Functional Properties of Gelatin Hydrolysate from Salmon Skin (*Salmo salar*)

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Abstract: Gelatin hydrolysate is derived from a hydrolysis of gelatin (denatured collagen) to expose the functional properties of the hydrolysate obtained. This study was carried out to determine functional properties of gelatin hydrolysate from salmon skin as influenced by the degree of enzymatic hydrolysis. Aqueous extraction of gelatin from salmon skin was done at 45°C for 60 minutes. The extracted gelatin was then hydrolysed using 1% alcalase at 54.5°C and pH 8. The functional properties determined were molecular weight, solubility, foaming capacity and stability, emulsifying activity and stability index were analysed. It was found that different time of hydrolysis (5, 15, 45 and 180 minutes) resulted in different degree of hydrolysis (DH) (10, 20, 30 and 40%, respectively). The gelatin hydrolysate at 40% DH showed the highest solubility ($p<0.05$). However, the foaming and emulsifying properties were the highest at 10% degree of hydrolysis ($p<0.05$).

Keywords: Gelatin hydrolysate, salmon skin, foaming properties, emulsifying properties.

1. INTRODUCTION

According to Food and Agriculture Organization (FAO) in 2006, more than 132 million tonnes of fish was captured every year around the world. From 70-85% wastes of the processed fish, 30% of it was skin and bones [1]. Salmon is a healthy source of food protein, and rich in omega-3 fatty acid, vitamin D and good cholesterol [2]. Increasing demand and consumption of salmon in the form of value added product (sushi, sashimi and steak) resulted in salmon skin as waste product.

Interest of recycling the wastes of processed fish is due to a high value of nutrition, a dynamic ability and also have a potential to be used in food system as a binder, an emulsion and a gelling agent. With the rising of technology, these wastes have been processed producing a high quality product. Collagen in bones and skin of animals can produce a high quality gelatin product after denaturation process [3]. Gelatin hydrolysate is produced due to its bioactive peptide which can potentially act as a modulation of physical metabolism, alternative used of fish gelatin [4].

Functional protein's ability can be altered by physical, chemical or enzymatic hydrolysis through changes of protein structure. Enzymatic hydrolysis is much preferred due to average processed condition requirement, easily controlled reaction and yield of wastes is minimum [5]. Enzyme also possess a critical point by affecting physicochemical, functional and sensory properties [1]. In hydrolysis process, the breakage of peptide bond will increase concentration of hydrolysates, free amino and carboxyl group of amino acid which may contribute to functional protein properties [1, 5, 6]. The properties involved include protein solubility, water holding capacity, oil holding capacity, emulsion and foaming characteristics [1].

Over the years, many studies have been done on sardine [7], capelin [8], shark protein [9], herring [10], salmon protein [11], soy protein [12] and whey protein [13] which proved that enzymatic hydrolysis had increased functional properties of protein. Research on gelatin hydrolysate from salmon skin at different degree of hydrolysis could be one of an interesting subject to be further studied. Therefore, this study aimed to produce gelatin hydrolysates from salmon skin at different degree of hydrolysis and study the effects on solubility, foaming and emulsion properties.

2. MATERIALS AND METHODS

2.1. Materials

Salmon skin was obtained in the frozen state from sashimi supplier in Penang, Malaysia. Chemicals used were of analytical grade, purchased from Sigma-Aldrich US.

2.2. Gelatin Preparation

Gelatin extraction from salmon skin was carried out according to Kolodziesjka *et al*. method [14]. The fish skin was cut into small pieces and immersed in 0.45 N NaCl solution with ratio 1:6 (w/v) at 4°C for 6 minutes.

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The sample was then washed with distilled water. Immersion and washing steps were repeated twice. Sample was filtered with cheese cloth. The skin cake was added with distilled water at ratio 1:10 (w/v) and incubated in waterbath shaker at 45°C, 200 rpm for 60 minutes. The sample was then centrifuged at 10000*xg*, 15°C for 15 minutes. The supernatant was concentrated using a rotary evaporator, then was freeze dried to be keep for further analysis.

