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Low Molecular Weight Collagen from Tilapia Fish Scales for Potential Cosmetic Application

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ABSTRACT

Low Molecular Weight (LMW) of collagen or hydrolyzed collagen is widely used in cosmetic industries due to high efficient penetration into skin and easily distributed in the human body. In this study, collagen from Tilapia (Oreochromis niloticus) fish scales was extracted and hydrolyzed using enzymatic hydrolysis technique. Gel Permeation Chromatography (GPC) analysis revealed that the molecular weight of extracted collagen, 12.5 kDa significantly decreased to 1.3 kDa with narrow distribution when hydrolyzed with 0.5 wt.% of alcalase enzyme. The effect of enzyme treatment on the pH, degree of hydrolysis, functional group, viscosity and amino acid content of collagen were studied using pH measurement, Kjeldahl method, Fourier Transform Infrared (FTIR), viscosity measurement and amino acid profile analysis, respectively. FTIR and amino acid analysis revealed that the functional groups were found unchanged and the profile content of amino acids in hydrolyzed collagen were similar to untreated collagen. The LMW of collagen prepared from Tilapia fish scales will be suitable for cosmetic ingredient.

Keywords: Collagen, Tilapia, Fish scales, Enzymatic hydrolysis

INTRODUCTION

Currently, collagen has become a valuable and well used component in cosmetic and beauty product as it can provide significant benefit to the skin [1]. Collagen is a natural protein that contributes to great skin strength and elasticity [2]. As one ages, the fibroblasts activities start to slow down in producing new collagen fibres in the body. Recent research showed that in cultured human dermal fibroblast, the addition of collagen-derived dipeptide, proline-hydrxyproline, was proved to stimulate the fibroblast cells to produce collagen matrix [3]. In addition, Shigemura et al. [4] proved that the existence of collagen dipeptides can stimulate directly fibroblasts in the mouse skin, led to a more efficient collagen synthesis in the dermis. Therefore, daily intake of hydrolyzed Type I collagen can improve skin moisture content and elasticity [5].

The main source for the world collagen is derived from pig skin which leads the highest about 46%, followed by bovine skin (29.4%), bones (23.1%), and other sources (1.5%) [6]. However, the outbreak of Bovine Spongiform Encephalopathy (BSE) and the spreading Foot-Mouth Disease (FMD) has caused considerations on the safety of using collagen extracted from animals especially pig and cow [7,8]. This problem has encouraged researcher to put great effort in the extraction of collagen from marine sources. It includes jellyfish, octopus, starfish, crab [9], fish skin and scales of all kinds [10,11]. Marine based collagen can be a potential alternative source as it is safe from any dangerous prion or animal virus. Huda et al. [12] reported that fish and fish products has been found to be safe as it does not contain toxins and poisons. The usage of fish scales to derive collagen will not only reduce pollution but may reclaim fish waste to high value added materials. Fish muscles and scales contains 0.2-10% of Type I collagen [13]. Tilapia fish scales can be one of the best alternative sources for collagen production as they consist of abundant Type I collagen fibre [14]. Sankar et al. [11] stated that the amounts of protein in fish scales are ranges from 41-84%. Therefore, the high amounts of crude protein (16-25%) and very low in fat (0.5-3.0%) of an adult Tilapia species [15] have reason to be chosen in this study.

However, the large molecular weight of collagen (300 kDa) [16] makes it impossible to be readily absorbed into the skin [17]. Therefore, the molecular weight of collagen should be reduced by producing collagen peptides, so that it can be absorbed easily. Variety of methods are used by researchers in producing low molecular weight of collagen such as direct thermal treatment, acidic or alkaline treatment, enzymatic treatment [18] and microwave assisted hydrolysis [19]. Hydrolyzed collagen is low molecular weight individual collagen strands that are broken down from triple-helical structure of collagen. Most researchers have succeeded in synthesizing hydrolyzed collagen with a molecular weight below 3000 Da [10,19,20]. Nevertheless, the production of the molecular weight distribution is also depending on the hydrolysis method. Chemical method such as acid and alkaline hydrolysis tends to reduce the amount of amino acid composition of the starting material and difficult process to control [18]. Therefore, enzymatic hydrolysis is preferable than the other methods as it could control the resulting molecular weight of hydrolyzed collagen.

