Enzymatic Extraction of Gelatin from Threadfin Bream (*Nemipterus japonicas*) Skin

*Normah Ismail, Izaty Hasni Ismail

Abstract— **Threadfin bream (*Nemipterus japonicus*) skin gelatin was enzymatically extracted for 6 or 12 hrs at 60°C, pH 8 in the presence of alcalase. The gelatin was analyzed for yield, gel strength, melting point, setting point, setting time, viscosity, solubility and molecular weight distribution. The gelatin was also compared with commercial gelatin. The 12 hrs extraction time yield 7.74% gelatin and the gelatin was more soluble while 6 hrs gelatin resulted in 5.69% yield. Gel strength of the extracted gelatin decrease with longer extraction time. The 6 hrs gelatin exhibited gel strength of 324.93g which was very close to the commercial gelatin (342.10g) while 12 hrs gelatin gel strength was 202.47g. Melting points of threadfin bream gelatin increased when the extraction time increased ranging from 25.53 to 29.42°C while commercial gelatin melted at 34.03°C. Threadfin bream gelatins set at lower temperature and longer time with increase in extraction time compared to commercial gelatin. The gelatin extracted for 6 hrs resulted in higher gel strength, viscosity, melting point, setting point and shorter setting time than the 12 hrs gelatin. This shows that the 6 hrs extracted gelatin is more suitable to be used in food production than the 12 hrs gelatin.**

***Index Terms*—Alcalase, Extraction, Gelatin, Threadfin Bream**

I. Introduction

Threadfin bream (*Nemipterus japonicus)* comes from the family Nemipteridae within the order Perciformes and also known as whiptail breams and false snappers [1]. There are thirteen species of threadfin bream from the genus *Nemipterus. Nemipterus japonicus* is a species that is normally sold in Malaysian market. It is one of the most captured fish in Malaysia based on the statistics released by Malaysia's Department of Fisheries [2]. The landing of threadfin bream in West Coast, East Coast, Sarawak, Sabah and Federal Territory of Labuan recorded a total of 154, 006 tons in 2007 to 2010. Threadfin bream is available all year. The flesh is widely used in the production of surimi and surimi-based products [3].

Gelatin derives by a partial hydrolysis of animal collagen [4]. The skin and bone are commonly used as raw materials for gelatin extraction [4]. Fish skins are especially suitable as a source of gelatin because it is easily extracted with high yield at relatively moderate temperature usually at or below 50 °C [5]. Gelatin have been extracted using hot water at 50 to 80°C for 12 hrs, 45°C for 4 to 20 hrs and 40 to 80°C for 2 to 4 hrs [6, 7, 8].

The gel strength and gel melting point are the major physical properties of gelatin [9]. Gelatin characteristic not only depends on the extraction condition but also on the raw materials used to extract the gelatin since intrinsic characteristics of the collagen molecules varies from each species especially the amino acid composition [10, 11]. Generally, gel strength of gelatin decrease as the extraction temperature and time increase [12, 6, 7].

The efficiency of gelatin extraction has been shown to improve in the presence of enzyme. Yan and Tiejin [13] obtained gelatin with high gel strength using pepsin (547 U/g) at pH 4, 46.98ºC and 1.27 hrs. Nalinanon et al., [14] used the extracted bigeye snapper pepsin at a concentration of 15 units/g alkaline treated skin for 48 hrs at 4 °C prior to hot water extraction. They found that the extraction efficiency was augmented in the presence of pepsin. In this study, threadfin bream skin gelatin was extracted by enzymatic method using alcalase and the resulting gelatin was compared with the commercial gelatin.

II. Procedure

Materials

All chemicals used were of analytical grade purchased from Sigma Aldrich, UK. Alcalase was purchased from Novo Nordisk Industries (AS, Bagsvaerd, Denmark). Threadfin bream was obtained from the nearby wet market in Shah Alam.

