

Effect of Legume Seed Protein Isolates on Autolysis and Gel Properties of Surimi from Sardine (*Sardinella albella*)

Tanaji Kudre and Soottawat Benjakul

Abstract— Effects of protein isolate from mung bean (MBPI), black bean (BBPI) and bambara groundnut (BGPI) at different levels (0-1.5%, w/w) on autolysis and gel properties of sardine (*Sardinella albella*) surimi were evaluated. Breaking force and deformation of both kamaboko (40/90 °C) and modori (65/90 °C) gels increased with increasing MBPI, BBPI and BGPI levels ($P<0.05$). When MBPI or BBPI or BGPI at a level of 1.5% was incorporated, breaking force of kamaboko and modori gels increased by 22.4-76.8, 24.9-95.3 and 90.0-135.3%, while deformation increased by 11.6-31.3, 13.1-42.7 and 26.4-61.39%, respectively. This was coincidental with lowered TCA-soluble peptide content. Myosin heavy chain (MHC) of modori gel was more retained as MBPI, BBPI and BGPI concentrations increased. The concomitant increase in water-holding capacity of resulting gel was obtained; however, the whiteness was slightly decreased ($P<0.05$). The microstructure study reveal that modori gel added with MBPI or BBPI or BGPI at 1.5% were comparatively denser and more ordered than without protein isolate. Generally BGPI exhibited the superior gel enhancing effect than BBPI and MBPI, regardless of level added. Therefore, BGPI at an appropriate level could be an alternative food grade protein additive to improve gel properties of surimi.

Keywords—Autolysis, Legume seeds, Protein isolates, Sardine, Surimi gel, Texture analysis

I. INTRODUCTION

SARDINE (*Sardinella albella*) is a small coastal pelagic fish species, which can be used for surimi production in Thailand. Apart from the darken colour, surimi from sardine has the poorer gel properties than those produced from lean fish. This leads to the less demand of surimi from sardine. In general, dark fleshed fish have been reported to contain high level of proteases, which induce the protein degradation [1]. Proteolytic disintegration of myofibrillar proteins has an adverse effect on gel-forming properties of surimi. The breakdown of myofibrillar proteins inhibits the development of three-dimensional gel network [2]. When the gel is heated at 50-70 °C, an irreversible proteolytic degradation of myofibrillar proteins occurs, resulting in the disintegration of

the gel structure named ‘modori’ [3]. Modori gel is mainly due to the autolysis caused by heat-stable proteases such as cathepsins, alkaline proteases, and calpains [4]. (Although some proteases are leached out during washing process, some proteases, especially those bound with myofibrillar proteins are retained. Reference [5] found that both endogenous sarcoplasmic and myofibril-associated proteinases play an important role in degradation of myofibrillar proteins in lizardfish muscle, particularly at 60-65 °C, leading to gel weakening.

To alleviate the problems associated with protein degradation caused by the endogenous proteases, several protein additives have been used in surimi to improve the properties of surimi gels. Whey protein concentrate (WPC), chicken plasma protein (CPP) beef plasma protein (BPP), porcine plasma protein (PPP), egg white (EW), and rainbow trout plasma protein (RPP) can be used as food grade protease inhibitors in surimi [6], [7]. However, the use of BPP and CPP has been forbidden, because of bovine spongiform encephalopathy (BSE) and outbreak of avian influenza (AI), respectively. Egg white is expensive and has an undesirable egg-like odor, whilst blood plasma is associated with off-colour and off-flavour of resulting surimi gel. Plant protein isolate, particularly soy protein isolate, has been used in surimi industry owing to its safety and reasonable price [8].

Due to a variety of legume seeds in Thailand, those seeds can be used for production of protein isolates, which can be used as an alternative protein additive for surimi gel improvement. It has been reported that several legume seed extract contained trypsin inhibitors and was able to inhibit protease activity of fish muscle and surimi [8], [9]. Reference [10] stated that vicilin and legumin a major legume seed storage proteins were act as a co-gelling agent or binder in surimi gels. Protein isolates from selected legume seed can be a source of protease inhibitors which could lower modori phenomenon in surimi, and/or act as binder or filler, thereby improving gel property. The purpose of this study was to investigate the preventive effects of protein isolate from black bean and mungbean seeds on the hydrolysis of myofibrillar protein by endogenous proteases in surimi made from sardine (*Sardinella albella*) and to study the associated effects on surimi gel improvement.

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II. MATERIALS AND METHODS

A Chemicals and Surimi

N- α -Benzoyl-DL-arginine-p-nitroanilide (BAPNA), trypsin from bovine pancreas (BAEE 10,200 units/mg), β -mercaptoethanol (β -ME), bovine serum albumin (BSA) and wide range molecular weight protein markers were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, sodium chloride, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, *N, N, N', N'*-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA).

Frozen surimi grade A from sardine (*Sardinella albella*) was obtained from Pacific Fish Processing Co., Ltd. (Songkhla, Thailand) and kept at -20 °C until use, but not longer than three months.

B. Preparation of Protein Isolates from Black Bean and Mungbean

Mungbean (*Phaseolus aureus*), black bean (*Phaseolus vulgaris* L.) and bambara groundnut (*Vigna subterranean*) were purchased from Thai Cereals World Co., Ltd. (Bangkok, Thailand). The samples were dehulled and ground using a blender (Moulinex, Type AY46, Shenzhen, Guangdong, China) to obtain the fine powder. The powder was screened using a mesh with an aperture size of 500 μ m, (35, ASTM E11, serial number 5666533, FRITTSCH GMBH, Idar-Oberstein, Germany).

Mungbean, black bean and bambara groundnut protein isolates were prepared according to the methods of [11] with a slight modification. Mungbean, black bean and bambara groundnut powder was suspended in 10 volumes of 0.2% NaOH solution (pH 12). The mixture was stirred continuously for 2 h at room temperature (28–30 °C), followed by centrifugation at 8000 \times g for 30 min at 25 °C (Avanti J-E centrifuge, Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was collected and pH was adjusted to 4.5 using 6 N HCl. The precipitate formed was recovered by centrifugation at 8000 \times g for 30 min. The pellet was washed with 10 volume of distilled water (pH 4.5), followed by centrifugation at 8000 \times g for 30 min. The resulting pellet was freeze-dried. Dried powder obtained was referred to as mung bean (MBPI), black bean (BBPI) and bambara groundnut proteins isolate (BGPI). MBPI, BBPI and BGPI were placed in polyethylene bag and stored at -40 °C until use. MBPI, BBPI and BGPI had the protein content of 88.73%, 87.80% and 86.2%, respectively, as determined by Kjeldahl method [12].

C. Trypsin Inhibitory Activity Assay

Trypsin inhibitory activity of MBPI, BBPI and BGPI was measured by the method of [13]. Trypsin inhibitors from all protein isolates were extracted by continuous stirring both

protein isolates (5 mg/ml) in 25 ml of distilled water at a speed of 300 rpm at room temperature (26–28 °C) for 90 min using magnetic stirrer (IKA® Model Colour Squid white, BEC THAI, Bangkok, Thailand). The mixtures were then subjected to centrifugation at 12000 \times g at 25 °C for 25 min. The supernatant was used for determination of trypsin inhibitors.