2.3. Gelatin Hydrolysate Preparation

Gelatin hydrolysate preparation was done according to Yang *et al*. method [15]. Sample of freeze dried gelatin weighing 2 g was dissolved in 200 ml 0.1 M sodium phosphate buffer (pH 9.3). The sample was incubated in waterbath at 45°C for 5 minutes to dissolve gelatin. The pH was adjusted to 7.8 and 1% alcalase enzyme was added to the sample, then incubated in 54.5°C, 100 rpm for 6 hours. The enzyme was inactivated by heat at 95°C for 10 minutes. The sample was then freeze dried to prepare gelatin hydrolysate's powder for further analyses. Isolated soy protein (ISP) and extracted gelatin without hydrolysation (G_o) were used as control samples.

2.4. Degree of Hydrolysis Measurement [16]

Hydrolysed gelatin solution was prepared by mixing gelatin hydrolysate powder with distilled water (1mg/ml). Then, 0.5 ml of the solution was mixed with 2.0 ml 1% sodium dodecyl sulphate (SDS). From the mixture, 0.125 ml was mixed with 2.0 ml sodium phosphate buffer (0.2 M, pH 8.2) and 1.0 ml of 0.01% TNBS solution. This mixture was then in a water bath at 50°C for 30 minutes. The reaction was terminated by adding 2.0 ml of 0.1 N sodium sulfite and allowed to cool at room temperature for 30 minutes. The degree of hydrolysis (DH) was determined using spectrophotometer (Shimadzu, Model UV-2450) at 420 nm. DH was calculated by using the following formula:

$$DH\ (\%) = 100 \times (A_{sample} - A_{control}/A_{TGH} - A_{control})$$

Where,

A_{sample}: sample absorption; $A_{control}$: control absorption;

A_{TGH}: total hydrolysed gelatin absorption

2.5. Molecular Weight Determination

Molecular weights of extracted gelatin and gelatin hydrolysates were determined using SDS-PAGE. The analysis was done in two parts, glycine and tricine system. The glysin system was done to measure the molecular weight of a wide range of sizes while the tricine system was to measure the molecular weight of polypeptide. SDS-PAGE was performed following the method described by Laemmli [17]. In glycine system, 12% (w/v) separating gel and 4% (w/v) stacking gel were used. In tricine system, 16% (w/v) separating gel and 5% (w/v) stacking gel were used.

2.6. Protein Content Determination

Protein content was determined using Kjeldahl method [18]. Approximately 1 g of each sample was digested using 12 ml of concentrated sulfuric acid, 7 g of potassium sulfate and copper in a digestion flask. The mixtures was then heated at 350°C-400°C until white smoke appeared 60-90 minutes. The flask was cooled and added with 75 ml distilled water. The mixture was then neutralised using 40% sodium hydroxide solution. Nitrogen in the form of ammonia was separated from the digestion mixture by distillation and the ammonia released was steam-trapped with 15 ml 4% boric acid containing methyl bromochrome red and green. The solution was titrated with 0.1 N HCl until the end point. Calculation of nitrogen content is as follows:

$$\text{mg of N / g of sample} = \frac{\text{* HCl (ml) xN standard acid x } 0.014}{\text{sample weight}} \times 1000$$

*mL of hydrochloric acid required for titrating sample solution

Protein (%) of sample = N (mg) x 6.25 x 100

2.7. Functional Properties Analysis

2.7.1. Protein Solubility

Protein solubility was determined according to Klompong *et al*. method [19] by dissolving 0.125 g sample in 25 ml of deionized water. pH was adjusted to pH 3, 5, 7 and 9 with 1 N HCl and 1 N NaOH. Each mixture was stirred at room temperature for 30 minutes, then centrifuged at 15000 rpm for 15 minutes. Protein content in the supernatant was determined by Kjeldahl method [18]. The protein solubility was determined using the following formula:

Protein solubility (%) = (protein content in supernatant / protein content in the sample) x 100