A. *Extraction of gelatin*

Gelatin was extracted according to Normah et al., [15]. The skin was removed from the fish and cleaned until there is no muscle or scales left, cut into 3 cm squares and washed under running tap water. Then, the skin was soaked in 0.8 M NaCl at the ratio of 1:6 (w/v) for two mins and rinsed under running water. To remove the non-collagenous proteins and pigments, the skin was soaked in 0.19 N of cold NaOH (~7°C) at the ratio of 1:3 w/v for 40 mins and rinsed under running water. This was followed by soaking in 0.12 N cold acetic acid solution (~7°C) at the ratio of 1:3 w/v for 40 mins and rinsed with distilled water until it is neutral (pH 7). Gelatin was extracted in distilled water at 1:3 (w/v) ratio of the skin to water at 60°C and pH 8 for 6 and 12 hrs in a shaking water bath. Alcalase was added to this mixture based on the amount of the skin which is 3g alcalase per 100g of skin. Subsequently, the mixture was filtered and the resulting filtrate was freeze-dried and ground into a powder.

Department of Food Technology, Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, 40450, Selangor,Malaysia

✉ Normah Ismail
*norismel@salam.uitm.edu.my

Received : 2 March 2016
Accepted : 14 May 2016
Published : 30 June 2016

B. *Analysis of gelatin*

1) Yield

The percentage of gelatin yield was obtained according to Binsi et al., [16] based on the wet weight of the skin using the following formula:

$$\text{Yield (\%)} = \frac{\text{weight of gelatin}}{\text{wet weight of skin}} \times 100$$

2) Gel strength

The gel strength was determined as in the previous methods [17, 18]. The gelatin powder 6.67% (w/v) was dissolved in distilled water and left at room temperature for 30 min to allow the gelatin to absorb water and swell. The mixture was then heated at 60ºC for 20 min to completely dissolve the gelatin and then poured into a bloom jar with dimensions of 3.3 cm diameter and 6 cm height (Schott Duran, 55122 Mainz, Germany). The bloom jar was covered and kept in a refrigerator for gel maturation at 7°C for 16 to 18 hrs for gel maturation. The gel strength at 7°C was determined by using Texture Analyzer Model TA-X2i (Stable Micro System, Surrey, UK) equipped with a flat-faced cylindrical Teflon plunger (1.27 cm in diameter) and load cell of 5 kN. The maximum force (in g) at the penetration depth of 4 mm was recorded at the rate of 2 mm/s. The measurement was performed in triplicate.

3) Melting point

The melting point was determined according to Muyonga et al., [12] by using thin wall screw-capped test tube (12 mm x 75mm). An amount of 6.67% (w/v) gelatin solution was filled into the test tube up to 15 mm headspace. The test tube was then kept in refrigerator at 7ºC for 16 to 18 hrs. Subsequently, it was transferred into water bath set at 10ºC in an inverted position so that the headspace was at the bottom. The temperature of the water bath was raised gradually at intervals of 1 min by adding warm water (~45°C). The gel melting point temperature was recorded when the gel started to slip down to the headspace.

4) Setting point and Setting time

The setting point was determined according to Muyonga et al., [12]. An amount of 10 % (w/v) gelatin solution was prepared where 30 ml of the gelatin solution was transferred into a test tube (12mm x 75mm) and placed in a water bath (40ºC). The water bath was cooled slowly by adding cold water (2ºC) at time intervals of 15s. A glass rod with diameter of 0.35 cm and length 20 cm was inserted into the solution and lifted every 15s. The setting point was recorded when the gelatin solution can no longer drip from the tip of the rod.

The setting time was determined at 4ºC [12, 41]. Samples were prepared similarly as setting point determination, where the aluminium needle (diameter, 0.1 cm and length, 8.5 cm) was inserted manually into the gelatin solution and raised at interval of 15s. The setting time was recorded when the needle could not detach from the gelatin.

5) Viscosity

The viscosity of the gelatin was measured at various temperatures according to Kittiphattanabawon et al., [19]. Gelatin solution was prepared at a concentration of 6.67% (w/v) gelatin powder in distilled water by heating at 60°C to dissolve the gelatin. The viscosity was measured using a rheometer (Model MCR 300, USA). The operating condition was 100 rpm and the temperature range of 10 to 60ºC at a rate of 1ºC/min. The viscosity was measured in triplicate.