To assay for trypsin inhibitor, the solution containing 200 μ l of inhibitor solution (protein isolates supernatant), 200 μ l of bovine pancreas trypsin (0.1 mg/ml) and 1000 μ l of 50 mM Tris-HCl, pH 8.5 containing 10 mM CaCl₂ was pre-incubated at 37 °C for 15 min. To initiate the reaction, 200 μ l of BAPNA (0.4 mg/ml in DMSO), pre-warmed to 37 °C were added and vortexed immediately to start the reaction. After incubating for 10 min, 200 μ l of 30% acetic acid (v/v) was added to terminate the reaction. The reaction mixture was centrifuged at 8000 \times g for 5 min at room temperature. For the control, distilled water was used instead of inhibitor solution. Activity of trypsin in presence or absence of inhibitor solution was determined by measuring the absorbance at 410 nm due to *p*-nitroaniline released. One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit ml⁻¹ min⁻¹ under the assay condition. One unit of trypsin inhibitory activity (TIU) was defined as the amount of inhibitor, which reduced trypsin activity by one unit.

D. Autolysis Study of Sardine Surimi

Defrosted surimi (3 g) with the physiological pH of approximately 7.0 was incubated at different temperatures (45, 50, 55, 60, 65, 70, 75 and 80 °C) in a temperature-controlled water bath (Mettmert, Schwabach, Germany) for 60 min. The autolytic reaction was terminated by addition of 27 ml of cold 5% trichloroacetic acid (w/v). The mixture was homogenised for 2 min at 11,000 rpm (IKA homogeniser, Labortechnik, Selangor, Malaysia). The homogenate was centrifuged at 8000 \times g for 10 min using a microcentrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Tuttlingen, Germany). TCA-soluble peptide content in the supernatant was determined by the Lowry method [14] using L-tyrosine as a standard and was expressed as μ mol tyrosine/g sample.

To determine the autolytic patterns of sardine surimi, another set (3 g) of samples incubated at different temperatures for 60 min were mixed with 27 ml of 5% SDS (85 °C) to terminate the autolytic reaction. The mixture was then homogenised at 11,000 rpm for 1 min. The homogenate was then incubated at 85 °C for 60 min to solubilise the sample. To remove undissolved debris, the mixture was centrifuged at 8000 \times g for 10 min. The supernatant was subjected to SDS-PAGE analysis.

E. Effect of MBPI, BBPI and BGPI on Gel Properties of Sardine Surimi

Frozen surimi was partially thawed at 4 °C for 2–3 h, cut into small pieces with an approximate thickness of 1 cm and then placed in the mixer (National Model MKK77, Tokyo, Japan). The mixture was chopped for 1 min, followed by

addition of 2.5% salt (w/w). The final moisture content of mixture was adjusted to 80% by adding iced water. MBPI, BBPI and BGPI, containing trypsin inhibitors at level of 2204 ± 153 , 2516 ± 119 and 11098 ± 64 units/g, respectively, at different final concentrations (0%, 0.25%, 0.5%, 1% and 1.5%, w/w), were added. Chopping was continued for additional 3 min. Temperature was maintained at below 7 °C during chopping. The sol was stuffed into a polyvinylidene chloride casing with a diameter of 2.5 cm and both ends were sealed tightly. Kamaboko and modori gels were prepared by incubating the sol at 40 and 65 °C for 30 min, followed by heating at 90 °C for 20 min, respectively. Subsequently, all gels were cooled in iced water for 30 min and stored at 4 °C overnight prior to analyses.

F. Analyses

1. Textural Analysis

Textural analysis of surimi gels was carried out using a texture analyser (Model TA-XT2, Stable Micro Systems, Surrey, UK). Gels kept at 4 °C were equilibrated at room temperature (28–30 °C) before analysis. Three cylindrical samples (2.5 cm in length) were prepared and subjected to determination. Breaking force and deformation were measured using the texture analyser equipped with a spherical plunger (diameter 5 mm; depression speed 60 mm min⁻¹).

2. Determination of Whiteness

All gels were subjected to whiteness measurement using a colorimeter (HunterLab, ColorFlex, Hunter Associates Laboratory, Reston, VA). Illuminant C was used as the light source of measurement. L^* , a^* and b^* values were measured and whiteness was calculated using the following equation [15]:

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

3. Determination of Expressible Moisture Content

Expressible moisture content was measured according to the method of [7]. Cylindrical gel samples were cut to a thickness of 5 mm, weighed (X) and placed between three pieces of filter papers (Whatman No.1, Whatman International Ltd., Maidstone, England) at the bottom and two pieces of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (Y). Expressible moisture content was calculated with the following equation and expressed as percentage of sample weight:

$$\text{Expressible moisture (\%)} = [(X-Y)/X] \times 100$$

4. Determination of TCA-Soluble Peptide Content

TCA-soluble peptides were determined according to the method of [6]. Gel sample (3 g) was homogenised with 27 ml of cold 5% (w/v) TCA for 2 min at speed of 11,000 rpm. The homogenate was allowed to stand in ice for 1 h and centrifuged at 8000×g for 10 min. TCA-soluble peptide

content in the supernatant was measured as previously described and expressed as $\mu\text{mol tyrosine/g sample}$.

5. SDS-Polyacrylamide Gel Electrophoresis

Protein patterns of surimi and gels were analysed by SDS-PAGE according to the method of [16]. To prepare the protein sample, 27 ml of 5% (w/v) hot SDS (85 °C) solution was added to the gel sample (3 g). The mixture was then homogenised at speed of 11,000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve proteins. The samples were then centrifuged at 8,000×g for 20 min to remove undissolved debris. Protein concentration in the supernatants was determined as per the method of Lowry et al. (1951). Solubilised samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10% β ME) and boiled for 3 min. The samples (15 μg protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protean III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% methanol (v/v) and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid.

6. Scanning Electron Microscopy (SEM)

Microstructure of surimi gels was determined using SEM. Kamaboko and modori gels (thickness of 2–3 mm) without and with 1.5% (w/w) MBPI, BBPI and BGPI were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with a serial concentration of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 15 kV.

7. Statistical Analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests [17]. Analysis was performed using a SPSS package (SPSS 17.0 for Window Inc., Chicago, IL, USA).

III. RESULTS AND DISCUSSION

A. Autolysis of Sardine Surimi at Different Temperatures

Autolysis of sardine surimi at various temperatures was examined and expressed in terms of TCA-soluble peptide content (Fig. 1a). TCA-soluble peptide content in sardine surimi increased as temperature increased, and reached the maximum at 65 °C ($p < 0.05$). TCA-soluble peptide content markedly decreased when incubated above 65 °C, probably

due to thermal denaturation of endogenous proteases. The result suggested the presence of heat activated proteases, which were able to hydrolyse the muscle proteins in surimi at high temperature. The maximum autolysis of mince and washed mince from two species of bigeye snapper (*P. macracanthus* and *P. tayenus*) was found at 60 °C [18]. Furthermore, heat-stable myofibril-bound serine and cysteine proteinases were responsible for protein degradation of lizardfish and goatfish muscle [5], [18]. The differences in autolysis profile of surimi from different species might be caused by the different types and amounts of proteases present in surimi.