2.7.2. Foaming Capacity and Stability

Foaming capacity and stability of gelatin hydrolysates at different degrees of hydrolysis (10, 20, 30 and 40%) and ph (3, 5, 7 and 9) were determined by the method of Shahidi *et al*. [8]. The samples were prepared at 0.5% (w/v) using distilled water and homogenized (Ultra Turrax Ika T 25) at 16000 rpm for 1 minute. The total volume of foam was determined after 0, 0.5, 1, 5 and 10 minutes. Foaming capacity was calculated by the formula below:

Foaming capacity (%) = (A-B) / B x 100

Where,

A = volume after foaming

B = volume before foaming

Foaming stability (%) = (A-B) / B x 100

Where,

A = volume after foaming were placed for 30 minutes

B = volume before foaming

2.7.3. Emulsification Properties

Emulsifying properties were determined by Pearce and Kinsella method [20]. Approximately 0.15 g of each sample was dissolved in 30 ml distilled water and 10 ml of soy oil. pH of the mixture was adjusted to pH 3, 5, 7 and 9. The mixture was homogenized at 20000 rpm for 1 minute. Then, 50 µl of the total mixture was pipetted out at 0 and 10 minutes, mixed with 5 ml 0.1% SDS and the absorbance was measured at 500 nm using a spectrophotometer (Shimadzu, Model UV-2450).

Emulsifying activity index (EAI) (m^2 / g) = (2 x 2303 x A) / (0.25 x protein weight, g)

Where,

A = absorption at 500 nm

Emulsification stability index (ESI) (min) = $(A_o \times \Delta_t)/\Delta_A$

Where,

Δ_t = 10 minutes

Δ_A = A_o - A_{10}, absorption after 10 minutes and 0 minutes at 500 nm.

2.8. Statistical Analysis

All data were analyzed using SAS software (version 6.12) using one-way Analysis of Variance (ANOVA) and Duncan Multiple Range Difference Test to determine whether there were significant differences between the samples. Significant level was set at $p < 0.05$. All experiments were carried out in triplicates.

3. RESULTS AND DISCUSSION

3.1. Degree of Hydrolysis

Based on preliminary data (results not shown), it was found that hydrolysates of salmon skin gelatin with different degrees of hydrolysis, 10, 20, 30 and 40% were produced at 5, 15, 45 and 180 minutes,

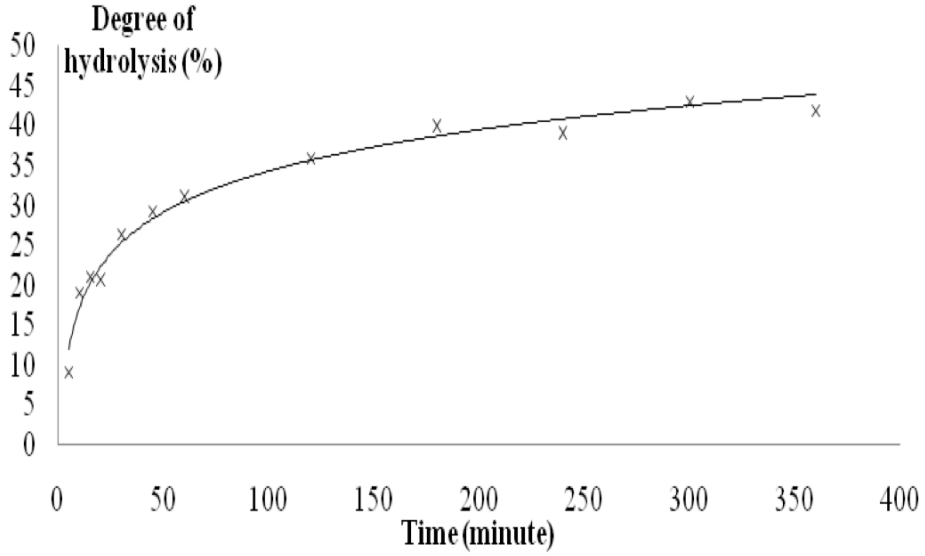

Figure 1: Degree of hydrolysis of salmon skin gelatin hydrolysate for 6 hours.

respectively. Figure **1** shows the hydrolysis curve of gelatin that exhibited typical curves. The profile of the degree of hydrolysis obtained from this study is similar to the classical hydrolysis of proteins induced by protease [21].