6) Solubility

Gelatin solubility was measured according to Normah et al., [15]. Powdered gelatin (0.5 g) was weighed and placed in glass beaker (W1). Then, 10 ml distilled water (40°C) was added. The gelatin powder was mixed until dissolved and then filtered through a pre-weighed Whatman filter paper No. 4 (W2). The filter paper was dried at 100°C for 4 hrs in a hot air oven (Memmert UNB 500, Germany). Finally, the dried filter paper was cooled in a dessicator and weighed again (W3) until constant weight is achieved. Solubility was calculated as follows:

$$\text{Solubility (\%)} = 100 - \frac{([W3 - W2]) \times 100}{W1}$$

7) Molecular weight distribution

The molecular weight determination of the gelatin was carried out according to Normah et al., [20] with slight modification using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE). Sample was prepared by mixing with 2X sample buffer at (1:1) followed by heating at 70°C for 10 mins. About 10 µl sample was loaded into each well on the gel comprising of 5% stacking and 10% resolving gel. Low molecular weight markers (SeeBlue Plus2 Prestained Standard) ranging from 220 to 10 kDa was used. Electrophoresis was performed with the XCell Surelock electrophoresis cell (Consort, Model EV231, Germany). The gel was then stained in Coomassie Brilliant Blue solution and destained in ultrapure water.

C. *Statistical analysis*

Data was analyzed using the Analysis of Variance (ANOVA) procedure of SAS [21] software by using Duncan's multiple range tests to determine the significant differences between means at significant level of 95%.

III. RESULT AND DISCUSSION

A. *Yield of gelatin*

Gelatin yield was expressed as grams of dry gelatin per 100 g of skin. The yield was significantly higher (7.74%) ($p<0.05$) at 12 hrs of extraction then the 6 hrs (5.69%) (Table 1). The different values of gelatin yield depend on differences in both the proximate composition of the skins and the amount of soluble components in the skins as these properties varies

with species and age of fish [22]. Furthermore, it has been stated that the yield and quality of gelatin are not only influence by the species or tissue from which it is extracted but also depend on the extraction process condition [10]. Low gelatin yield has been suggested to be due to the loss of extracted collagen through leaching during the series of washing steps [23]. In this study, threadfin bream gelatin was subjected to alkali pre-treatment, acid pre-treatment and hot water extraction. In addition, the lower yield is also attributed to the insufficient denaturation of soluble collagen during extraction [24]. According to Karim and Bhat [25], hydrogen bonding stabilizing triple helix of mother collagen was destroyed during heating. This led to cleavage of peptide bonds, helix-to-coil transition and the conversion of collagen to soluble gelatin. Longer extraction time allows sufficient time in disrupting the bond within the collagen.

Table 1. Yield, gel strength and melting point of commercial gelatin and threadfin bream gelatin extracted for 6 and 12 hrs. $^{a-b}$ Means ± standard deviation of triplicate determinations. Means in the same column with different superscript letters were significantly different ($p<0.05$).

Gelatin	Yield (%)	Gel strength (g)	Melting point (°C)
6 hrs	$5.96^{b} \pm 0.215$	$324.93^{b} \pm 1.777$	$29.42^{b} \pm 0.458$
12 hrs	$7.74^{a} \pm 0.585$	$202.47^{c} \pm 1.803$	$25.53^{c} \pm 0.305$
Commercial		$342.10^{a} \pm 1.873$	$34.03^{a} \pm 0.208$