Autolysis patterns of sardine surimi are shown in Fig. 1b. Degradation was more pronounced at temperatures higher

than 50 °C, as evidenced by the marked disappearance in myosin heavy chain (MHC) band. For actin, slight changes in band intensity were observed at the same temperature range. Thus, it was noted that MHC was more susceptible to degradation than other proteins. At temperature higher than 70 °C, MHC and actin were more retained, mainly due to heat inactivation of endogenous proteases. Therefore, the degradation of MHC was maximised at 65 °C, which was more likely a temperature causing gel weakening (modori) of surimi from this species.

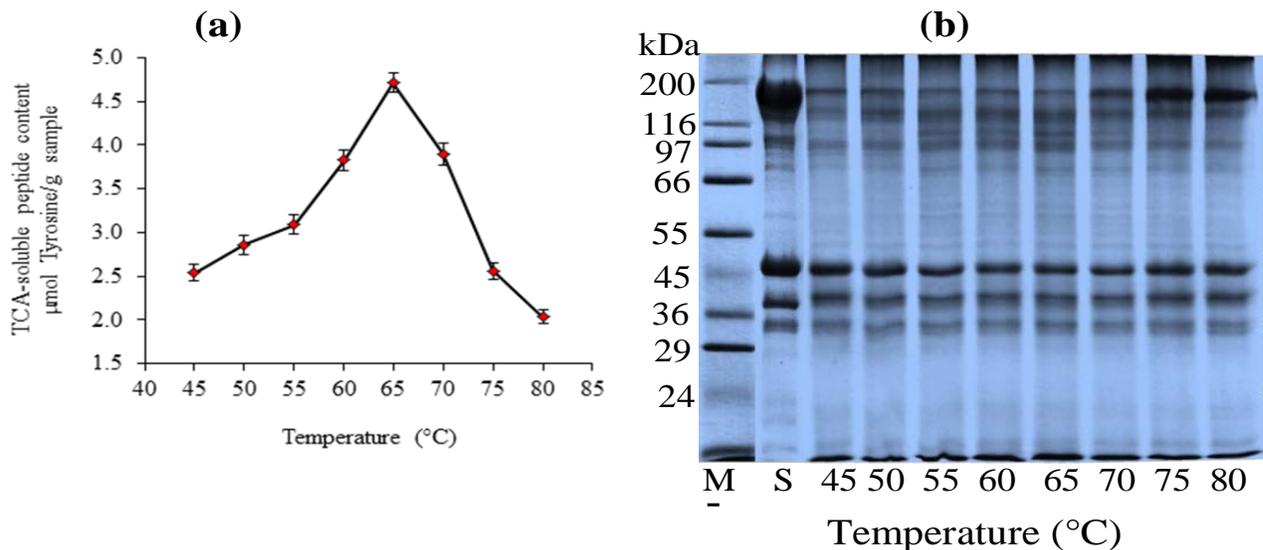


Fig. 1 TCA-soluble peptide contents (A) and protein pattern (B) of sardine surimi incubated at different temperatures. Autolysis was conducted for 60 min. MHC: myosin heavy chain; AC: actin; TM: tropomyosin. Bars represent the standard deviation (n=3).

B. Effect of MBPI, BBPI and BGPI on Gel Properties of Sardine Surimi

1. Textural Properties of Surimi Gel

Breaking force and deformation of sardine surimi gels added with MBPI, BBPI and BGPI at different levels (0-1.5%, w/w) are shown in Fig. 2 (a & b). Both modori (65/90 °C) and kamaboko (40/90 °C) gels had the increases in breaking force and deformation with increasing levels of MBPI or BBPI or BGPI up to 1% ($p < 0.05$). However, no marked increase in breaking force and deformation was observed when all protein isolates at a level above 1% were added ($p > 0.05$). When MBPI or BBPI or BGPI at a level of 1.5% was incorporated, breaking force of kamaboko and modori gels increased by 22.4-76.8, 24.9-95.3 and 90.0-135.3%, while deformation increased by 11.6-31.3, 13.1-42.7 and 26.4-61.39%, respectively, compared with the control (without protein isolate addition). The results suggested that all protein isolates (BGPI BBPI and MBPI) were effective in enhancing the gel strength for both gels, modori and kamaboko gels. This might

be associated with the presence of trypsin inhibitors in MBPI (2204±153 units/g), BBPI (2516±119 units/g) and BGPI (11098±64 units/g). Therefore, MBPI, BBPI and BGPI more likely played an essential role in inhibiting endogenous heat-activated proteases in sardine surimi gels, which showed the optimal autolytic activity at 65 °C (Fig. 1). However BGPI showed higher breaking force and deformation than BBPI and MBPI might be due to containing higher trypsin inhibitors. Apart from protease inhibitory activity, vicilin and legumin from legume seeds were reported to act as a co-gelling agent or filler or binder in surimi gels [8], [10]. Those proteins from MBPI, BBPI and BGPI at appropriate levels possibly enhanced protein-protein interaction, thereby form the strong gel network with myofibrillar proteins. Those interactions might also lower the availability of proteinaceous substrate toward hydrolysis caused by proteases. Vicilin-rich protein isolates from various Phaseolus legumes exhibited thermo-irreversible gelation mediated by the formation of disulfide bonds [10]. Reference [15] reported that conglycinin and glycinin played a major role in the gel formation of soy

proteins and can form aggregates or gels at 85 °C in the presence of salt. Therefore, addition of MBPI or BBPI or BGPI could improve the properties of gels via protease inhibition and its binding or gelling effects.