3.2. Molecular Weight

Based on the results of glycine system, molecular weight of all gelatin hydrolysates were less than 21.5 kDa regardless the degree of hydrolysis. Thus, SDS-PAGE tricine system was used to determine the molecular weights of the hydrolysates. Figure **2** showed the molecular weight distribution from tricine system. It was found that hydrolysed gelatin at 10,20 and 30% DH was in the range of 26.6-6.5, 17.0-6.5 and 14.4-6.5 kDa, respectively. However, at 40% DH, there was no clear band observed, probably due to complete hydrolysis of the gelatin.

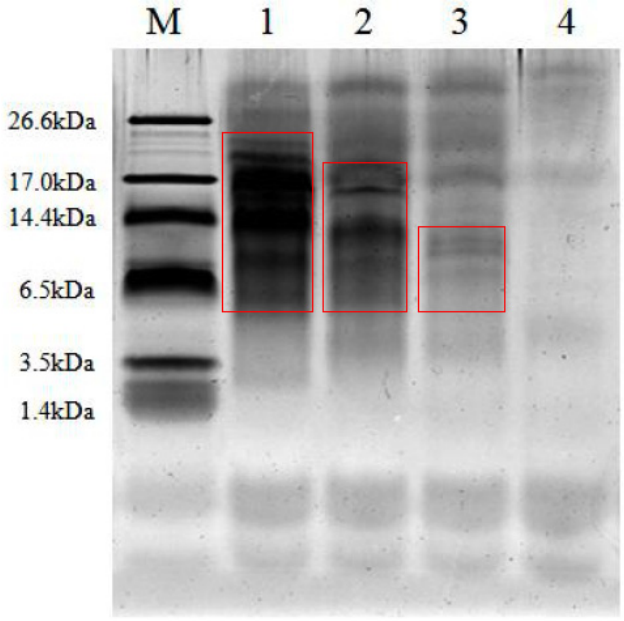

Figure 2: SDS-PAGE trisin system. M: standard; 1: D_H10%; 2: D_H20%; 3: D_H30%; 4: D_H40%.

According to Gbogouri *et al*. [21], the low molecular weight of peptide at high DH have higher peptides polarity. Subsequently, it forms strong hydrogen bonding with water molecule which makes the peptides more soluble, compared to polypeptides with higher molecular weight at low DH. For foaming activity, the presence of a surface active species (surfactant) that adsorbs at the bubble surface reduces the surface energy or interfacial tension, necessary for the formation of foam [22], [23]. Low molecular weight of peptides adsorb at the air–water interface rapidly in comparison to much larger protein molecules. For a protein to have good emulsion property, it has to have low molecular weight, a balance charge of amino acid composition and high solubility properties [24].

3.3. Solubility of Protein

It was observed that gelatin hydrolysates with different degree of hydrolysis display protein solubility from 20% and above. Gelatin hydrolysates with different degree of hydrolysis had higher protein solubility compared to isolated soy protein (ISP). Gbogouri *et al*. [21] showed that salmon hydrolysates has higher solubility with higher degree of hydrolysis, which is similar as the results obtained in this study. At higher degree of hydrolysis, the peptides have a higher polarity and can form stronger hydrogen bonds with water when compared to the polypeptide present in the gelatin or hydrolysates with low degree of hydrolysis. It was found that the hydrolysed gelatin has the lowest protein solubility at pH 5 ($p < 0.05$) (Figure **3**). These data indicate that hydrolysed gelatin with high molecular weight will precipitate at pH approaching the isoelectric point (pI) after the hydrolysis of protein. At pH values below or above pI of the protein the solubility increases due to repelling of the positive or negative ions, as well as due to increased interaction of the charged polypeptide chains with water dipoles [25]. Protein solubility plays an important role in the functional properties of gelatin hydrolysates such as foaming and emulsification properties due to the rapid migration and adsorption of peptides between the surfaces [26].

