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**Molecular characteristics and properties of gelatin
from skin of seabass with different sizes**

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ABSTRACT

Gelatin was extracted from the skin of seabass (*Lates calcarifer*) with different average sizes (2, 4 and 6 kg/fish), termed G2, G4 and G6, respectively and their characteristics and functional properties were determined. Yields of G2, G4 and G6 were 38.22, 40.50 and 43.48% (based on dry weight), respectively. G2 contained α -chains as dominant component, whilst G4 and G6 comprised α -, β - and γ -chains with a larger content of high MW cross-links. All gelatins had the similar imino acid (hydroxyproline and proline) content. Net charge of G2, G4 and G6 became zero at pHs of 6.73, 6.41 and 7.12, respectively. Amongst all gelatin samples, G6 exhibited the highest gel strength (321.5 g) ($p < 0.05$), but had the lowest turbidity ($p < 0.05$). Gels of G6 sample had the lower L^* -value but higher a^* -, b^* - and ΔE^* -value, compared with others. Gelling and melting temperatures of all gelatins were 17.09-19.01 and 26.92-28.85 °C, respectively. Furthermore, all gelatins were able to set at room temperature, regardless of size of seabass used. G6 had the shorter setting time at room temperature than others. Therefore, size of seabass, in which skin was used for gelatin extraction, had the impact on yield, composition and properties of resulting gelatin.

Keywords: Seabass, Gelatin, Size, Gel strength, Setting time, Gelling temperature

1. Introduction

Gelatin is the denatured or partially hydrolysed form of collagen [1]. It represents a major biopolymer with several applications in food, materials, pharmacy and photography industries [2]. Due to its gelling property and surface behaviour (e.g., formation and stabilisations of foams and emulsions), gelatin has been widely used to enhance the elasticity, consistency and stability of food products [3-5].

In general, the main sources of gelatin are skins and bones of pig and cow obtained from processing by-products. Gelatin from those sources can be a problem for certain consumers, e.g., Muslims and Jews, in which porcine gelatin is prohibited. Occurrence of bovine spongiform encephalopathy (BSE) has led to awareness for consumption of bovine gelatin [3]. Nowadays, an increasing interest has been paid to alternative sources of gelatin, especially from the skins and bones from fish processing by-products [6, 7]. Fish gelatin can be extracted from skin of several fish species including skipjack tuna, dog shark [8], cobia [9], farmed Amur sturgeon [10], seabass [6], and brownbanded bamboo and blacktip shark [7]. It has been known that the extraction conditions including temperature, time as well as pretreatment affect the functional properties of gelatin from fish processing by-products [6, 11, 12]. Additionally, characteristics and properties of gelatin vary with species and age of raw material [13, 14]. Muyonga, Cole and Duodu [15] reported that gelatin from adult Nile perch skin exhibited the better gel properties than young Nile perch skin when the same extraction condition was used.

Seabass is one of economically important fish in Thailand. A number of seabass farms are located in the south of Thailand, especially in the lake of Songkhla. Due to its delicacy, a large amount of seabass has been exported as well as domestically consumed. During processing, particularly fillet production, skin is

generated as byproduct. Skins from seabass has been used as raw material for collagen and gelatin extraction with higher yield [16]. Gelatin from seabass skin had higher gel strength than bovine gelatin and could be set at room temperature within 30 min [6]. Size or age of seabass, in which skin is used for gelatin extraction, can have the impact on composition and properties of gelatin. Nevertheless, no information regarding gelatin extracted from skin of seabass with different sizes has been reported. Therefore, the aims of this investigation were to extract and determine the chemical characteristics and functional properties of gelatin from the seabass skin as affected by sizes of fish.

2. Materials and methods

2.1 Chemicals

All chemicals were of analytical grade. Sodium dodecyl sulphate (SDS), Coomassie blue R-250 and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). High-molecular-weight markers were purchased from GE Healthcare UK Limited (Buckinghamshire, UK). Fish gelatin produced from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy).

2.2 Fish skin preparation

Fresh seabass (*Lates calcarifer*) with different sizes of 1.7-2.3, 3.7-4.3 and 5.7-6.2 kg/fish, equivalent to average size of 2, 4 and 6 kg/fish, respectively, were obtained from a farm in Koyo Island, Songkhla, Thailand. The fish were kept in ice with a fish/ice ratio 1/3 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h after capture. Fish were washed

using cold tap water. Skins were then removed, descaled, and cut into small pieces (0.5 cm x 0.5 cm) using a scissor. The skin was placed in polyethylene bags and stored at -20 °C until used, but not longer than 2 months.

2.3 Extraction of gelatin from the skin of seabass

Gelatin was extracted from seabass skin according to the method of Sinthusamran, Benjakul and Kishimura [6]. Before gelatin extraction, skin was soaked in 0.1 M NaOH with a skin/solution ratio of 1/10 (w/v) to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28-30 °C) using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany). The alkaline solution was changed every 1 h for totally 3 times. The pretreated skin was washed with tap water until neutral or faintly basic pH was obtained. Then, the washed skin was mixed with 0.05 M acetic acid at a skin/solution ratio of 1/10 (w/v) to swell collagenous material in the fish skin matrix. The mixture was stirred at room temperature for 2 h. The skin was washed using tap water until neutral or faintly acidic pH of wash water was obtained. Finally, the swollen skin was mixed with distilled water at a ratio of 1/10 (w/v) at 45 °C for 3 h with continuous stirring. The mixtures were filtered using a Buchner funnel with Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). Then, the filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The freeze-dried gelatins extracted from seabass skin with an average size of 2, 4 and 6 kg/fish were referred to as 'G2', 'G4' and 'G6', respectively. Gelatin samples were subsequently subjected to analyses.