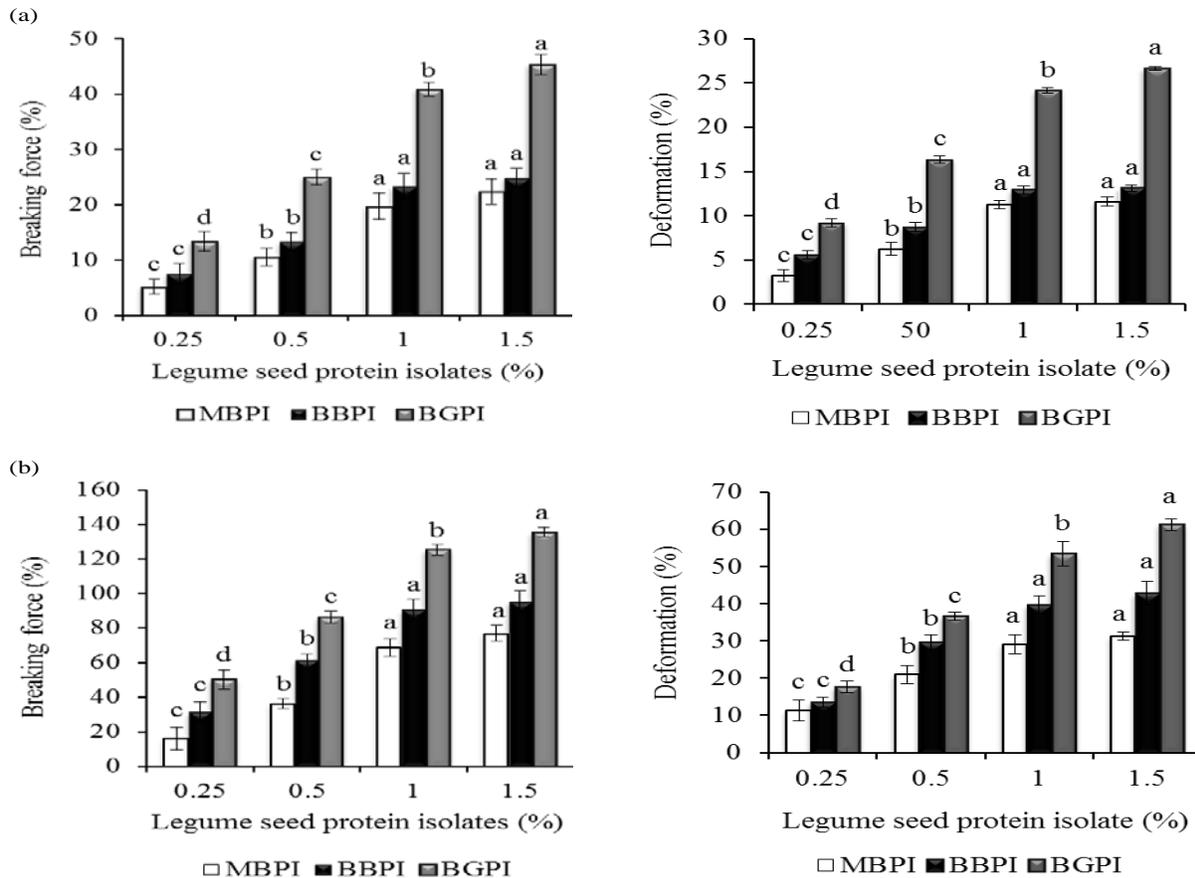


Fig. 2 Breaking force and deformation of kamaboko (a) and modori (b) gels from sardine surimi added with MBPI or BBPI or BGPI at different levels. Bars represent the standard deviation (n=3). Different letters on the bars within the same gel indicate significant differences ($p < 0.05$).

2. Whiteness and Expressible Moisture Content of Surimi Gel

Whiteness of kamaboko and modori gels added with MBPI or BBPI or BGPI at different levels is shown in Table I. The slight decrease in whiteness was observed in all gels, as MBPI or BBPI or BGPI levels increased ($p < 0.05$). When MBPI or BBPI or BGPI at a level of 1.5% was incorporated into modori and kamaboko gels, the whiteness decreased by 0.9-2.2, 1.3-2.5 and 1.7-2.6%, respectively, compared with control gels ($p < 0.05$) (Table I). This result was in accordance with [19] who observed that the addition of partially purified protease inhibitor from cowpea, pigeon pea and bambara groundnuts, noticeably decreased whiteness of gel from threadfin bream surimi. Decrease in whiteness was probably due to presence of pigments in legume seed protein isolate. However, whiteness of surimi gels added with BGPI and BBPI was lower than MBPI, when the same level was used ($p < 0.05$). This might be due to the differences in pigments in

seed coat or meal of protein isolates, which might be contaminated into protein isolate during extraction. Colour characteristic of surimi gels were largely dependent on the types and amounts of additives added [7], [19]. Additionally, a slightly higher whiteness value was observed in modori gels when compared with kamaboko gels, regardless of MBPI or BBPI or BGPI levels used.

The expressible moisture content of kamaboko and modori gels added with MBPI or BBPI or BGPI at different concentrations is presented in Table I. The expressible moisture content of all gels significantly decreased as the levels of MBPI or BBPI or BGPI added increased ($p < 0.05$). The highest expressible moisture was found in modori gel without MBPI or BBPI or BGPI ($p < 0.05$), indicating a poor gel matrix with low water-holding capacity owing to proteolysis caused by heat activated endogenous proteases. Expressible moisture content of kamaboko and modori gels decreased by 23.3-37.3, 23.1-46.8 and 26.6-51.5% when 1.5% MBPI or BBPI or BGPI was added, respectively (Table I).

Modori and kamaboko gels containing BGPI had higher water-holding capacity than those added with MBPI and BBPI ($p < 0.05$). However no difference was observed in kamaboko gels ($p > 0.05$), irrespective of BBPI or MBPI levels incorporated into gel ($p < 0.05$). For modori gel, MBPI or BBPI or BGPI effectively prevented the degradation of MHC. As a consequence, gel network was retained and was able to imbibe more water as indicated by less expressible moisture content. This result was in accordance with [6] and [7] who reported that addition of WPC and CPP or other protein additives resulted in less expressible moisture in modori gel. Moreover, some proteins in protein isolates might possess

high water holding capacity. The lowered expressible moisture content was coincidental with the increased breaking force and deformation of resulting modori and kamaboko gels (Fig. 2). Expressible moisture content of the control kamaboko gel was lower than that of the control modori gel. This reflected less degradation of MHC by endogenous protease in kamaboko gel. Thus, the kamaboko gel showed the higher water-holding capacity than the modori gel. The released water from gel matrix to the gel surface of modori gel more likely contributed to the light scattering, which led to the increased whiteness of modori gel (Table I).

TABLE I
WHITENESS AND EXPRESSIBLE MOISTURE CONTENT OF KAMABOKO AND MODORI GELS FROM SARDINE SURIMI ADDED WITH MBPI, BBPI AND BGPI AT DIFFERENT LEVELS

Protein isolate (PI)	PI level (%)	Whiteness		Expressible moisture (%)	
		Kamaboko	Modori	Kamaboko	Modori
MBPI	0	69.90±0.35 ^a	6.77±0.50 ^a	6.77±0.50 ^a	10.86±0.94 ^a
	0.25	69.50±0.17 ^{ab}	6.28±0.51 ^{ab}	6.28±0.51 ^{ab}	7.83±0.31 ^b
	0.5	69.08±0.32 ^{bc}	6.01±0.41 ^{abc}	6.01±0.41 ^{abc}	7.69±0.37 ^{bc}
	1.0	68.73±0.23 ^{cd}	5.72±0.21 ^{bc}	5.72±0.21 ^{bc}	7.41±0.24 ^{bc}
	1.5	68.39±0.26 ^d	5.19±0.61 ^c	5.19±0.61 ^c	6.81±0.18 ^c
BBPI	0	69.54±0.19 ^a	7.16±0.93 ^a	7.16±0.93 ^a	11.47±1.09 ^a
	0.25	68.76±0.21 ^b	6.47±0.64 ^{ab}	6.47±0.64 ^{ab}	7.58±0.90 ^b
	0.5	68.50±0.13 ^{bc}	6.12±0.71 ^{ab}	6.12±0.71 ^{ab}	6.84±0.51 ^{bc}
	1.0	68.19±0.20 ^c	5.32±0.43 ^b	5.32±0.43 ^b	6.36±0.21 ^{bc}
	1.5	67.80±0.17 ^d	5.51±0.23 ^b	5.51±0.23 ^b	6.10±0.31 ^c
BGPI	0	69.61±0.15 ^a	7.06±0.36 ^a	7.06±0.36 ^a	11.96±0.40 ^a
	0.25	69.28±0.20 ^a	6.66±0.37 ^a	6.66±0.37 ^a	7.87±0.43 ^b
	0.5	68.62±0.13 ^b	6.04±0.48 ^b	6.04±0.48 ^b	7.33±0.46 ^{bc}
	1.0	68.18±0.29 ^c	5.46±0.38 ^{bc}	5.46±0.38 ^{bc}	6.99±0.54 ^c
	1.5	67.82±0.40 ^c	5.18±0.38 ^c	5.18±0.38 ^c	5.80±0.37 ^d