3.4. Foaming Capacity and Stability

Protein hydrolysates led to a decrease in surface tension between water and air space, causing the existence of foaming capacity [9]. Foaming capacity and foaming stability of gelatin hydrolysates at different degrees of hydrolysis was lower than ISP and gelatin, ($p < 0.05$) as shown in Figures **4** and **5**, respectively. It was found that hydrolysed gelatin with higher degree of hydrolysis has lower foaming capacity. Gelatin with 10 and 20% degree of hydrolysis showed foaming activity and stability but the foam start to decrease at 30 and 40% of degree of hydrolysis. Klompong *et al*. [19] reported that the foaming capacity for protein hydrolysates from capelin fish is also less at high degree of hydrolysis. This phenomenon is caused by the lack of alignment of small peptides on the surface of water and air [27]. According to Zhu and Damodaran [28], the addition of ions such as Ca^{2+} or Mg^{2+} might cause unfolding and polymerization of the proteins at

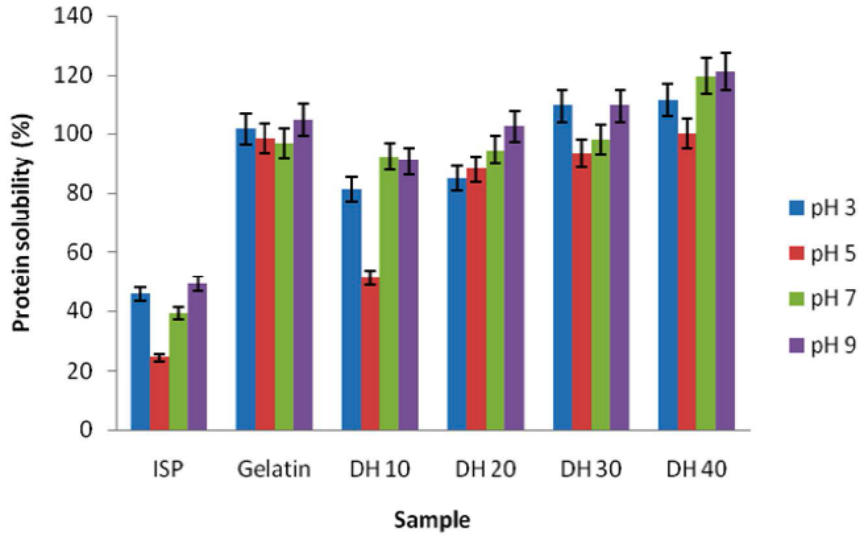

Figure 3: Protein solubility for gelatin hydrolysate at 10-40% degree of hydrolysis, pH 3-9.

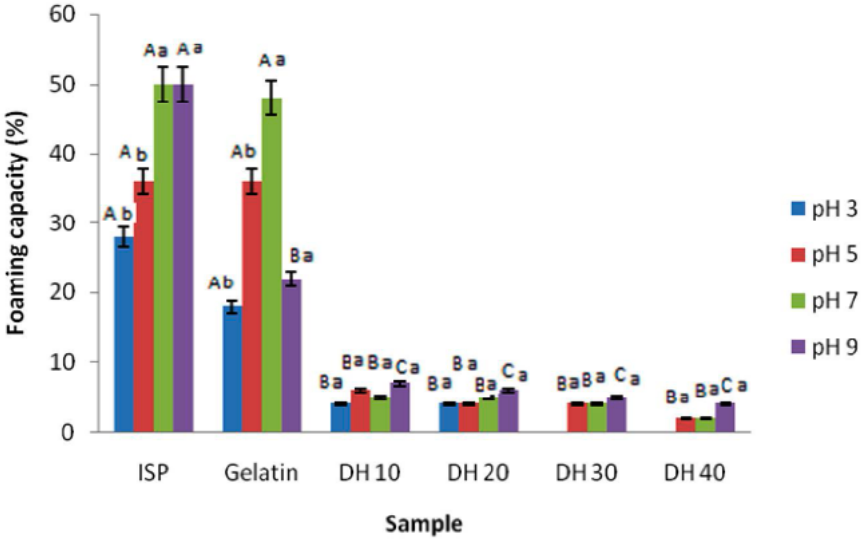

Figure 4: Foaming capacity at 10 minutes for gelatin hydrolysate, 10-40% degree of hydrolysis, pH 3-9.
Different capital letters indicate significant differences (P < 0.05) of hydrolysates with different sample.
Different lowercase letters indicate significant differences (P < 0.05) of hydrolysates with different pH.

the interface *via* ionic linkages so that the foaming can be improved.