B. *Gel strength*

Gelatin was known to be highly capable of forming hydrogen bonds with water molecules to form a stable three-dimensional gel [26]. Gel strength or bloom strength is a measure of hardness, stiffness, strength, firmness and compressibility of the gel at a particular temperature and is influenced by the concentration and molecular weight [27]. Gel strength depends on several factors such as pH, molecular weight distribution and amino acid content [28, 14]. The gel strength of 6 hrs gelatin was significantly higher ($p<0.05$) than those extracted for 12 hrs (Table 1). Commercial gelatin had significantly ($p<0.05$) higher gel strength (342.10g) compared to threadfin bream gelatin. The addition of 1.0 mg/g transglutaminase has been shown to be one of the effective methods to increase the gel strength of fish gelatin resulting in the gel strength value of 101.10g compared to the control sample which was only 69.03 mg/g [29]. Gelatin derives from collagen which consists of three chain types; the , and -chains that form a triple helix with counter clockwise or left-handed orientation [30]. Higher amount of , and components would allow for more organized structure with higher gel strength [31]. Gelatin extracted at 12 hrs gives lower gel strength because the bonds of collagen was probably degraded further in the presence of alcalase and long extraction time. In addition, it has been reported that some heat-stable proteases endogenous to the skin are involved in the degradation of gelatin molecules specifically the and -chains which contribute to the low bloom strength [32]. According to See et al., [18], gel strength varies due to difference arises from the proline and hydroxyproline content in collagen of different species. The main difference between fish and mammalian gelatins is the imino acid content, where the mammalian gelatins have higher amounts [28]. Muyonga et al., [12] reported that proline and hydroxyproline contents of approximately 30% for mammalian gelatins, 22 to 25% for warm water fish gelatin and 17% for cold water fish gelatins. Sarabia et al., [33] stated that the higher the hydroxyproline content, the higher the gel strength of the gelatin. The hydroxyl groups of hydroxyproline play a part in the stability of the helix by inter-chain hydrogen bonding via a bridging water molecule as well as direct hydrogen bonding to the carbonyl group [34]. Shyni et al., [35] stated that the quality of gelatin also depends on the isoelectric point which could be controlled by pH adjustment. During the extraction of threadfin bream gelatin, the pH was controlled and maintained to pH 8 to ensure that alcalase works at optimum condition. As a result, the gel was compact and became stiffer when the pH were adjusted close to the isoelectric point which make protein chain become natural and thus gelatin polymer were close to each other [18].

C. *Melting point*

Melting properties of gelatin are fundamental for their functionality and the temperature at which a gel becomes liquid is known as the melting point [27]. The melting point of 6 hrs gelatin was significantly higher ($p<0.05$) than those from 12 hrs (Table 1). However, commercial gelatin showed highest melting point. It is generally known that fish gelatin had lower melting point than mammalian gelatin [25, 31]. Choi and Regenstein [36] reported that bovine and porcine gelatin have considerably higher melting points than most fish gelatin. The melting point for porcine and bovine gelatin range from 28 to 31°C compared to fish gelatin which ranged from 11 to 28°C [25]. The melting point of gelatins was also closely related to the fish species [37]. Gilsenan and Ross-Murphy [38] compared the rheological properties and melting points of mammalian gelatin with gelatin from different types of fish. They observed that the gelatin from cold-water fish have lower melting point than mammalian gelatin due to the lower imino acid contents. Warm-water fish gelatin however, have properties that are quite similar to mammalian gelatin [33]. The melting point observed in this experiment are higher than those reported for cold-water fish such as cod 13.8°C, hake 14°C and hoki 16.6°C [17, 39]. Based on the result, threadfin bream which is a warm-water fish had a melting point from 25 to 29°C. The melting points of black tilapia gelatin which is also a warm water fish and tuna skin gelatin were 28.9°C and 25.8°C, respectively [23, 35]. According to Mohtar et al., [39], proline and hydroxyproline are responsible for the melting point of different types of gelatin. The lower the proline and hydroxyproline contents, the lower are the melting temperatures of a gelatin [40]. Mammalian gelatin contains approximately 30% proline and hydroxyproline while warm water fish gelatin contain 22 to 25% and cold water fish gelatin contain 17% [12]. The lower content of proline and hydroxyproline gives fish gelatin a low gel modulus and low gelling and melting temperatures [30].