2.4 Analyses

2.4.1. Yield

The yield of gelatin was calculated based on dry weight of starting material.

$$\text{Yield (\%)} = \frac{\text{Weight of freeze-dried gelatin (g)}}{\text{Weight of initial dry skin (g)}} \times 100$$

2.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli [17]. Gelatin samples were dissolved in 5% SDS solution. The mixtures were then heated at 85 °C for 1 h using a temperature controlled water bath model W350 (Mettler, Schwabach, Germany). Solubilised samples were mixed at a 1/1 (v/v) ratio with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 5% SDS and 20% glycerol). Samples were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 20 mA/gel. After electrophoresis, gels were stained with 0.05% (w/v) Coomassie blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min. Finally, they were destained with the mixture of 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min and destained again with the mixture of 5% (v/v) methanol and 7.5% (v/v) acetic acid for 1 h. High-molecular-weight protein markers were used to estimate the molecular weight of proteins.

2.4.3 Amino acid analysis

Amino acid composition of gelatin samples was analysed using an amino acid analyser. The samples were hydrolysed under reduced pressure in 4 M methanesulphonic acid containing 0.2% (v/v) 3-(2-aminoethyl) indole at 115 °C for 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M

citrate buffer (pH 2.2). An aliquot of 0.04 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

2.4.4 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectra of gelatin samples were obtained using a FTIR spectrometer (EQUINOX 55, Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate (DLATGS) detector. The horizontal attenuated total reflectance accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm⁻¹ and the measurement range was 4000–650 cm⁻¹ (mid-IR region) at room temperature. Automatic signals were collected in 32 scans at a resolution of 4 cm⁻¹ and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

2.4.5 Measurement of ζ -potential

Gelatin samples were dissolved in distilled water at a concentration of 0.5 mg/ml. The mixture was stirred at room temperature for 6 h. The Zeta (ζ) potential of each sample (20 ml) was measured using a zeta potential analyser (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). ζ -Potential of samples adjusted to different pHs with 1.0 M nitric acid or 1.0 M KOH using an autotitrator (BIZTU, Brookhaven Instruments Co., Holtsville, New York, USA) was determined. The pI was estimated from pH rendering ζ -potential of zero.

2.4.6 Determination of gel strength

Gelatin gel was prepared by the method of Kittiphattanabawon, Benjakul, Visessanguan and Shahidi [7]. Gelatin was dissolved in distilled water (60 °C) to obtain a final concentration of 6.67% (w/v). The solution was stirred until gelatin was solubilised completely and transferred to a cylindrical mold with 3 cm diameter and 2.5 cm height. The solution was incubated at the refrigerated temperature (4 °C) for 18 h prior to analysis.

Gel strength was determined at 8–10 °C using a texture analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon[®] plunger. The maximum force (grams), taken when the plunger had penetrated 4 mm into the gelatin gels, was recorded.

2.4.7 Determination of gel colour

The colour of gelatin gels (6.67% w/v) was measured by a Hunter lab colourimeter (Colour Flex, Hunter Lab Inc., Reston, VA, USA). L^* , a^* and b^* values indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. The colourimeter was warmed up for 10 min and calibrated with a white standard. Total difference in colour (ΔE^*) was calculated according to the following equation [18]:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^* , Δa^* and Δb^* are the differences between the corresponding colour parameter of the sample and that of white standard ($L^* = 90.77$, $a^* = -1.27$ and $b^* = 0.50$).

2.4.8 Measurement of turbidity

Turbidity of gelatin solution (6.67 %, w/v) was determined as per the method of Fernández-Díaz, Montero and Gómez-Guillén [19] with a slight modification. Gelatin solution was preheated at 40 °C for 15 min. The turbidity of gelatin solutions was measured by reading the absorbance at 360 nm using a double-beam spectrophotometer (model UV-1601, Shimadzu, Kyoto, Japan).

2.4.9 Measurement of setting time

Setting time of gelatin solution was determined at 4 °C and room temperature (26±2 °C) according to the method of Kittiphattanabawon, Benjakul, Visessanguan and Shahidi [7]. Gelatin solution (6.67%, w/v) was prepared in the same manner as described previously. The solution (2 ml) was transferred to thin wall (12 mm x 75 mm) test tubes (PYREX[®], Corning, NY, USA) and preheated at 60 °C for 10 min, followed by incubation in an ice bath (4 °C) or at room temperature. An aluminium needle with the diameter and length of 0.1 and 25 cm, respectively, was inserted manually in the gelatin solution and raised every 10 s. The time at which the needle could not detach from the gelatin sample was recorded as the setting time. The setting time was expressed as min.

2.4.10 Determination of gelling and melting temperatures

Gelling and melting temperatures of gelatin samples were measured following the method of Sinthusamran, Benjakul and Kishimura [6] using a controlled stress rheometer (RheoStress RS 75, HAAKE, Karlsruhe, Germany). Gelatin solution (6.67%, w/v) was prepared in the same manner as described previously. The solution was preheated at 35 °C for 30 min. The measuring geometry used was 3.5 cm parallel

plate and the gap was set to 1.0 mm. The measurement was performed at a scan rate of 0.5 °C/min, a frequency of 1 Hz, oscillating applied stress of 3 Pa during cooling from 35 to 5 °C and heating from 5 to 35 °C. The gelling and melting temperatures were calculated, where $\tan \delta$ became 1 or δ was 45°.