Mean ± S.D (n=3). Different superscripts in the same column within the same gel indicate significant differences ($P < 0.05$)

3. TCA-Soluble Peptide Content of Surimi Gel

TCA-soluble peptide content in both modori and kamaboko gels added with different levels of MBPI, BBPI and BGPI are depicted in Fig. 3. The highest TCA-soluble peptide content was obtained in both gels (modori and kamaboko gels) without MBPI or BBPI or BGPI addition, indicating the pronounced degradation caused by endogenous proteases in both gels. This result was in accordance with the lowest breaking force and deformation in the control gels (without

MBPI or BBPI or BGPI). Reference [20] reported that proteolysis in surimi from some tropical fish occurred during setting at 40°C. Thus, proteolysis contributed to poorer gel quality. Inhibition of proteolysis should be an effective means for controlling the undesirable gel weakening of surimi from tropical fish during setting at high temperatures. TCA-soluble peptide content of modori and kamaboko gels decreased as the concentration of MBPI or BBPI or BGPI increased up to 1% level ($p < 0.05$). This result suggested that MBPI, BBPI and BGPI exhibited the inhibitory activity toward degradation of

muscle proteins in a dose dependent manner. However, MBPI or BBPI or BGPI at a concentration higher than 1% were not able to lower TCA-soluble peptide content in all gels ($p > 0.05$). Therefore, the addition of MBPI or BBPI or BGPI at levels greater than 1% was not necessary for the prevention of proteolysis.

Generally, TCA-soluble peptide content in modori gels was higher than that of the kamaboko gels, indicating the greater degradation of surimi proteins in the former. Reference [4] stated that degradation occurred during heat-induced gelation is considered to result from the action of endogenous proteases, especially heat activated proteases. Nevertheless, the degradation could be prevented to same degree with the addition of MBPI or BBPI or BGPI for both gels.

Comparatively, higher decrease in TCA-soluble peptide contents in both modori and kamaboko gels were observed with the addition of BGPI, compared with BBPI and MBPI.

The result was in agreement with the low inhibitory activity of BBPI and MBPI toward autolysis of surimi gels, compared with BGPI (Fig. 3). Thus, protein degradation in surimi gels could be decreased by the addition of BGPI or BBPI or MBPI. The result was in agreement with the low inhibitory activity of BBPI and MBPI toward autolysis of surimi gels, compared with BGPI (Fig. 3). Thus, protein degradation in surimi gels could be decreased by the addition of BGPI or BBPI or MBPI.

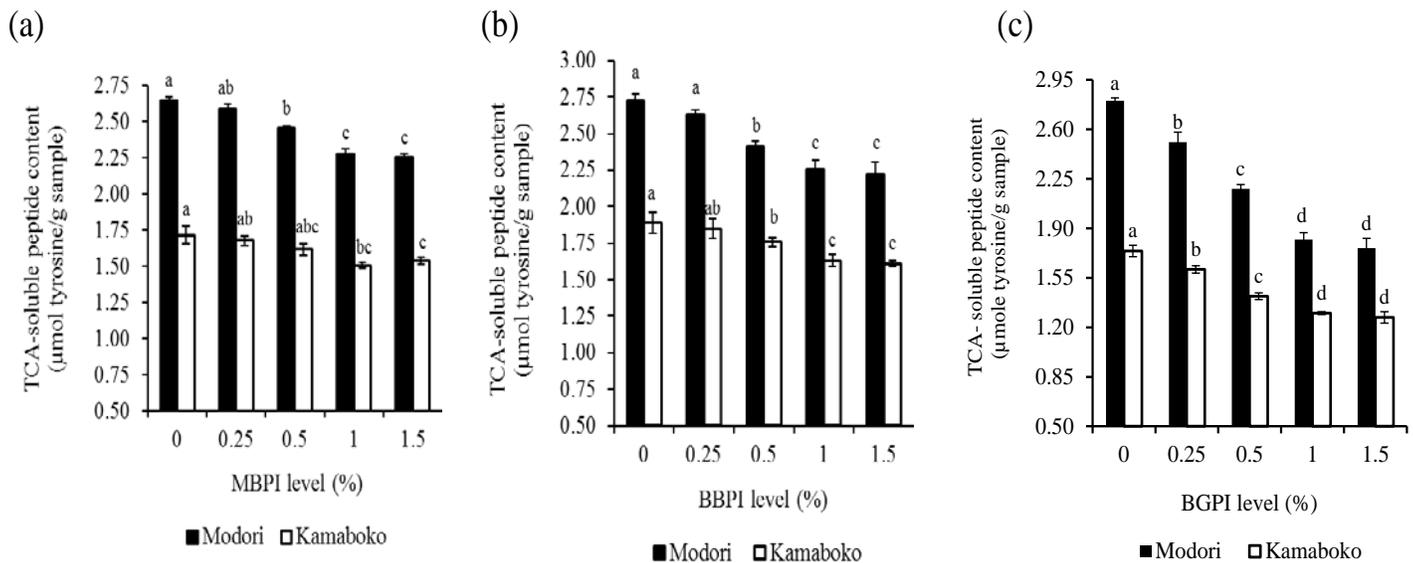


Fig. 3 TCA-soluble peptide content of modori and kamaboko gels from sardine (*Sardinella albella*) added with MBPI (a), BBPI (b) and BGPI (c) at different levels. Bars represent the standard deviation (n=3). Different letters on the bars within the same gel indicate significant differences ($P < 0.05$).