Foaming capacity was left for 30 minutes to observe the foaming stability. The stability and strength of the foam, measured by the rate of drainage and the resistance to compression, respectively, depend on the flexibility and the rheological properties of the film. The results show gelatin hydrolysates at high degrees of hydrolysis has low foaming stability. Foaming stability is dependent on the nature of the film and limit the interaction between proteins in the matrix. Foaming stability can be enhanced by flexible proteins that increase protein concentration and film thickness [29]. Thus, gelatin hydrolysates with a low molecular weight are unable to maintain proper orientation of molecules on the surface.

3.5. Emulsification Properties

Emulsifying activity index (EAI) and emulsifying stability index (ESI) gelatin hydrolysate produced by enzymatic hydrolysis alcalase at different degrees at pH (3-9) are shown in Figures **6** and **7**, respectively. Both are determined based on emulsion turbidity at a wavelength of 500 nm [20]. Hydrolysed gelatin with a high degree of hydrolysis has low EAI and ESI due to the size of small peptides (p <0.05). According to

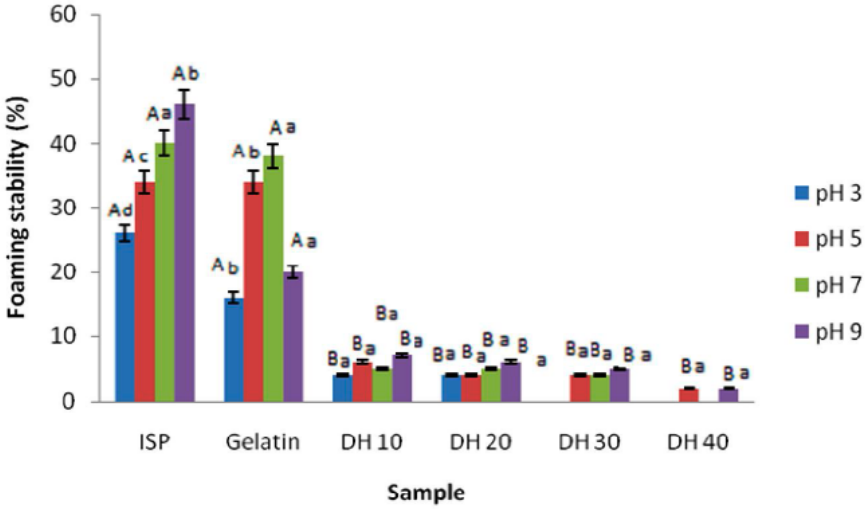

Figure 5: Foaming stability for gelatin hydrolysate at 10-40% degree of hydrolysis, pH 3-9.
Different capital letters indicate significant differences (P < 0.05) of hydrolysates with different sample.
Different lowercase letters indicate significant differences (P < 0.05) of hydrolysates with different pH.