2.4.11 Microstructure analysis of gelatin gel

Microstructure of gelatin gel was visualised using scanning electron microscopy (SEM). Gelatin gels having a thickness of 2-3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. The samples were then rinsed with distilled water for 1 h and 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, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 20 kV.

2.5 Statistical analysis

All experiments were run in triplicate using three different lots of skin samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by using Duncan's multiple range test [20]. Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1 Extraction yield

The yields of gelatin extracted from the skin of seabass with different sizes are shown in Table 1. Extraction yields of G2, G4 and G6 were 38.22, 40.50 and 43.48% (based on dry weight), respectively. The highest yield was found for G6 sample extracted from the skin of largest size, in comparison with those of smaller sizes ($p < 0.05$). Difference in yield can be associated with varying extraction processes as well as the species or tissue used [13]. The result was in accordance with Muyonga, Cole and Duodu [15] who reported that the lower yield of gelatin was obtained from young Nile perch skins, compared to adult Nile perch skin. The age of animal used as raw material for gelatin extraction has the influences on the connective tissues and protein compositions [21]. The number of cross-links in collagen increased with increasing age of the animal [22]. The result suggested that the lower yield of gelatin from seabass with smaller size might be associated with lower cross-linking in collagen molecule. Leaching of collagen might take place to a higher extent during the swelling process. As a result, the lower yield was gained when skin of seabass with smaller size was used.

On the other hand, the cross-links might be attached tightly in the skin matrix. Thus, the loss during swelling could be lower. The yields of fish skin gelatin varied among species, e.g. dog shark (62.3%), tuna (48.1%), rohu (39.55%) [8], cobia (24.1%), croaker (30.3%) [9], tiger-toothed croaker (36.8%) and pink perch (27.3%) [23]. Therefore, size or age of seabass directly affected the extraction yield of gelatin.

3.2 Protein patterns

The protein patterns of gelatin extracted from the skin of seabass with different sizes are illustrated in Fig. 1. Gelatins from seabass skin had the typical molecular distribution of collagen type I, in which the ratio of α_1 -chain and α_2 -chain was approximately 2/1. Apart from α -chain, β - and γ -chains with MW of 195 and 125-110 kDa, respectively, were obtained. It was noted that G2 sample had α -chains as the major constituents. Commercial fish gelatin also contained α -chains as major components with some degraded proteins. The band intensity of β -chains, γ -chain and high molecular weight cross-link increased when skin from larger size seabass was used for extraction. The increase in band intensity of proteins larger than α -chains was in agreement with the lower degradation. Generally, G2 samples exhibited more degraded peptides with the range of 100-25 kDa. Cross-links with higher MW found in skin of seabass with the average size of 4 and 6 kg/fish might be more resistant to degradation during extraction at 45 °C, compared with small MW components present in skin of smallest size seabass (2 kg/fish). It was noted that the ratio of α_2/α_1 band intensity of G4 and G6 became lower than that of G2. It was presumed that α_2 might under cross-linking to form a larger MW component such as β -, γ -chains as well as high MW cross-links [7]. The protein components of gelatin as well as degradation of proteins contribute to gelling behaviour of gelatin, especially gel strength, setting time and gelling temperature [7]. Therefore, the components of gelatin from seabass skin were affected by size of seabass used.

3.3 Amino acid compositions

Amino acid compositions of gelatin extracted from the skin of seabass with different sizes are presented in Table 2. All gelatins had glycine as the major amino

292 acid (22.57-23.33 g/100 g) and had a relatively high content of alanine (11.17-11.38
293 g/100 g). The amino acid composition of all gelatins showed very low contents of
294 cysteine (0.09-0.10 g/100 g), tyrosine (0.57-0.60 g/100 g), histidine (0.74-0.75 g/100
295 g) and hydroxylysine (0.79-0.81 g/100 g). In general, cysteine and tryptophan are not
296 found in collagen and gelatin [4, 6, 7]. For imino acids, all gelatins contained proline
297 and hydroxyproline contents of 11.95-13.60 and 9.57-9.76 g/100 g, respectively. The
298 imino acid content of G2 (23.36 g/100 g) and G4 (23.12 g/100 g) was higher than that
299 of G6 (21.57 g/100 g). Commercial fish gelatin had imino acid of 22.89 g/100 g.
300 Gelatin of skin from young Nile perch (21.55 g/100 g) showed similar imino acid
301 content to that of skin from adult Nile perch (21.63 g/100 g) [15]. The imino acid
302 content of seabass skin gelatin was higher than that of farmed Amur sturgeon skin
303 (18.90 g/100 g) [10], dog shark skin (19.75 g/100 g), rohu skin (18.38 g/100 g), tuna
304 skin (17.96 g/100 g) [8] and carp skin (19.47 g/100 g) [24]. Mammalian gelatins
305 generally contain 30% imino acids, which was higher than fish gelatin [8]. The
306 species, environment living habitat and body temperature of fish are the main factors
307 governing hydroxyproline and proline content [25]. Moreover, hydroxyproline
308 content of gelatin was also affected by the extraction conditions [10]. The stability of
309 triple-helix in collagen molecule correlated with imino acid content [16]. The
310 difference in imino acid content, especially hydroxyproline, was considered as the
311 important factor influencing viscoelastic properties and gel formation of gelatin [4, 8].
312 In the present study, there was similar hydroxyproline content amongst all samples,
313 but G6 showed the lowest proline content. The difference in amino acid composition
314 could be due to the differences in cross-links in the starting skin matrix. With
315 increasing fish age, the cross-linking of collagen in skin can be enhanced [20]. Those
316 imino acids might contribute to cross-links. As a consequence, the chains rich in

proline were not leached out during extraction as indicated by the lower proline in the extracted gelatin. This result suggested that size or age of fish raw materials might determine the amino acid composition of the resulting gelatin.