D. Setting point and setting time

The temperature at which the gelatin solution forms into a gel is known as the setting point [40]. The setting point of gelatin solution depends on the conditions used during the extraction [41]. In this experiment, the threadfin bream gelatin extracted for 6 hrs has significantly ($p<0.05$) higher setting point compared to 12 hrs of extraction (Table 2). Commercial gelatin sets at 34.20°C. The setting point of porcine and bovine gelatin was in range of 20 to 25°C and 28 to 35°C, respectively, while warm water fish (tilapia spp) gelatin and cold water fish (Alaskan pollock spp) were between 8 to 25°C and 11 to 28°C, respectively [25]. Setting time of threadfin bream skin gelatin was determined at 4°C. Threadfin bream gelatins set at significantly ($p<0.05$) longer time than commercial gelatin (Table 2). This result was in agreement with previous report where the lower the extraction temperature (45°C<60°C< 75°C) and the shorter the extraction time (6 hrs<12hrs) the shorter was the setting time [41]. A negative relationship between the setting time and the bloom strength has also been reported [41, 42]. Furthermore, it has been suggested that differences in setting time might be due to the differences in molecular weight distribution [12]. Generally, gelatins extracted at higher temperature contained a higher amount of low molecular weight components [41].

Table 2. Setting point and setting time of threadfin bream skin gelatin and commercial gelatin extracted at different time.

Gelatin	Setting point (°C)	Setting time at 10°C (min)	Setting time at 4°C (min)
6 hrs	18.96^{b} ± 0.666	12.87^{b} ± 0.602	8.63^{b} ± 0.379
12 hrs	14.83^{c} ± 0.702	16.74^{c} ± 0.310	11.19^{a} ± 0.380
Commercial	34.20^{a} ± 0.881	9.98 ^{a} ± 0.645	6.72^{c} ± 0.676

$^{a-c}$ Means ± standard deviation of triplicate determinations. Means in the same column with different superscript letters were significantly different ($p<0.05$).

E. Viscosity

Viscosity is the second most important commercial property of gelatin after gel strength [43]. Viscosity is the properties of fluids indicate resistance to flow [28]. Figure 1 shows that the viscosity decrease with increase in extraction time. It was observed that the 6 hrs gelatin are more viscous compared to 12 hrs gelatin. Threadfin bream skin gelatins started to gel at the range of 14 to 18°C. The gelatin extracted for 6 hrs gel quickly compared to 12 hrs gelatin. At 18°C, the viscosity of 6 hrs gelatin was 7.55cP while 12 hrs gelatin was 5.84cP. Commercial gelatin was more viscous (8.68cP) than both of the extracted gelatin. The physical properties of gelatin are influenced by the extraction condition which is the time, temperature and pH of extraction [31]. In this study, the viscosity of gelatin decreases significantly ($p<0.05$) with increase in extraction time. Large amount of and -chain have been shown to negatively affect some of the functional properties of fish gelatin such as reducing the viscosity, melting point and setting points and a longer setting time [12]. Gel strength, gelling point, melting point and viscosity are associated with the contents of hydroxyproline and proline in collagen of different species [44]. Based on Pranoto et al., [37], fish skins from yellowfin tuna, red snapper and white cheek shark pretreated with 0.05M acetic acid, followed by extraction at 80°C for 2 hrs gives viscosity range between 6.64 to 8.00 cP at room temperature. Jamilah and Harvinder [23] who extracted gelatin by soaking the skins in 0.2% (w/v) NaOH, followed by 0.2% sulfuric acid, 1% citric acid and a final extraction in water at 45°C for 12 hrs reported that black tilapia gelatin viscosity was doubled (7.12cP) when compared to red tilapia which was only 3.20 cP at room temperature.

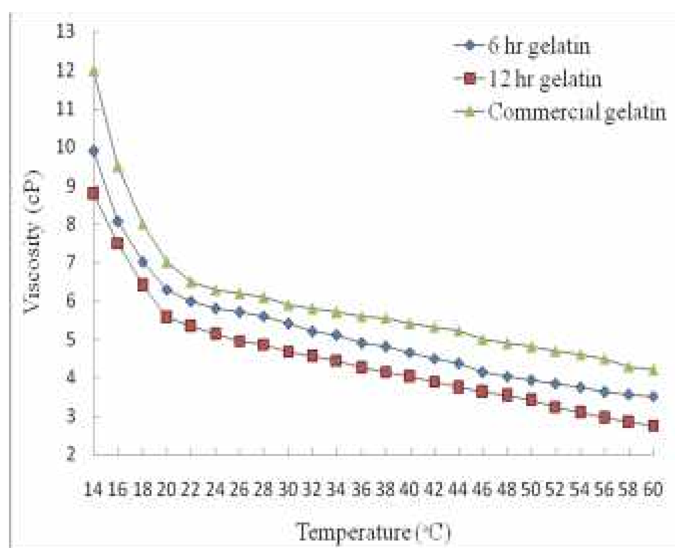

Figure 1. Viscosity of commercial and threadfin bream skin gelatin extracted at 6 and 12 hrs.