Preparing low molecular weight of collagen with narrow molecular weight distribution is important to ensure the effectiveness of collagen absorption through the skin. It is reported that the enzymatic hydrolysis process of collagen from Tilapia skin produced peptides with average molecular weight below 1700 Da compared to thermal hydrolysis, above 3000 Da [21]. In addition, enzymatic hydrolysis is the most appropriate method for the preparation of tailor-made peptides [22].

In the present study, Tilapia fish scales collagen was hydrolyzed with Alcalase 2.4L, is a food-grade enzyme preparation that was produced by a selected strain of *Bacillus licheniformis*. Alcalase enzyme was used by most researchers in enzymatic hydrolysis [10,20,21] of protein as it has a high degree of hydrolysis reaction rate [22]. Furthermore, alcalase has been reported to be one of the highly efficient on hydrolyzing fish protein based on enzyme cost per activity [23]. It is inexpensive and it used would be economical. Though numerous studies have reported on hydrolysis of fish collagen, only few of them have been focusing on hydrolyzing fish scale collagen using enzymatic technique. Therefore, the main objective of this study is to prepare hydrolyzed collagen from Tilapia fish scales using Alcalase and characterize the suitability of LMW fish scale collagen for potential cosmetic application.

EXPERIMENTAL

Materials

Tilapia fish scales (*Oreochromis niloticus*) were obtained from the local market at Tanjung Malim, Perak (Malaysia) whereas Alcalase 2.4L (declared activity of 2.4 AU/kg and density of 1.18 g/ml), an endoproteinase from *B. licheniformis*, was purchased from Brentag specialities, (USA). Collagen extraction was prepared by using deionized water. All reagents used were of analytical grade.

Methods

Extraction of collagen from Tilapia fish scales

Collagen was extracted from Tilapia (*O. niloticus*) fish scales according to the method of Zainol et al. using hydrothermal method [24]. The fish scales were collected and washed several times with deionized water to remove dirt. This process is repeated until the fish scales were cleaned before being dried. The dried fish scales were ground into powder using domestic grinder, Panasonic MX-337. One hundred grams of fish scales powder was mixed with 1000 ml of deionized water and extracted hydrothermally at 80°C for 8 h.

Enzymatic hydrolysis

After 8 h of extraction, the pH value of the mixture was measured and further hydrolyzed with different concentrations of Alcalase 2.4 L, i.e., 0.5, 1.0, 1.5 and 2.0 wt.%. The mixture was hydrolyzed at 55°C which is the optimum temperature for Alcalase [21,25]. The hydrolysis process was monitored for 60 min with continuous stirring. The resulting hydrolyzed collagen solution was filtered and heated at 90°C for 5 min to deactivate the alcalase enzyme. The hydrolysate was allowed to cool down and 50 ml of solution was removed from each sample to measure the pH value using pH meter (Thermo Scientific Orion 2-star benchtop pH meter, Cole-Parmer Instruments, USA) at room temperature. Then, the remaining samples were frozen and freeze-dried for 72 h by using freeze dryer (Freeze Dryer Scanvac Labogene Cool Safe 95-15, Denmark) without further purification.

Characterizations

Molecular weight analysis

The average molecular weight of collagen and hydrolyzed collagen were measured by using Gel Permeation Chromatography (GPC), model HLC-8320GPC equipped with a UV, dual flow Refractive Index (RI) detector and TSKgel GMPWXL column. The UV absorbance was monitored at a wavelength of 248 nm. The solvent consisted of 0.2 M $NaNO_3$ and acetonitrile (9:1) was maintained at a flow rate of 1 ml/min. The standards PEG 400, PEG 7400 and polyethylene oxide (molecular weight 27000, 44900, 101000, 146000, 394000 and 580000) were used to construct a calibration curve. All the results obtained were recorded using the GPC Workstation EcoSEC-WS software GPC, version 1.08.