4. Protein Patterns of Surimi Gel

Protein patterns of modori and kamaboko gels added with MBPI or BBPI or BGPI at different levels are depicted in Fig. 4 (a & b). For modori gels, the marked degradation of MHC was observed in the sample without the addition of MBPI or BBPI or BGPI as indicated by the complete disappearance of MHC. Nevertheless, actin was still retained, regardless of protein isolate addition. The result indicated that MHC is the primary substrate for proteases. Reference [4] reported that MHC, β -tropomyosin and troponin-T were more susceptible to degradation than actin. MHC band intensity of modori gels

generally increased as the concentrate BGPI or BBPI or MBPI increased. The result indicated that BGPI or BBPI or MBPI could inhibit the degradation in a concentration dependent manner. This result was agreement with the decrease in TCA-soluble peptide content of modori gels (Fig. 3) and the increase in breaking force and deformation (Fig. 2). It was noted that BGPI showed high efficacy in preventing the degradation in MHC of surimi gels than BBPI and MBPI. The result was in accordance with higher decrease in TCA soluble peptide content in BGPI added gels than that containing BBPI and MBPI. This was also reconfirmed by higher inhibitory activity of BGPI than BBPI and MBPI against autolysis of surimi.

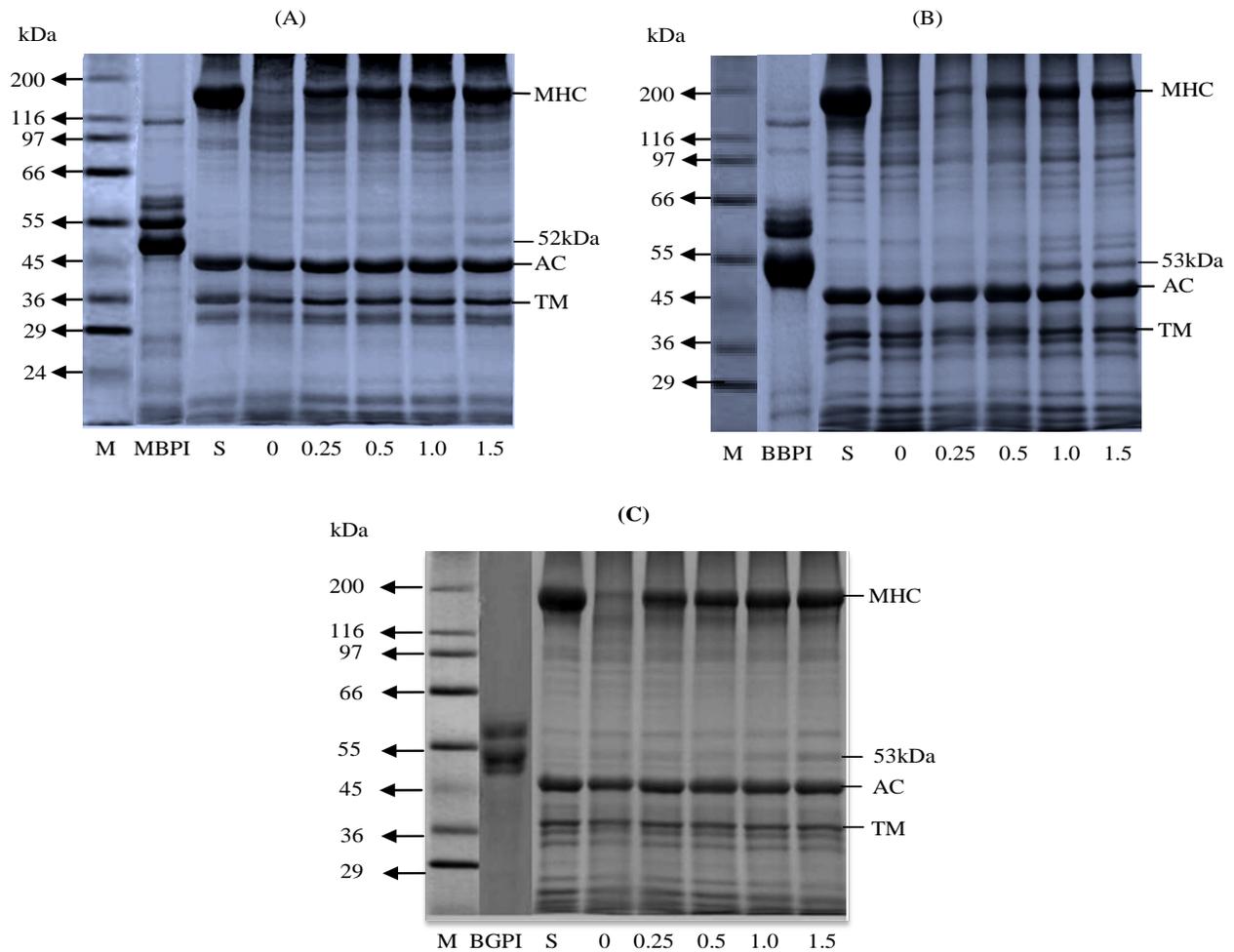
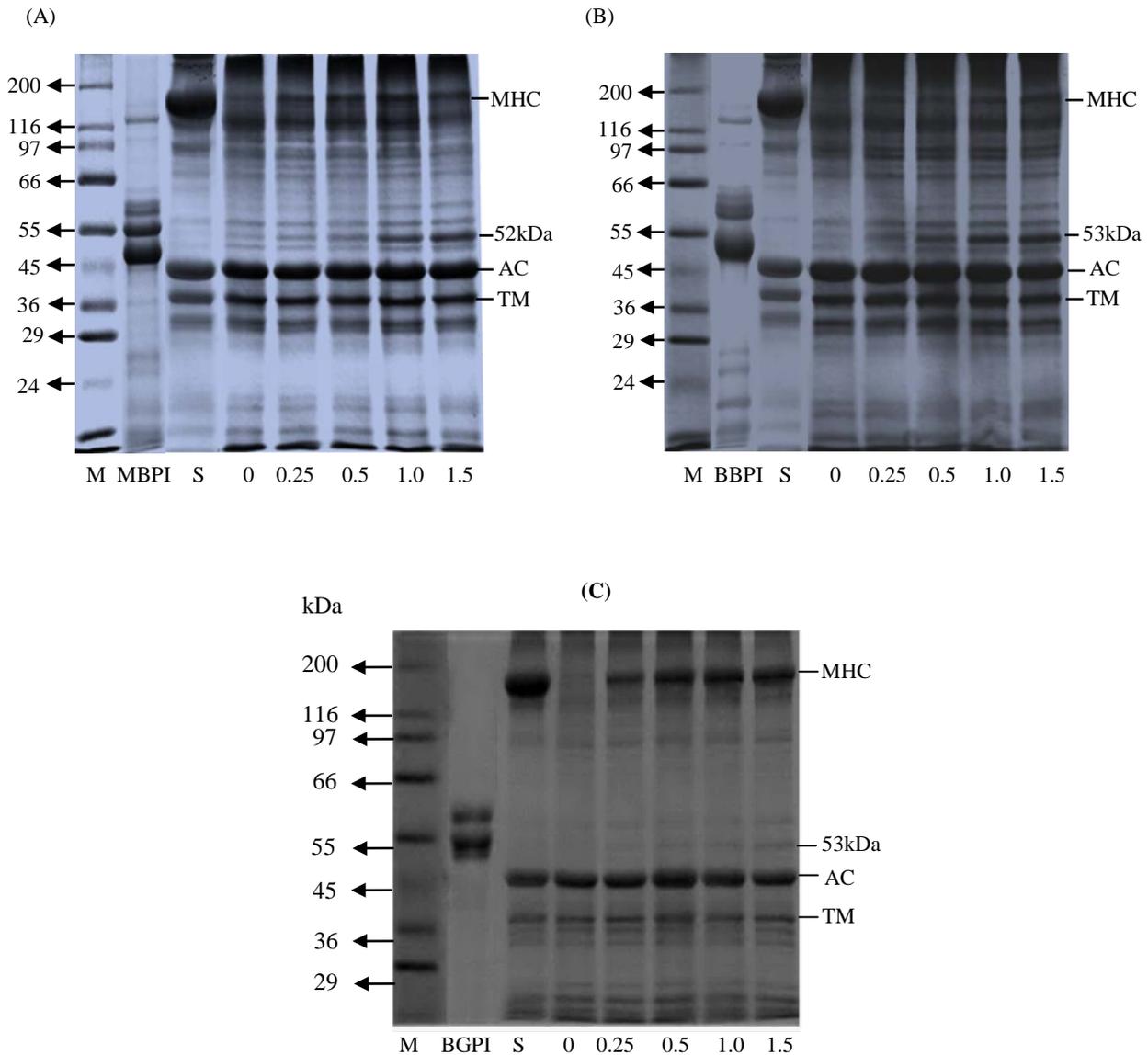