3.4 Fourier transform infrared (FTIR) spectra

FTIR spectra of gelatin extracted from the skin of seabass with various sizes are depicted in Fig. 2. FTIR spectroscopy has been used to study the changes in functional groups and the secondary structure of gelatin [10]. All gelatin samples showed similar spectra, which had the major peaks in amide region. G2, G4 and G6 samples exhibited the amide I bands at the wavenumber of 1637, 1638 and 1637 cm^{-1} , respectively. Normally, amide I band of gelatin appeared at 1700-1600 cm^{-1} , which was associated primarily with a C=O stretching vibration/hydrogen bonding coupled with COO [26]. The absorption in the amide I is probably the most useful for infrared spectroscopic analysis of the secondary structure of protein [7]. In the present study, the amide I peak (1637-1638 cm^{-1}) of all samples was in agreement with Yakimets, Wellner, Smith, Wilson, Farhat and Mitchell [27] who reported that the absorption peak at 1633 cm^{-1} was the characteristic of coiled structure of gelatin. The amide I bands of all gelatin samples were shifted to the higher wavenumber, compared with seabass skin collagen [16]. Loss of triple helix occurred during heating via breaking down hydrogen-bonds between α -chains [15, 28]. The Amide II band of G2, G4 and G6 appeared at 1540, 1536 and 1536 cm^{-1} , respectively. The amide II vibration modes are related to an out-of-phase combination of a CN stretch and in-plane NH deformation modes of the peptide group [26, 29]. In addition, amide III was detected around the wavenumber of 1236, 1235 and 1235 cm^{-1} for G2, G4 and G6, respectively, more likely associated with N-H deformation and C-N stretching vibration as well as

the absorptions arising from wagging vibrations of CH₂ groups in the glycine backbone and proline side-chains [22, 30]. It was noted that the amide III band of gelatins extracted from skin of seabass with different sizes showed similar spectra.

The amide A band of G2, G4 and G6 samples was found at 3294, 3299 and 3293 cm⁻¹, respectively. A free NH stretching vibration is found in the range of 3400-3440 cm⁻¹. However, when NH group of peptide is involved in hydrogen bond, its position is shifted to lower frequencies, usually 3300 cm⁻¹ [31]. The amide B band was observed at 2918, 2922 and 2923 cm⁻¹ for G2, G4 and G6, respectively. It represents CH stretching vibrations of the -CH₂ groups [11]. Amplitude of Amide B peak was lower in G6 sample and the peak was shifted to the lower wavenumber, compared with other samples. This suggested that CH₂ groups were more likely interacted via cross-linking. This was in accordance with the higher MW cross-links in G6 sample. Therefore, gelatins from seabass skin showed slight difference in the secondary structure, as affected by size of raw material used.

3.5 Zeta-potential

The ζ-potential of gelatins extracted from the skin of seabass having different sizes tested at different pHs is shown in Fig. 3. Generally, gelatin samples showed positive charge at acidic pH ranges and became negatively charged under alkaline conditions. Zero surface net charge was obtained at pH 6.73, 6.41 and 7.12 for G2, G4 and G6, respectively, which were estimate to be their isoelectric points (pI). At pI, the positive charges are balanced out by the negative charges [32]. Gelatin from G6 had higher pI (7.12) than other gelatins extracted from skin of seabass with smaller sizes (G2 and G4). Gelatin from skin of different fish showed varying pIs, e.g 6.65-7.15 for unicorn leatherjacket [33], 8.8 for young Nile perch and 9.4 for adult Nile perch [15].

Thus, the differences in pI of all gelatin samples might be caused by the difference in their amino acid compositions and distribution of amino acid residues, which were more likely influenced by size or age of raw material used.

3.6 Gel strength of gelatin

Gel strength of gelatin from the skin of seabass with different sizes is shown in Fig. 4A. Gel strength of gelatin increased as the size of fish increased ($p < 0.05$). G6 had the highest gel strength, compared with others ($p < 0.05$). All gelatin gels from seabass skin exhibited higher bloom strength (223-322 g) than that of commercial fish gelatin from tilapia skin (212 g) ($p < 0.05$). Muyonga, Cole and Duodu [15] reported that gelatin from young Nile perch skins (222 g) had slightly lower gel strength than adult Nile perch skin gelatin (229 g). The result suggested that gelatin with greater ratio of high-molecular weight components showed the better gelling properties via higher stabilising interactions [34]. G4 and G6 showed higher band intensity of β -chain, γ -chains and high MW cross-links than G2 (Fig. 1). Those large components might serve as the strong strands in gel network. Gelatins with different gel strength were reported for farmed Amur sturgeon skin (141 g) [10], cobia skin (232 g), croaker skin (212 g) [9], dog shark skin (206 g), tuna skin (177 g) [8], tiger-toothed croaker (170 g), pink perch (140 g) [23] and grey triggerfish (168 g) [2]. The difference in gel strength amongst species was mainly due to the different amounts of the β - and α -components and the amino acid composition of gelatin [6]. Furthermore, imino acids, especially hydroxyproline, are involved in gel formation of gelatin via hydrogen bonding through hydroxyl group [5]. However, similar hydroxyproline contents were observed amongst all gelatin samples as shown in Table 2. The result suggested that

components of gelatin, particularly cross-links, most likely played an essential role in gel formation and determined gel strength of gelatin.