Degree of hydrolysis (DH)

DH was calculated according to the percentage of Trichloroacetic Acid (TCA) ratio method as described by Hoyle and Merritt [26]. After enzymatic hydrolysis, 20 ml of hydrolyzed collagen solution was added to 20 ml of 20% (w/v) TCA to produce 10% TCA soluble material. The mixtures were centrifuged at 7800 g for 15 min. The supernatant was analyzed for the protein content by using Kjeldahl method (AOAC 16th Edi.981.10). The DH was calculated using the Eqn. 1:

$$DH\ (\%) = \frac{10\ \%\ TCA\ soluble\ nitrogen\ in\ the\ sample}{Total\ nitrogen\ in\ the\ sample} \times 100\ \% \qquad (1)$$

Fourier Transform Infrared (FTIR) analysis

FTIR analysis (Thermo Nicolet 6700, USA) was conducted to characterize and compare the functional group in Tilapia fish scales and extracted collagen (untreated collagen). The presence of collagen functional groups such as amide A, amide I, amide II and amide III in collagen (0 wt.% Alcalase) and hydrolyzed collagen at different concentrations of Alcalase, 0.5, 1.0, 1.5 and 2.0 wt.% were also determined. The samples were directly scanned from 500-4000 cm^{-1} using a diamond tip Horizontal Attenuated Total Reflectance (HATR) accessories.

Viscosity measurement

The viscosity of collagen and hydrolyzed collagen with 0.5, 1.0, 1.5 and 2.0 wt.% of enzymes were determined using a computerized Brookfield digital viscometer (Model DV-II) together with spindle LV 2 at 30 rpm. Collagen solution of 5% (w/v) was prepared by dissolving the collagen powder in deionized water at room temperature. The viscosity of sample was monitored continuously at 25°C for 15 min.

Amino acid profile analysis

The amount of amino acid contents in the collagen and hydrolyzed collagen were analyzed by High Performance Liquid Chromatography (HPLC) Alliance 2695 (Waters, USA), equipped with 2475 Multi wavelength Fluorescence Detector and AccQ. Tag Column (3.9 × 150 mm), respectively. Amino acid compositions were determined as gram (g) amino acid per 100 g protein.

RESULTS AND DISCUSSION

According to Kristinsson and Rasco [22], the effective parameter for the pH to extract proteins from Mackerel Fish waste by using alcalase was 7.5. However, Chabeaud et al. [27] reported that the optimal working pH range of Alcalase was 8 to 10. All these pH ranges were agreed as the Alcalase is an alkaline protease. In this hydrolysis study, the pH of the mixture was not adjusted to desired value. The collagen was enzymatic hydrolyzed using Alcalase under working pH of the mixture near to seven (pH≈7). The neutral medium in preparation of sample is suitable and safe for topical application especially in cosmetic industry.

In addition, the average pH values of hydrolyzed collagen prepared using different concentration of alcalase (i.e., 0.5, 1.0, 1.5 and 2.0 wt%.) are between 6.15-6.58 (Figure 1). This pH range is within the level of being buffered by the skin [28]. In addition, the pH of a product for skin application is required to be in accord with the pH of skin in order to ensure that the product will be well tolerated [29]. Therefore, the resulting pH of hydrolyzed collagen prepared in this study will be suitable to be applied at human skin.

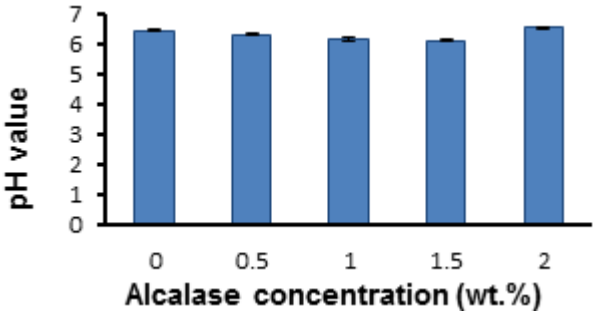

Figure 1: pH value of hydrolyzed collagen prepared at different Alcalase concentration (0, 0.5, 1.0, 1.5 and 2.0 wt%)