F. Solubility

Table 3 shows the solubility of threadfin bream skin gelatin extracted at 6 and 12 hrs. Solubility of threadfin bream skin gelatin increased with increase in extraction time. The gelatin extracted for 12 hrs had significantly ($p<0.05$) higher solubility compared to the 6 hrs. Commercial gelatin exhibited the highest solubility (64.5%). The stability of triple helical structure in denatured gelatin is proportional to the total content of imino acids (proline and hydroxyproline) [31]. Hydroxyproline via its hydrogen bonding property through its –OH group has a major role in the stabilization of triple helical structure of collagen [17]. As heating time increased, the rate of collagen break down increased and more soluble protein formed [17]. Sukkwai et al., [45] reported that hot water extraction breaks the triple helix structure producing smaller gelatin molecules and different extraction time slightly affected the degree of hydrolysis of gelatin.

Table 3. Solubility (%) of threadfin bream skin gelatin extracted at 6 and 12 hrs and commercial gelatin.

Gelatin	Solubility (%)
6 hrs	38.36 [c]± 0.611
12 hrs	55.37 [b]± 0.451
Commercial	64.50 [a]± 0.519

[a-c] Means ± standard deviation of triplicate determinations. Means in the same column with different superscript letters were significantly different (p<0.05).

G. *Molecular weight distribution*

Threadfin bream skin has a wide molecular weight bands approximately from 220 kDa to lower than 10 kDa (Figure 2). The extracted gelatins exhibited bands from 160 to more than 220 kDa. However, the bands of 6 hrs gelatin are more intense compared to the 12 hrs gelatin. Commercial gelatin showed bands with the same range, however the bands were very faint. The absence of bands less than 160 kDa indicates that protein below this range was hydrolyzed or denatured during the extraction process. Muyonga et al., [12] stated that the disappearance of low molecular weight bands might be due to the degradation of protein during the extraction process. The and -chains were observed in the extracted gelatin while for commercial gelatin only -chain is present. High molecular weight distribution gives negative effect on some of the functional properties of the gelatin such as lower gel strength and melting point [25]. Zhang et al., [46] stated that the content of - and -chain may be associated with the gel strength of gelatin. This may explain the 6 hrs gelatin with higher gel strength compared to 12 hrs. Gomez-Guillen and Montero [4] reported that the difference in gel strength, viscosity and melting point was explained based on the imino acid composition, the 1/ 2 collagen-chain ratio and the molecular weight distribution. Cho et al, [9] reported that viscosity is partially controlled by molecular weight and molecular size distribution. The presence of imino acid hydroxyproline has strong effect on viscosity of gelatin sample [33, 9].

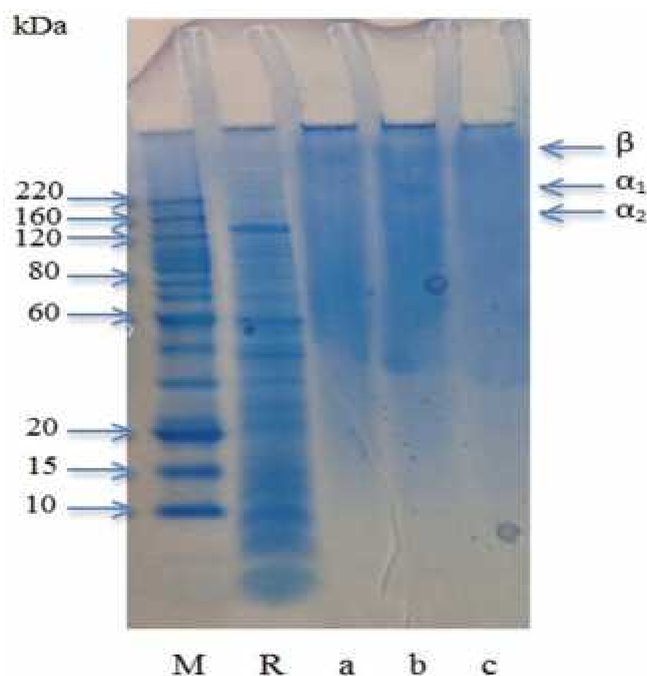

Figure 2. Electrophoretic profiles of threadfin bream skin gelatin. M) is protein marker, R) is raw threadfin bream skin, a) 12 hrs extracted gelatin, b) 6 hrs extracted gelatin and c) commercial gelatin.