3.7 Colour and turbidity of gelatin gel

Colours of different gelatin gels from skin of seabass and commercial fish gelatin expressed as L^* , a^* , b^* and ΔE^* are shown in Table 1. Gelatin gel from G2 showed the higher L^* -value (lightness) than others (G4 and G6) ($p < 0.05$). The L^* -value of gelatin gels slightly decreased when skin from seabass with larger sizes was used ($p < 0.05$). G2 sample had the lower redness value (a^* -value), compared to commercial fish gelatin ($p < 0.05$). No differences in a^* -value were found amongst gelatins from seabass with different sizes ($p > 0.05$). Additionally, gelatin gels from seabass had higher b^* -value than that from commercial fish gelatin ($p < 0.05$). Slight differences in b^* -value were found amongst gelatin gels from seabass skin with different sizes ($p < 0.05$). For total difference in the colour value (ΔE^*), commercial fish gelatin showed the highest ΔE^* -value (91.27), compared with gelatin gels from seabass skin. ΔE^* -value increased as size of seabass used for gelatin extraction increased. This trend was in agreement with the decrease in L^* -value as the size of fish used increased. Thus, size or age of seabass as raw material had the impact on colour of gelatin and their gels.

Turbidity of solutions of gelatin extracted from skin with different sizes expressed as A_{360} is shown in Fig. 4B. Gelatin solution exhibited the lower turbidity when size of seabass used for extraction increased ($p < 0.05$). The highest turbidity was found in solution of G2 sample ($p < 0.05$). Turbidity of gelatin solution from commercial fish gelatin was much lower, compared with all gelatins from seabass skin ($p < 0.05$). The turbidity of gelatin solution is dependent on species of raw

material, gelatin extraction process and clarification/filtration process [15, 23]. In this study, only filtration process was implemented, whilst the clarification process has been used for commercial gelatin to remove both light- and heavy-impurities [15]. Koli, Basu, Nayak, Patange, Pagarkar and Gudipati [23] reported that inorganic, protein and muco-substance contaminants mainly affected turbidity and dark colour of gelatin solution. Both G4 and G6 samples contained high MW components at higher level than G2 (Fig. 1). Solution of G2 sample with smaller peptides or proteins might prevent transmission of light more effectively, thereby increasing the light scattering. This might contribute to higher turbidity of gelatin solution obtained from skin of fish with smaller sizes. These results showed that the size or age of raw materials had the impact on turbidity of gelatin solution.

3.8 Setting time for gel formation

The setting times required for the gel formation of gelatin extracted from the skin of seabass having different sizes at 4 °C and room temperature (26±2 °C) are presented in Fig. 5A and 5B, respectively. The setting times at 4 °C of G2, G4 and G6 were 2.13, 2.80 and 3.60 min, respectively. The setting time at 4 °C of gelatin increased as seabass size used for gelatin extraction increased ($p<0.05$). G2 and G4 samples had a shorter setting time at 4 °C than the commercial fish gelatin ($p<0.05$). Due to the higher proportion of high MW peptide chains of G6, the arrangement of those chains formation of gel network plausibly took a longer time as indicated by longer setting time. In addition, the nucleation zone during gel formation required hydroxyproline [35]. With high proportion of cross-links or β - and γ -chains, hydroxyproline residues could be localized inside the molecules. This could lower the

accessibility or exposure of hydroxyproline to form H-bond. Thermal degradation of peptides in gelatin during heating extraction affected the setting time of gelatin gel [6].

When setting was carried out at room temperature, setting times of 32.0, 28.83 and 19.13 min were found for G2, G4 and G6, respectively. Gelatin samples from seabass skin had the longer setting time when small size of seabass was used ($p < 0.05$). All gelatins from seabass were able to undergo setting at room temperature. This result was in agreement with Sinthusamran, Benjakul and Kishimura [6] who reported that gelatin from seabass skin was able to set at room temperature within 30 min. Conversely, commercial fish gelatin was not able to set within 3 h at room temperature. Setting phenomenon of gelatin samples at room temperature was different from that occurred at lower temperature (4 °C). At higher setting temperatures, higher entropy of gelatin molecules was presumed. Those γ - and β -components found in G4 or G6 samples might promote the formation of gel network with rapid gelation [36]. When those high MW cross-links aligned themselves under the high entropy conditions, they could form gel easily as evidenced by lower setting time. Different extraction condition, especially extraction temperature and time also influenced the setting time of gelatin [7, 15]. Furthermore, difference in setting time of fish gelatin might be due to different age or size of fish used as raw material [8]. Thus, setting time could be affected by size of seabass used as raw material. Additionally, setting temperature was another factor affecting time employed for gel formation.