Fig. 4a SDS-PAGE pattern of modori gel from sardine (*S. albelli*) surimi add with MBPI (A), BBPI (B) and BGPI (C) at different levels. M: marker; S: surimi sol; MHC: myosin heavy chain; TM: tropomyosin. Numbers designate the level of legume seed protein isolates added (% w/w).

For kamaboko gel, slight increase in MHC band intensity was observed when BGPI or BBPI or MBPI at higher levels were added. This result was anticipated with the lower degradation of MHC in kamaboko gel in the presence of higher levels of protein isolates. Additionally, endogenous proteases were more likely less active at 40 °C than 65 °C, used for preparation of kamaboko and modori gels, respectively. It was noted that MHC was less retained in kamaboko gel even though the proteolysis was lower. The decrease in MHC band intensity of kamaboko gel was more likely due to the polymerisation mediated by endogenous transglutaminase. Reference [21] suggested that the decreases

in MHC band intensity in bigeye snapper surimi gels were most likely caused by polymerisation of MHC, especially during setting. However, the degradation proteins with molecular weight (MW) ranging from 70 to 190 kDa were also observed in kamaboko gels. This was in agreement with TCA-soluble peptide content, which was detectable in all kamaboko gel samples (Fig. 3). It was found that protein with MW of 52-53 kDa was observed in both kamaboko and modori gels, and the intensity increased with increasing levels of BGPI or BBPI or MBPI.



5. Microstructure of Surimi Gel

Microstructure of modori and kamaboko gels from sardine surimi without and with 1.5% MBPI, BBPI or BGPI addition are illustrated in Fig. 5 (a & b). Modori control gels (without MBPI, BBPI and BGPI addition) had a coarser network containing coagulated proteins arranged in clusters (Fig 5). A large porous matrix with some cavities was also observed. When modori gels were added with 1.5% MBPI or BBPI or BGPI, more compact structure with smaller voids was formed. This result coincidental with the higher breaking force and deformation of modori gel added with 1.5% MBPI or BBPI or BGPI (Fig. 2). Surimi gels added with 1.5% BGPI exhibited slightly higher interconnected three-dimensional protein network structure with smaller voids, compared with that containing 1.5% BBPI and MBPI.

Kamaboko control gels (without MBPI, BBPI and BGPI addition) showed the higher interconnected three-dimensional protein network, compared with modori gels (Fig. 5). Kamaboko gels added with 1.5% BGPI or BBPI or MBPI were more compact and finer with smaller voids, compared with control gels. These observations suggested that BGPI or BBPI or MBPI might distribute uniformly as the filler in the ordered network and impeded the degradation of muscle protein mediated by endogenous proteases. Reference [7] reported that addition of 3% WPC in kamaboko gel from bigeye snapper was more compact with smaller clusters of aggregated protein than that of the control gel (without WPC). Thus, the addition of 1.5% BGPI or BBPI or MBPI resulted in the formation of fine gel network with improved gel strength and water holding capacity.