IV. CONCLUSION

Gelatin was successfully extracted from threadfin bream skin by using alcalase at different time and compared with commercial gelatin. Longer extraction time resulted in gelatin with higher yield. Gelatin extracted for 12 hrs was more soluble than the 6 hrs. However, the 6 hrs extracted gelatin had higher gel strength, viscosity and melting point. The gel strength and melting point of gelatin extracted for 6 hrs exhibited properties close to the commercial gelatin. The 6 hrs gelatin set faster and at higher temperature. This shows that the gelatin extracted for 6 hrs are more suitable to be used in the food production than the 12 hrs gelatin.

REFERENCES

[1] [1] D. Golani, O. Sonin. J. Fish Bio. 2006; 68(3), 940-943.
[2] [2] Department of Fisheries Malaysia (2010). Performance and achievements of fisheries sectors. Malaysia Department of Fisheries.
[3] [3] M. T. Morrissey, J. Lin, A. Ismond. In Surimi and Surimi Seafood (2nd edition, Ed J. W. Park), Florida : CRC Press, Taylor and Fracnis Group, 2005; pp 279-323.
[4] [4] M. C. Gomez-Guillen, P. Montero. J. Fd Sci. 2001; 66, 213-216.
[5] [5] I. Kołodziejska, E. Skierka, M. Sadowska, W. Kołodziejska, C. Niecikowska. Fd. Chem. 2008; 107 (2), 700-706.
[6] [6] N. Muralidharan, B. Soottawat, P. Thummanoon, S. Ponusa, K. Hideki. Fd Hydrocolloids. 2012; 29 (2), 389 – 397.
[7] [7] I. Normah, S. Zainal, M.F. Nurul Fatihah. Sci. Lett. 2013a; 7 (1), 1-8.
[8] [8] F. Mahmoodani, V.S. Ardekani, S.F. See, S.M. Yusop, A.S. Babji. Sains Malaysiana. 2014; 43(7), 995–1002.
[9] [9] S.M. Cho, K.S. Kwak, D.C. Park, Y.S. Gu, C.I. Ji, D.H. Jang. Fd Hydrocolloids. 2004; 18(4), 573-579.
[10] [10] P. Montero, M.C. Gomez- Guillen. J. Fd. Sci. 2000; 65 (3), 434-438.
[11] [11] B. Gimenez, J. Turnay, M.A. Lizarbe, P. Montero, M.C. Gomez-Guillen. Fd Hydrocolloids. 2005;19 (6), 941-950.

[12] [12] J.H. Muyonga, G.B. Cole, K.G. Duodu. Fd. Hydrocolloids. 2004; 18, 581-592.

[13] [13] T. Yan, Y. Tiejin, Y. Fd. Agric. and Env. 2013; 11(1), 146-150.

[14] [14] S. Nalinanon, S. Benjakul, W. Visessanguan, H. Kishimura. Fd. Hydrocolloids. 2008; 22(4), 615-622.

[15] [15] I. Normah, S. Najihah, S. Zainal. Scientific Res. J. 2013b; 10(1).

[16] [16] P.K. Binsi, B.A. Shamasundara, A.O. Dileepa, F. Badii, N.K. Howell. Fd. Hydrocolloids. 2009; 23, 132-145.

[17] [17] M.C. Gomez-Guillen, J. Turnay, M.D. Fernandez-Diaz, N. Ulmo, M.A. Lizarbe, P. Montero. Fd. Hydrocolloids. 2002; 16, 25-34.

[18] [18] S.F. See, P.K. Hong, K.L. Ng, W.M. Wan Aida, A.S. Babji. Int. Fd. Res. J. 2010; 17, 809-816.