3.9 Gelling and melting temperatures

Gelling and melting temperatures of gelatin extracted from the skin of seabass with different sizes are presented in Table 1. Thermal transition of gelatin solution

were monitored by changes in phase angle (δ) during cooling (35-5 °C, 0.5 °C/min) and subsequent heating (5-35 °C, 0.5 °C/min). The formation of junction zones in the three-dimensional network of gelatin gel can be monitored in term of changes of phase angle [6]. The gelling temperatures were 17.09, 18.43 and 19.01 °C for G2, G4 and G6, respectively, which were higher than commercial fish gelatin (15.84 °C) ($p<0.05$). The higher gelling temperatures were observed in G4 and G6 samples extracted from the skin of larger size, in comparison with that of smaller size (G2) ($p<0.05$). The gelling temperature of gelatin tended to increase with increasing amount of cross-links. This result indicated that the molecular weight distribution in gelatin was associated with gelation. Muyonga, Cole and Duodu [15] also reported that chemical compositions, especially the content of γ -, β -, and α -chain components influenced gelling point of gelatin gel. Additionally, different gelling temperature was related to amino acid composition, especially proline and hydroxyproline, which is governed by temperatures of habitat, where fish live [10, 37]. The imino acid content is normally available to form hydrogen bonds with water [13]. In general, warm water fish or mammalian gelatin has higher gelling and melting temperatures than the cold water fish gelatin [10]. Most of fish gelatin had lower gelling temperature than mammalian gelatin [37]. Varying gelling temperatures were reported for gelatin from different fish, e.g. cobia skin (19.9 °C), croaker skin (17.8 °C) [9], dog shark skin (20.8 °C), tuna skin (18.7 °C) [8], farmed Amur sturgeon skin (14.2 °C) [10], red snapper bone (16.0 °C) and grouper bone (16.0 °C) [37].

Melting temperatures of G2, G4 and G6 were 26.92, 27.62 and 28.85 °C, respectively. The melting temperatures of gelatin from seabass skin were higher than that of commercial fish gelatin (25.10 °C) ($p<0.05$). Nevertheless, the melting temperature of gelatins from seabass skin were lower than those of bovine bone

(29.7 °C) and porcine skin (32.3 °C) [38]. It was noted that the melting temperatures of all gelatins from seabass skin were higher than those of gelatins from the dog shark skin (25.8 °C), tuna skin (24.2 °C) [8], farmed Amur sturgeon skin (22 °C) [10], tiger-toothed croaker skin (20.36 °C), pink perch skin (19.23 °C) [23] and tilapia skin (22.4 °C) [39]. Since seabass used in the present study was tropical fish (25-32 °C), gelatin gel might be more stable than those from temperate zone (15-25 °C) [40]. Thermal stability of gelatin gel is associated with the temperature of habitat of the animals [13]. G4 and G6 samples had higher melting temperature than G2 ($p < 0.05$). High MW components might contribute to the stability and stronger network of gel.

In the present study, G6 showed the higher gelling as well as melting temperatures, although it showed the lower imino acid content than G2 and G4. It was noted that G6 had the higher band intensity of β -chains, γ -chain and high molecular weight cross-link, compared with G2 and G4 (Fig. 1). Due to the higher percentage of β - and γ -components, a shorter maturation time might be required since the entanglement of chain took place with ease. Also, those large components might serve as the strong strands in gel network, leading to the increased thermal stability of the gelatin gel [41] as indicated by the higher melting temperature. As a consequence, G6 had higher gelling and melting temperatures. Gelatin extracted from tropical fish has higher gelling and melting temperature than that gelatin extracted from temperate fish. Thus, the differences in gelling and melting temperature of seabass gelatin might be governed by the differences in components, especially those with high MW.

3.10 Microstructures of gelatin gels

Microstructures of the gel from commercial fish gelatin and gels from gelatin extracted from skin of seabass with different sizes are shown in Fig. 6. Generally, all

gelatin gels from seabass skin were sponge or coral-like in structure. The gel from G2 had a looser network with larger voids and thinner strands, compared with gels from other gelatin samples (G4 and G6). G6 showed thicker strands in gel network, compared with others. The gelation process was governed by cross-linked polypeptide network stabilized mainly by hydrogen-bonded junction zones [5]. Gelatin with high molecular weight distribution could form the junction zones with ordered gel structure, leading to higher gel strength. Sinthusamran, Benjakul and Kishimura [6] reported that gelatin extracted from the seabass skin at milder condition (45 °C for 3 h) had a higher gel strength with the finest structure and smaller voids in gel matrix, compared with gelatin extracted under the harsher conditions. Zhang, Duan, Wang, Yan and Xue [25] reported that the denser strands of gel structure were governed by greater content of high molecular weight peptides (γ - and β -chains) in gelatin, whilst looser strands in gel matrix were found in gelatin sample containing smaller and shorter peptides. Based on protein pattern (Fig. 1), it was found that gelatin extracted from skin of seabass with the larger sizes contained higher content of longer or larger peptides chains. Those chains could build up the strong strands, which could strengthen the network as evidenced by the stronger gel strength.

4. Conclusions

Yield and gelling properties of gelatin were influenced by the size of seabass. Gelatin obtained from the skin of seabass with 6 kg/fish had the highest yield and better gelling properties. This was associated with the higher content of high MW components or cross-links. Nevertheless, gels of all gelatins were able to set at room temperature. The gelling and melting temperatures were in the range of 16.49-18.64 °C and 26.92-27.69 °C, respectively, which were higher than that of

commercial fish gelatin. Thus, skin from seabass with different size used as raw material for gelatin extraction had the influence on chemical compositions and properties of gelatin.