Molecular weight analysis

Weight-average molecular weight is a molecular weight measurement that takes into consideration the contributions of molecules according to their size. From the elution time of different size molecules, the molecular weight of collagen sample was calculated through the calibration curves of standard. Overlaid of GPC chromatograms for both samples, collagen and hydrolyzed collagen is given in Figure 2. The single sharp peak of hydrolyzed collagen at periods of 17-22 min appeared later than collagen peak, resulting in a low molecular weight of hydrolyzed collagen with a narrower molecular weight distribution compared to collagen. It shows that the hydrolyzed collagen produced were monodisperse.

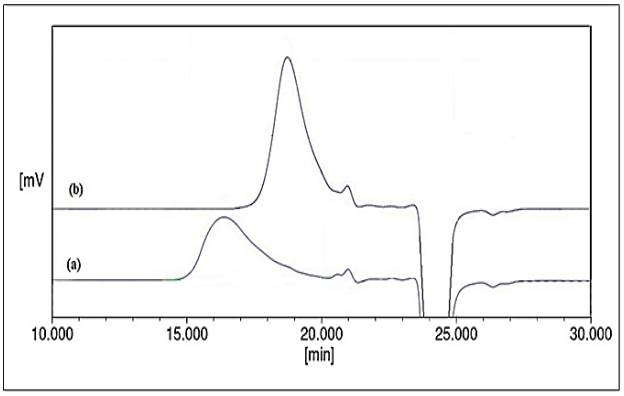

Figure 2: GPC chromatograms of (a) collagen (b) hydrolyzed collagen (0.5 wt.% alcalase)

As shown in Figure 3, the molecular weight of collagen have significantly reduced from 12.5 kDa to 1.3 kDa when it was hydrolyzed with 0.5 wt.% alcalase. The significant reduction is due to the intra-chain cleavage of collagen peptide bonds by the enzyme which had taken place during hydrolysis process [30]. The small size of collagen structure produced in this study can pass through easily into the skin**.** Chai et al. proposed that the transdermal efficiency of collagen is positively correlated to collagen sizes [31]. They stated that the low molecular weight of collagen below 4.5 kDa can penetrate successfully into skin. In addition, Potts and Guy stated that the diffusivity of a drug in skin is correlated to molecular features of the drug [32].

Increasing concentration of alcalase above 0.5 wt.% shows insignificant reduction in molecular weight. The result shown by further increasing the concentration of alcalase until 2.0 wt.%, the molecular weight produced is still around 1000 Da. This is probably due to the fact that the enzyme concentration of 0.5 wt.% has achieved the optimum enzyme-to-substrate ratio. Mechanism of the enzyme to the substrate could be described as a lock and key hypothesis [33]. The intra-chain cleavage reactions took place when an enzyme captured one or more collagen substrate to form an active enzyme-substrate complex. Increasing the concentration of Alcalase enzyme from 1.0-2.0 wt.%, led to excessive enzyme to the substrate. Hence, there was no more remaining substrate to be captured by the enzyme. It was assumed that if the Alcalase concentration was increased above 0.5 wt.%, the molecular weight produced will only decrease slightly or there will be no reducing in weight.

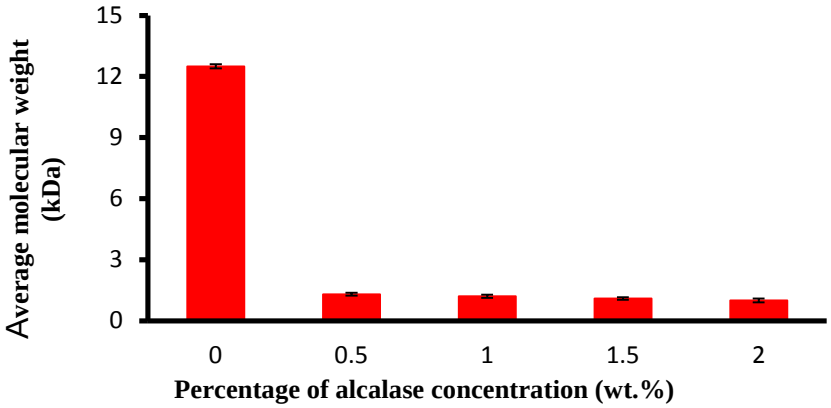

Figure 3: Average molecular weight of collagen (0 wt.% alcalase) and hydrolyzed collagen at 0.5, 1.0, 1.5 and 2 wt.% of alcalase