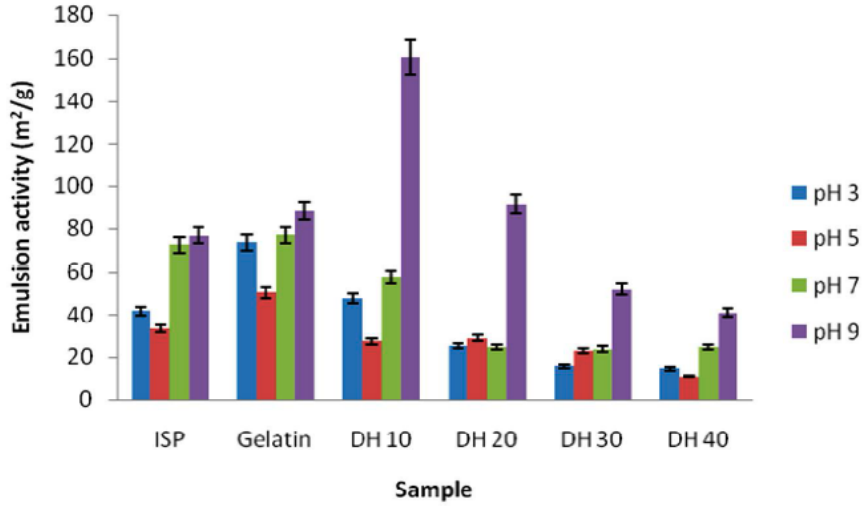

Figure 6: Emulsification activity index for gelatin hydrolysate at 10-40% degree of hydrolysis, pH 3-9.

Klompong *et al*. [19], protein hydrolysates from the yellow stripe fish showed lower both EAI and ESI properties when the degree of hydrolysis increases. At a lower degree of hydrolysis of 10%, hydrolysed gelatin showed strong emulsifying properties compared to the degree of hydrolysis 40% ($p < 0.05$).

The peptides with higher molecular weight or more hydrophobic will contribute to the stability of emulsification [30]. EAI and ESI values increased when the pH is away from pH 5. These effects result from amino acid sequence and composition of the various charges which was brought at a certain pH. The efficiency of proteins as emulsifiers depends upon their surface hydrophobicity and charge, stearic effects, elasticity or rigidity, and viscosity in solution. The pH of the environment affects the emulsifying properties by changing the solubility and surface hydrophobicity of proteins, as well as the charge of the protective layer around the lipid globules. Ions may alter the electrostatic interactions, conformation, and solubility of the proteins [25].

CONCLUSION

High solubility of protein hydrolysates obtained showed a potential on functional properties of gelatin hydrolysate. Gelatin hydrolysates from salmon skin demonstrated lower foaming ability compared to ISP (R) and gelatin (C). Higher DH resulted in lower foaming stability. Gelatin hydrolysate produced at lower DH is more suitable for emulsifying properties,

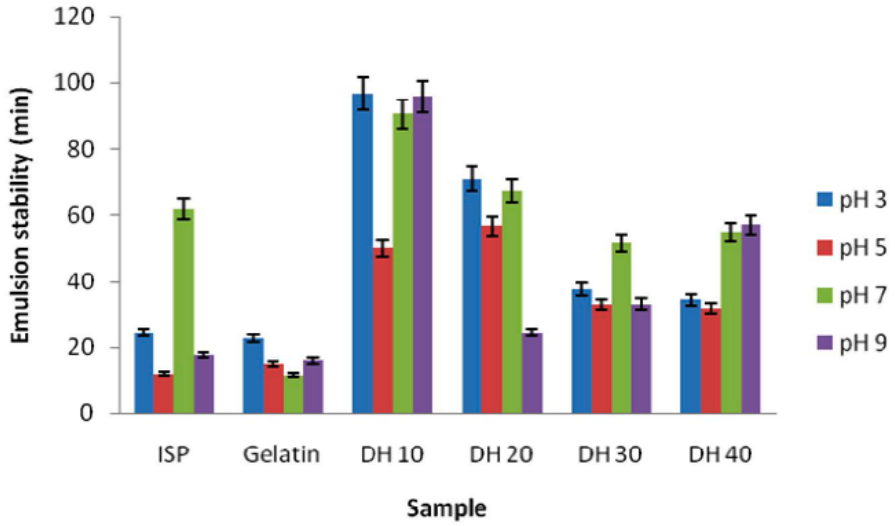

Figure 7: Emulsification stability index for gelatin hydrolysate at 10-40% degree of hydrolysis, pH 3-9.

compared to the higher DH. Gelatin and gelatin hydrolysates at lower DH illustrated the potential of peptide surfactants in improvement of foaming and emulsifying properties. Foaming is responsible for desirable rheological properties of many foods, such as texture of bread, cakes, whipped cream and ice cream. Emulsifying properties of protein is important for food products, mainly like comminuted meat batters, to form a continuous phase of emulsion. This new foaming and emulsifying agents may contribute towards improvement of food ingredients and food products.

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