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References

- [1] L. Chen, L. Ma, M. Zhou, Y. Liu, Y. Zhang, Food Hydrocoll. 36 (2014) 316-322.
- [2] K. Jellouli, R. Balti, A. Bougatef, N. Hmidet, A. Barkia, M. Nasri, LWT-Food Sci. Technol. 44 (2011) 1965-1970.
- [3] T. Nur Azira, Y.B. Che Man, R.N. Raja Mohd Hafidz, M.A. Aina, I. Amin, Food Chem. 151 (2014) 286-292.
- [4] S. Benjakul, K. Oungbho, W. Visessanguan, Y. Thiansilakul, S. Roytrakul, Food Chem. 116 (2009) 445-451.
- [5] S. Benjakul, P. Kittiphattanabawon, J.M. Regenstein, Fish gelatin, in: B.K. Simpson, L.M.L. Nollet, F. Toldrà, S. Benjakul, G. Paliyath, Y.H. Hui (Eds.), Food Biochemistry and Food Processing, John Wiley & Sons, Inc., Ames, 2012, pp. 388-405.
- [6] S. Sinthusamran, S. Benjakul, H. Kishimura, Food Chem. 152 (2014) 276-284.

- 564 [7] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, F. Shahidi, Food Hydrocoll.
565 24 (2010) 164-171.
- 566 [8] K. Shyni, G.S. Hema, G. Ninan, S. Mathew, C.G. Joshy, P.T. Lakshmanan, Food
567 Hydrocoll. 39 (2014) 68-76.
- 568 [9] R.S.G. Silva, S.F. Bandeira, L.A.A. Pinto, LWT-Food Sci. Technol. 57 (2014)
569 580-585.
- 570 [10] M. Nikoo, S. Benjakul, M. Bashari, M. Alekhorshied, A. Idrissa Cissouma, N.
571 Yang, X. Xu, Food Bioscience. 5 (2014) 19-26.
- 572 [11] M. Nagarajan, S. Benjakul, T. Prodpran, P. Songtipya, H. Kishimura, Food
573 Hydrocoll. 29 (2012) 389-397.
- 574 [12] P. Kaewruang, S. Benjakul, T. Prodpran, Food Chem. 138 (2013) 1431-1437.
- 575 [13] A.A. Karim, R. Bhat, Food Hydrocolloid. 23 (2009) 563-576.
- 576 [14] P. Singh, S. Benjakul, S. Maqsood, H. Kishimura, Food Chem. 124 (2011) 97-
577 105.
- 578 [15] J.H. Muyonga, C.G.B. Cole, K.G. Duodu, Food Hydrocoll. 18 (2004) 581-592.
- 579 [16] S. Sinthusamran, S. Benjakul, H. Kishimura, Food Chem. 138 (2013) 2435-2441.
- 580 [17] U.K. Laemmli, Nature. 227 (1970) 680-685.
- 581 [18] A. Gennadios, C.L. Weller, M.A. Hanna, G.W. Froning, J. Food Sci. 61 (1996)
582 585-589.
- 583 [19] M.D. Fernández-Díaz, P. Montero, M.C. Gómez-Guillén, Food Chem. 74 (2001)
584 161-167.
- 585 [20] R.G.D. Steel, J.H. Torrie, Principles and Procedures of Statistics: A Biometrical
586 Approach., second ed., McGraw-Hill, New York, 1980.
- 587 [21] E. Foegeding, T.C. Lanier, H.O. Hultin, Characteristics of edible muscle tissue,
588 in: O.R. Fennema (Ed.) Food Chem, Marcel Dekker., New York, 1996, pp. 879-942.

- 589 [22] J.H. Muyonga, C.G.B. Cole, K.G. Duodu, *Food Chem.* 85 (2004) 81-89.
- 590 [23] J.M. Koli, S. Basu, B.B. Nayak, S.B. Patange, A.U. Pagarkar, V. Gudipati, *Food*
591 *Bioprod. Process.* 90 (2012) 555-562.
- 592 [24] L.M. Kasankala, Y. Xue, Y. Weilong, S.D. Hong, Q. He, *Biores. Technol.* 98
593 (2007) 338-343.
- 594 [25] J. Zhang, R. Duan, Y. Wang, B. Yan, W. Xue, *Food Hydrocoll.* 29 (2012) 100-
595 105.
- 596 [26] J. Bandekar, *Biochimica et Biophysica Acta (BBA) - Protein Structure and*
597 *Molecular Enzymology.* 1120 (1992) 123-143.
- 598 [27] I. Yakimets, N. Wellner, A.C. Smith, R.H. Wilson, I. Farhat, J. Mitchell,
599 *Polymer.* 46 (2005) 12577-12585.
- 600 [28] P. Kaewruang, S. Benjakul, T. Prodpran, S. Nalinanon, *Food Bioscience.* 2
601 (2013) 1-9.
- 602 [29] F. Lavialle, R.G. Adams, I.W. Levin, *Biochemistry.* 21 (1982) 2305-2312.
- 603 [30] M. Jackson, L.P. Choo, P.H. Watson, W.C. Halliday, H.H. Mantsch, *Biochimica*
604 *et Biophysica Acta Molecular Basis of Disease.* 1270 (1995) 1-6.
- 605 [31] B.B. Doyle, E.R. Blout, E.G. Bendit, *Biopolymers.* 14 (1975) 937-957.
- 606 [32] P.L.R. Bonner, *Protein purification*, UK: Taylor & Francis Group, Cornwall,
607 2007.
- 608 [33] M. Ahmad, S. Benjakul, *Food Hydrocoll.* 25 (2011) 381-388.
- 609 [34] H.S. Tabarestani, Y. Maghsoudlou, A. Motamedzadegan, A.R. Sadeghi
610 Mahoonak, *Biores. Technol.* 101 (2010) 6207-6214.
- 611 [35] D.A. Ledward, Gelation of gelatin, in: J.R. Mitchell, D.A. Ledward (Eds.),
612 *Functional properties of food macromolecules*, Elsevier Applied Science., London,
613 1989, pp. 171-201.