[19] [19] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, T. Nagai, M. Tanaka. Fd. Chem. 2005; 89, 363-372.

[20] [20] I. Normah, M.S. Siti Hafsah, A. Nurul Izzaira. Int. Fd. Res. J. 2013c; 20(5), 2261-2268.

[21] [21] SAS Institute Inc. 2004. SAS user's guide to the statistical analysis system. SAS Institute Inc. Raleigh, NC.

[22] [22] A.M. Abdalbasit, F.A. Hadia. Acta Scientiarum Polonorum Technologia Alimentaria*,* 2013; 89(3), 185-193.

[23] [23] B. Jamilah, K.G. Harvinder. Fd Chem. 2002; 77, 81 – 84.

[24] [24] J.M. Koli, S. Basu, B. B. Nayak, S. B., Patange, A.U. Pagarkar, V. Gudipati. Fd Bioprod. Proc. 2012; 90(3), 555-562.

[25] [25] A.A. Karim, R. Bhat. Fd Hydrocolloids. 2009; 23(3), 563-576.

[26] [26] V.C. Killekar, J.M. Koli, S.T. Sharangdhar, S. Y. Metar. Indian J. Fundamental Applied Life Sci. 2012; 2(3), 106-116.

[27] [27] H.W. Ockerman, C.L. Hansen. New York: Ellis Horwood 1988; 133-157.

[28] [28] M. Gudmundsson. J. Fd. Sci. 2002; 67 (6), 2172-2176.

[29] [29] M.H. Norziah, A. Al-Hassan, A. B. Khairulnizam, M.N. Mordi, M. Norita. Fd. Hydrocolloids. 2009; 23, 1610-1616.

[30] [30] N.K. Te. Adv. Polymer Sci. 1997; 130, 1-267.

[31] [31] M.C. Gomez-Guillen, B. Gimenez, M.E. Lopez-Caballero, M. P. Montero. Fd. Hydrocolloids. 2011; 25, 1813-1827.

[32] [32] R. Intarasirisawat, S. Benjakul, W. Visessaguan, T. Prodpran, M. Tanaka, N.K. Howell. Fd Hydrocolloids. 2007; 21, 537-544.

[33] [33] A.L. Sarabia, M.C. Gomez-Guillen, P. Montero. Fd. Chem. 2000; 70, 71-76.

[34] [34] D.W.S. Wong. 1989; Van Nostrand Reinhold, New York.

[35] [35] A. Shyni, G.S. Hema, G. Ninan, S. Mathew, C.G Joshy, P.T. Lakshmanan. Fd. Hydrocolloid, 39, 68-76.

[36] [36] S.S. Choi, J.M. Regenstein. J. Fd. Sci. 2000; 65, 194-199.

[37] [37] Y. Pranoto, D.W. Marseno, H. Rahmawati. Int. Fd. Res. J. 2011; 18(4), 1335-1342.

[38] [38] P.M. Gilsenan, S.B. Ross Murphy. Fd. Hydrocolloids. 2000; 14, 191-195.

[39] [39] N.F. Mohtar, C. Perera, S. Queck. Fd. Chem. 2010; 122(1), 307-313.

[40] [40] I.J. Haug, K.I. Drajet, O. Smidsrød. Fd. Hydrocolloids. 2004; 18 (2), 203-213.

[41] [41] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, F. Shahidi. Fd Hydrocolloids. 2010; 24, 164-171.

[42] [42] P. Kaewruang, S. Benjakul, T. Prodpran. Fd. Hydrocolloids. 2014; 35, 694-699.

[43] [43] A.G. Ward, A. Courts. London: Academic Press. 1997.

[44] [44] K. Ilona, K. Kaczorowski, B. Piotrowska, M. Sadowaska. Fd. Chem. 2004; 86, 203-209.

[45] [45] S. Sukkwai, K. Kijroongrojana, S. Benjakul. Int. Fd. Res. J. 2011; 18(3), 1129-1134.

[46] [46] F. Zhang, S. Xu, Z. Wang. Fd. Bioprod. Proc. 2011; 89(3), 185-193