Degree of hydrolysis

Figure 4 shows degree of hydrolysis in hydrolyzed collagen at Alcalase concentration of 0, 0.5, 1.0, 1.5 and 2.0 wt.%. According to the results, degree of hydrolysis significantly increases when collagen was first treated with 0.5 wt.% of alcalase. This observation is due to the peptides released that were hydrolyzed by the enzymes into amino acids and smaller peptides. The degree of hydrolysis do not show any significant difference when the Alcalase concentration is increased up to 1.0, 1.5 and 2.0 wt.%, respectively. This might be due to the small percentage of 0.5 wt.% alcalase concentration have resulted the optimal hydrolysis process.

This was supported by the molecular weight analysis result in which the molecular weight of extracted collagen, 12,530 Da significantly decreased to 1,282 Da when first hydrolyzed with 0.5 wt.% of alcalase. Meanwhile, there were no significant difference in molecular weight of hydrolyzed collagen when the concentration was increased above 0.5 wt.% of alcalase. Based on the results, it has been shown that a low concentration of 0.5 wt.% alcalase already produce the low molecular weight of fish scale collagen (<1 kDa). In industries like cosmetics, low percentage of enzyme used is recommended to reduce cost.

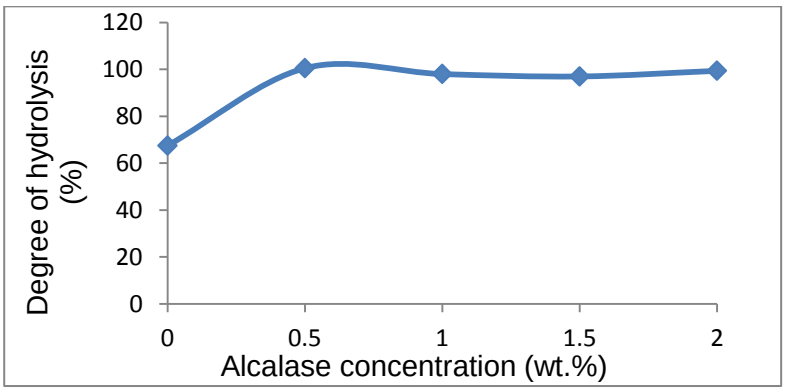

Figure 4: Percentage of degree hydrolysis (DH) in collagen prepared at different alcalase concentration (0, 0.5, 1.0, 1.5 and 2.0 wt.%)

IR spectra analysis

The spectra in the range of 500-4000 cm^{-1} for Tilapia fish scales and extracted Tilapia fish scale collagen (untreated collagen) is shown in Figure 4. IR spectra show the difference of functional groups presence in both samples. The collagen amide group peaks were seen at 3303-3293 cm^{-1}, 1675-1642 cm^{-1} and 1601-1536 cm^{-1}, respectively for both samples whereas the appearance of prominent peak at 614 cm^{-1} is shown only in Tilapia fish scales spectra. This peak represents the symmetric stretching vibration of P-O band of PO_4^{3-} ions, indicates the presence of phosphate groups from hydroxyapatite, HAp ($Ca_5(PO_4)_3OH$) [34]. The characteristics FTIR peaks confirmed the Type I collagen and HAp as main compositions in Tilapia fish scales (Figure 5a). The existence of P-O band disappears in extracted Tilapia fish scale collagen due to the removal HAp component during the filtration (Figure 5b).