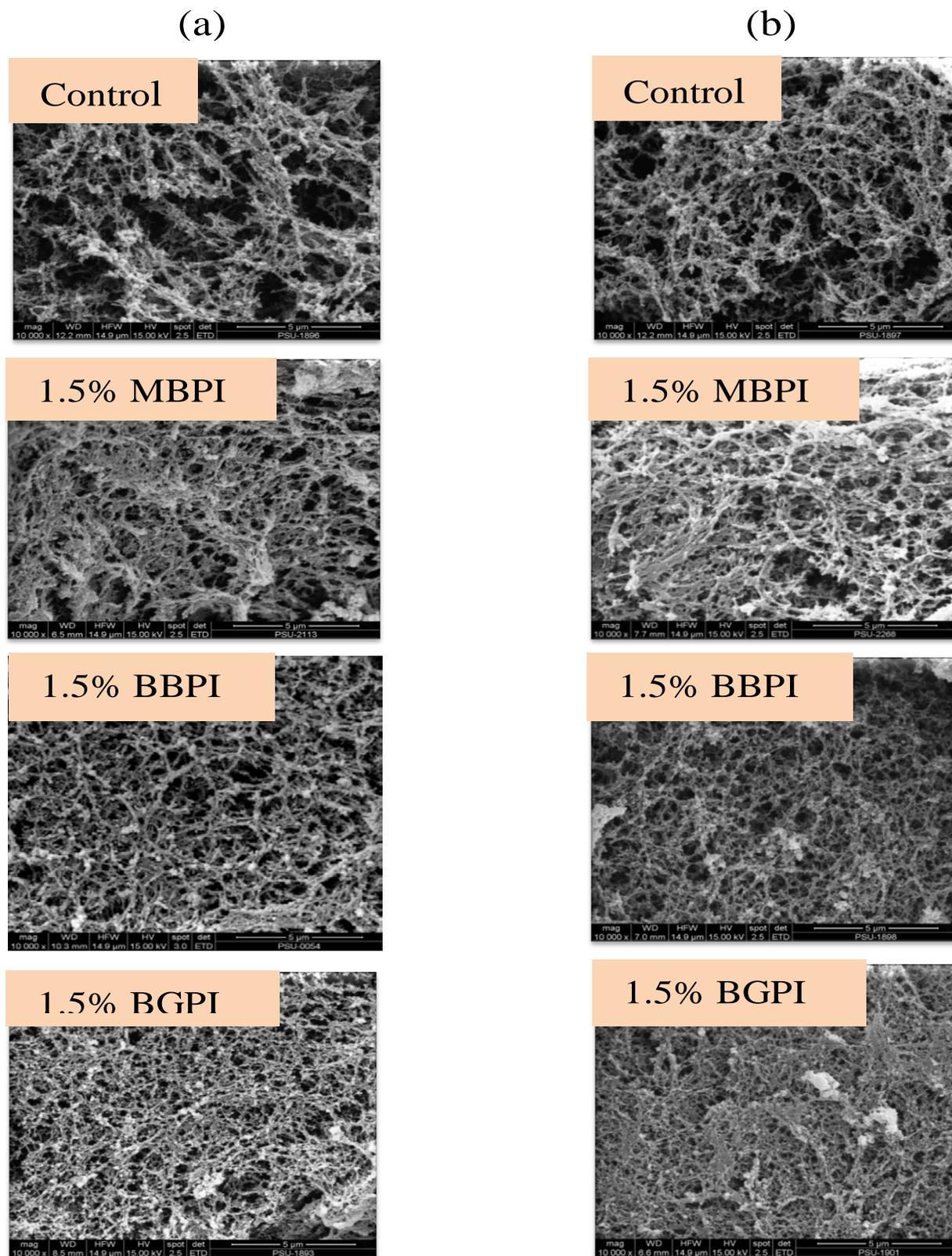


Fig. 5 Microstructures of kamaboko (a) and modori (b) gels without MBPI/BBPI/BGPI (control) and with 1.5% MBPI, 1.5% BBPI and 1.5% BGPI addition from sardine surimi. Magnification: $\times 10,000$

IV. CONCLUSION

Proteolysis of sardine surimi caused by heat activated proteases could be partially inhibited by the addition of BGPI or BBPI or MBPI. The increased breaking force and deformation of sardine surimi gel with higher water holding capacity was obtained when BGPI or BBPI or MBPI at a level of 1.5% was added. However, BGPI or BBPI or MBPI addition caused slightly decreased whiteness. Therefore, BGPI or BBPI or MBPI at an appropriate level could be an alternative food grade protease inhibitor to improve gel properties of surimi, especially those facing with gel weakening.

ACKNOWLEDGMENT

The authors would like to express their sincere thanks to Graduate School, Prince of Songkla University and the TRF senior research scholar program for the financial support.

REFERENCES

- [1] Y. Shimizu, H. Toyohara, and T. C. Lanier, "Surimi production form fatty and dark fleshed fish species," in *Surimi Technology*: T. C. Lanier, C. M. Lee, Eds. New York: Marcel Dekker, USA, 1992, pp. 181-207.
- [2] M. T. Morrissey, J. W. Wu, D. Lin, and H. An, "Protease inhibitor effects on torsion measurements and autolysis of pacific whiting surimi," *J. Food Sci.*, vol. 58, pp. 1050-1054, 1993.
- [3] C. Alvarez, I. Couso, and M. Tejada, "Thermal gel degradation (modori) in sardine surimi gel," *Journal of Food Science*, vol.64, pp. 633- 637, 1999.
- [4] H. An, M. Y. Peters, and T. A. Seymour, "Role of endogenous enzymes in surimi gelation," *Trends Food Sci. Technol.*, vol.7, pp. 321-326, 1996.
- [5] S. Benjakul, W. Visessanguan, and J. Turksuban, "Heat activated proteolysis in lizardfish (*Saurida tumbil*) muscle," *Food Res. Int.*, vol. 36, pp. 1021-1028, 2003a.
- [6] S. Benjakul, S. Yarnpakdee, W. Visessanguan, and S. Phatcharat, "Combination effects of whey protein concentrate and calcium chloride on the properties of goatfish surimi gel," *J. Texture Stud.*, vol. 41, pp. 341-357, 2010.
- [7] S. Rawdkuen, S. Benjakul, W. Visessanguan, and T. C. Lanier, "Effect of chicken plasma protein and some protein additives on proteolysis and gel-forming ability of sardine (*Sardinella gibbosa*) surimi," *J. Food Process. Preserv.*, vol. 31, pp. 492-516, 2007.
- [8] Y. K. Luo, R. Kuwahara, M. Kaneniwa, Y. Murata, and M. Yokoyama, "Effects of soy protein isolate on gel properties of Alaska pollock and common carp surimi at different setting conditions," *J. Sci. Food Agric.*, vol. 84, pp. 663-671, 2004.
- [9] L. C. Sun, A. Yoshida, Q. F. Cai, G. M. Liu, L. Weng, K. Tachibana, W. J. Su, and M. J. Cao, "Mung bean trypsin inhibitor is effective in suppressing the degradation of myofibrillar proteins in the skeletal muscle of blue scad (*Decapterus maruadsi*)," *J. Agric. Food Chem.*, vol. 58, pp. 12986-12992, 2010.
- [10] C. H. Tang, "Thermal denaturation and gelation of vicilin-rich protein isolates from three *Phaseolus* legumes: A comparative study," *LWT-Food Sci. Technol.*, vol. 41, pp. 1380-1388, 2008.
- [11] M. S. Hoque, S. Benjakul, and T. Prodpran, "Properties of blend film based on cuttlefish (*Sepia pharaonis*) skin gelatin and mungbean protein isolate," *Int. J. Biol. Macromol.*, vol. 49, pp. 663-673, 2010.
- [12] AOAC, 1984, *Official Methods of Analysis*, 14th ed. Association of Official Analytical Chemists, Washington, DC.
- [13] S. Benjakul, W. Visessanguan, and P. Thummaratwasik, "Isolation and characterisation of trypsin inhibitors from some Thai legume seeds," *J. Food Biochem.*, vol. 24, pp. 107-127, 2000a.
- [14] O. H. Lowry, N. J. Rosebrough, L. A. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent," *J. Biol. Chem.*, vol. 193, pp. 265-275, 1951.
- [15] J. W. Park, "Ingredient technology and formulation development," in *Surimi and Surimi Seafood* J.W. Park, Ed. New York: Marcel Dekker, 2000, pp. 343-392.
- [16] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage," *Nature*, vol. 227, pp. 680-685, 1970.
- [17] R. G. D. Steel, and J. H. Torrie, *Principles and procedures of statistics: A biometrical approach*. 2nd ed. New York: McGraw-Hill, 1980, pp. 173-393.
- [18] S. Yarnpakdee, S. Benjakul, W. Visessanguan, and K. Kijroongrjana, "Autolysis of goatfish (*Mulloidichthys martinicus*) mince: Characterisation and effect of washing and skin inclusion," *Food Chem.*, vol. 114, pp. 1339-1344, 2009.
- [19] S. Benjakul, W. Visessanguan, and P. Thummaratwasik, "Inhibition of gel weakening of threadfin bream surimi using Thai legume seed proteinase inhibitors," *J. Food Biochem.*, vol. 24, pp. 363-380, 2000b.
- [20] S. Benjakul, W. Visessanguan, and C. Chantarasuwan, "Effect of high-temperature setting on gelling characteristic of surimi from some tropical fish," *Int. J. Food Sci. Technol.*, vol. 39, pp. 617-680, 2004.
- [21] S. Benjakul, and W. Visessanguan, "Transglutaminase-mediate setting in bigeye snapper surimi," *Food Res. Int.*, vol. 36, pp. 253-266, 2003.