flavour compounds and that BML decreased the amounts of hexanal, hexadecane, 2,6,10,14-tetramethylpentadecane and 1-octen-3-ol to 2.11, 25.97, 16.07 and 1.59%, respectively, and increased heptadecane to 38.23% in the GP + 0.4% BML fish balls, making their flavour similar to that of YF fish balls. The results suggest that a 0.4% BML (*w*/*w*) solution can tenderise GP to produce quality fish balls with desirable texture and flavours, with a similar nanostructure and protein composition compared with YF fish balls. This tenderisation method could be applied to the fish ball manufacture using locally farmed GP on a commercial scale, as part of the efforts to enhance Singapore's food supply resilience in the long term. Furthermore, the results might also offer an insight into the potential application of BML in general fishery products, which remains a developing research field in need of further exploration.

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