The effect of enzymatic hydrolysis using difference concentration of alcalase on collagen structure is shown by the FTIR spectra (Figure 6). All of the prominent peaks corresponding to Type I collagen functional groups such as amide A (3291 cm^{-1}), amide I (1645 cm^{-1}), amide II (1538 cm^{-1}) and amide III (1241 cm^{-1}) were present in all hydrolyzed sample. This observation proved that the enzymatic hydrolysis had not altered the main functional groups of collagen such as N-H, C=O and C-N.

Viscosity measurement

Figure 7 shows the viscosity trend of collagen sample prepared. The average viscosity of collagen (0 wt.% alcalase) obtained from this study was 6.4 cP which is similar to the viscosity values of gelatin extracted from silver carp, which are between 2.5 to 13.5 cP [35]. Zhou and Regenstien [36,37] also reported that the viscosity for the gelatin extracted from Alaska Pollock is 6.62 cP.

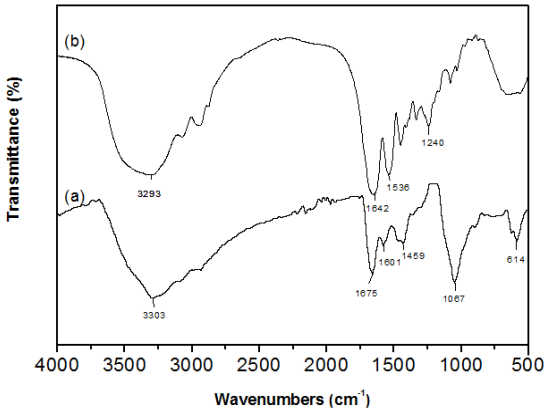

Figure 5: FTIR spectra of (a) Tilapia fish scales and (b) Tilapia fish scale collagen

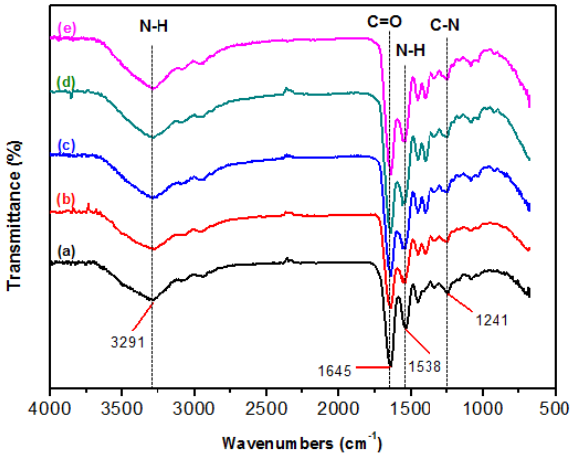

Figure 6: Overlaid of FTIR spectra of fish scale collagen: (a) collagen (0 wt.% alcalase) and hydrolyzed collagen at Alcalase concentration of (b) 0.5 wt.%, (c) 1.0 wt.%, (d) 1.5 wt.% and (e) 2.0 wt.%

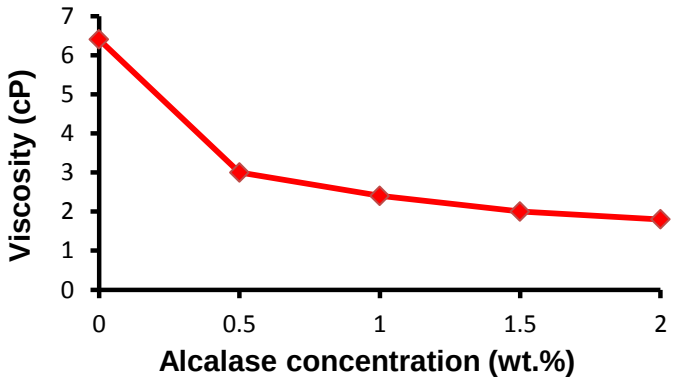

Figure 7: Viscosity values of collagen prepared at different alcalase concentration (0, 0.5, 1.0, 1.5 and 2.0 wt.%)

In this study, the average viscosity of the hydrolyzed collagen samples prepared is between 1.8-3.0 cP, which is similar to previous report, i.e., between 2.27-2.54 cP [37]. The viscosity of the hydrolyzed collagen solution shows decreasing trend as the concentration of enzyme used is increased. This probably is due to the amide-bond cleavage which occurred during the enzyme reaction, producing the low molecular weight collagen. Hydrolysates with low molecular weight tend to produce a low viscous solution [38].