- 614 [36] D. Fernández, amp, x, M.D. az, P. Montero, M.C. Gómez-Guillén, Food
615 Hydrocoll. 17 (2003) 281-286.
- 616 [37] R. Jeya Shakila, E. Jeevithan, A. Varatharajakumar, G. Jeyasekaran, D. Sukumar,
617 LWT-Food Sci. Technol. 48 (2012) 30-36.
- 618 [38] M. Gudmundsson, J. Food Sci. 67 (2002) 2172-2176.
- 619 [39] S. Zeng, X. Yan, W. Cao, P. Hong, C. Zhang, L. Li, Int. J. Food Sci. Technol. 45
620 (2010) 1807-1813.
- 621 [40] T.O. Haugen, A.C. Sanger, Am. Fish. Soc. Symp. 76 (2011), 1-24.
- 622 [41] S. Wangtueai, A. Noomhorm, J.M. Regenstein, J. Food Sci. 75 (2010) 731-739.
- 623
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- 626
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Figure Legends

Fig. 1. SDS-PAGE patterns of gelatin extracted from the skin of seabass with different sizes. M and CF denote high molecular weight markers and commercial fish gelatin.

Fig. 2. FTIR spectra of gelatin extracted from the skin of seabass with different sizes.

Fig. 3. Zeta-potential of gelatins extracted from the skin of seabass with different sizes. Bars represent the standard deviation (n=3).

Fig. 4. Gel strength (A) and turbidity (B) of gelatin extracted from the skin of seabass with different sizes. CF denotes commercial fish gelatin. Bars represent the standard deviation (n=3). Different lowercase letters on the bars indicate significant differences ($p < 0.05$).

Fig. 5. Setting time of gelatin extracted from the skin of seabass with different sizes at 4 °C (A) and room temperature (26 ± 2 °C) (B). NS: Gel was not set within 3 h. Bars represent the standard deviation (n=3). Different lowercase letters on the bars indicate significant differences ($p < 0.05$).

Fig. 6. Microstructures of gel from the commercial fish gelatin (CF), gelatin extracted from the skin of seabass with the average size of 2 kg/fish (G2), gelatin from the skin of seabass with the average size of 4 kg/fish (G4) and gelatin from the skin of seabass with the average size of 6 kg/fish (G6). Magnification: 3000x.

Molecular characteristics and properties of gelatin

from skin of seabass with different sizes

Sittichoke Sinthusamran¹ and Soottawat Benjakul^{1,*} and Hideki Kishimura²

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Table 1. Extraction yield, gel colour, gelling and melting temperatures of gelatin extracted from seabass skin with different sizes

Gelatin samples	Yield (%, dry wt basis)	L^*	a^*	b^*	ΔE^*	Gelling temperatures (°C)	Melting temperatures (°C)
CF	NM	4.17±0.37a	-0.18±0.36b	-3.35±0.40a	91.27±0.25d	15.84±0.28a	25.10±0.57a
G2	38.22±0.21c	17.07±0.49d	-0.47±0.11a	-1.31±0.14b	73.09±0.68a	17.09±0.42b	26.92±0.35b
G4	40.50±0.31b	16.33±0.34c	-0.43±0.07ab	-0.91±0.11c	74.25±0.44b	18.43±0.29c	27.62±0.63bc
G6	43.48±1.27a	14.46±0.04b	-0.33±0.14ab	-1.19±0.16bc	76.86±0.08c	19.01±0.63c	28.85±0.62c

Mean±SD (n = 3).

Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

NM: Not mentioned

Table 2. Amino acid compositions of gelatins extracted from seabass skin with different sizes

Amino acids (g/100 g)	CF	G2	G4	G6
Alanine	10.08	11.17	11.18	11.38
Arginine	8.59	8.26	8.19	8.58
Aspartic acid/asparagine	5.25	5.31	5.34	5.36
Cysteine	0.06	0.09	0.10	0.10
Glutamine/glutamic acid	9.68	9.50	9.49	9.60
Glycine	22.90	22.57	22.65	23.33
Histidine	0.64	0.74	0.74	0.75
Isoleucine	1.30	1.11	1.11	1.10
Leucine	2.58	2.14	2.17	2.14
Lysine	3.63	3.56	3.55	3.64
Hydroxylysine	0.80	0.79	0.80	0.81
Methionine	1.31	1.85	1.86	1.88
Phenylalanine	1.84	1.93	1.95	1.94
Hydroxyproline	9.89	9.76	9.57	9.62
Proline	12.99	13.60	13.55	11.95
Serine	3.31	2.55	2.58	2.57
Threonine	2.49	2.39	2.43	2.44
Tyrosine	0.43	0.57	0.58	0.60
Valine	2.26	2.13	2.15	2.21
Imino acid	22.89	23.36	23.12	21.57

Research highlights

- ♦ Gelatin from seabass skin had higher gel strength than commercial fish gelatin.
- ♦ Gelatin from skin of larger size seabass exhibited the better gelling properties.
- ♦ Higher cross-links were found in gelatin from skin of seabass with larger sizes.