The active site of the enzyme was attached to a collagen molecule during the reaction forming an enzyme-collagen complex. Subsequently, the enzyme caused certain covalent bond to weaken, resulting in a breakdown (hydrolysis) of the collagen into a smaller molecule which is called hydrolyzed collagen. The enzyme was unaltered during the reaction and free to catalyze the breakdown of another collagen molecule.

Amino acid compositions

The distribution patterns of amino acid compositions for collagen and hydrolyzed collagen samples that were prepared in this study differ insignificantly and shows similar amino acid profile with native collagen that was extracted from the scales of red Tilapia (*Oreochromis niloticus*), blue Tilapia (*O. aureus*) and Carp fish by Ikoma et al. [14], Zubia et al. [39] and Zhang et al. [40], respectively.

The similarities in the amino acid profiles proved that the chemical compositions of native collagen that was extracted from Tilapia fish scales were conserved among the samples prepared. The presence of cysteine and thyrosine in the least amount for all samples profiles was indicated that the existence of Type I collagen in scales of this species [14,41]. As shown in Table 1, the amount of amino acid composition per 100 total residues was found to be similar with the collagen peptides that were extracted from Chum Salmon skin [42]. The highest amount of glycine in amino acid composition of all samples was due to the characteristics of collagen triple helical structure, which have regular arrangement of glycine in each of the three chains of collagen subunits.

Table 1: Comparison between the amino acid compositions of collagen, hydrolyzed collagen (treated with 0.5 wt.% and 2.0 wt.% alcalase) and collagen (Chum Salmon skin) [14], residues/100 [42]

S. No.	Amino acid	Amount % (w/w)			
		Collagen	Hydrolyzed collagen (0.5 wt.% alcalase)	Hydrolyzed collagen (2.0 wt.% alcalase)	Collagen (Chum Salmon skin)
1	Hydroxyproline	10.2	10.9	11.3	7.51
2	Aspartic acid	5.6	5.2	5.0	7.29
3	Serine	4.1	4.0	4.2	4.23
4	Glutamic acid	9.6	9.3	9.0	12.22
5	Glycine	21.6	21.4	21.3	23.77
6	Histidine	1.4	1.5	1.7	1.61
7	Arginine	9.3	9.1	8.9	6.08
8	Threonine	3.2	3.4	3.5	2.53
9	Alanine	9.2	8.9	8.4	6.59
10	Proline	11.1	10.8	10.6	9.79
11	Cysteine	0.1	0.1	0.1	0
12	Thyrosine	0.6	0.7	0.9	0.03
13	Valine	2.4	2.5	2.6	2.94
14	Methionine	1.6	1.9	2.4	0.03
15	Lysine	3.7	3.4	3.2	5.66
16	Isoleucine	1.3	1.5	1.5	2.57
17	Leucine	2.8	3.0	2.9	4.64
18	Phenylalanine	2.2	2.4	2.5	2.51

CONCLUSIONS

The average molecular weight of Tilapia fish scale collagen was significantly reduced from 12.5 kDa to 1 kDa by enzymatic hydrolysis treatment. The low enzyme concentration of 0.5 wt.% alcalase can produce low molecular weight of collagen which is below < 1 kDa. In addition, the enzymatic hydrolysis did not alter the functional group and profile of amino acid content in collagen. Hydrolyzed fish scale collagen prepared in this study is rich in the amino acids glycine; proline and hydroxyproline as well as a variety of peptides may have the ability to stimulate and enhance the fibroblast cell to synthesis more collagen in the dermis. The resulting characteristic behaviours of the prepared hydrolyzed collagen have a great potential to be used as an ingredient in cosmetic product.

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