Properties and Characteristics of Acid-Soluble Collagen from Salmon Skin Defatted with the Aid of Ultrasonication

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Abstract: Salmon skin, a byproduct from the deskinning process, can be used as an alternative source of collagen. Due to the high fat content in skin, the defatting process is required prior to extraction. The properties and characteristics of acid-soluble collagen (ASC) from salmon skin (Oncorhynchus nerka), defatted using isopropanol without and with ultrasonication (70% amplitude for 10 min), were investigated. The ASC from the skin that was defatted with aid of ultrasonication (U-ASC) exhibited lower (p < 0.05) fat content (1.86%) with extraction yield (23.18% w/w, dry weight basis) than C-ASC (collagen extracted from skin defatted without ultrasonication). Both of the ASCs were classified as type I collagen. C-ASC and U-ASC had isoelectric points of 7.17 and 7.40, respectively. Fourier transform infrared (FTIR) and circular dichroism spectra reconfirmed the triple-helix structure of both ASCs. The major amino acid of both collagens was glycine (297–308 residues/1000 residues). A high amount of imino acid (191–193 residues/1000 residues) was also found. After gastrointestinal digestion, the degree of hydrolysis of the digested U-ASC (23.19%) was slightly higher than that of the digested C-ASC (22.31%). However, both digests had no differences in antioxidant activities. Both of the ASCs could be therefore used as functional ingredient.

Keywords: salmon skin; collagen; defatting process; ultrasonication; digestion

1. Introduction

Collagen, especially fish collagen, has drawn augmenting attention in several areas. There are at least 29 types of collagens, which have various molecular properties [1]. Among all of the collagens, type I collagen is dominant in fish skin. Collagen has a triple-helical structure, in which hydrogen bonds between glycine and amide groups are mainly involved in the stabilization of polypeptide chains [2]. Type I collagen is generally used in food, nutraceutical, pharmaceutical, and biomedical industries [3–5]. Native collagen is organized in fibers and is, therefore, insoluble, hence appropriate pretreatment is required before extraction [6]. Fish collagen has been used for Halal and Kosher products served for Muslim and Jewish communities, respectively. Due to the outbreak of bovine spongiform encephalopathy and bird flu, fish collagen has inevitably gained augmenting demand [2,4].

Globally, salmon is of high demand owing to its high nutritive value, with its attractive orange-red color mediated by pigment, namely astaxanthin. Salmon meat has high-quality protein comprising essential amino acids at high content [7]. The salmon processing industry generates a huge amount of by-products, particularly skins (about 7%) [7]. These underutilized bio-resources have been exploited as a starting material for collagen and gelatin production [8,9]. Fish collagen can increase the revenue for the producer and serves as alternative biomaterial for several purposes. Nevertheless, salmon skin has a high fat content (23.3–61.5%, dry basis) and is rich in polyunsaturated fatty acids (PUFA) [7,10]. The oxidation of PUFA distributed in the skin matrix is a problem, causing an undesirable
fishy odor/flavor of the resulting collagen or gelatin [11]. Numerous techniques have been implemented to lower or remove the fat from fish skin. Citric acid pretreatment, with subsequent defatting using 30% isopropanol, was able to remove fats and lower the fishy odor of seabass skin gelatin [11]. Nevertheless, the fat retained in the skin after defatting can lower the purity of the collagen, along with the undesirable fishy odor. In order to enhance the fat removal, ultrasonication could be employed. The cavitation effect is the main mechanism of ultrasound in a liquid system [12]. When the wave generates the regions with high and low pressure, this results in the rapid formation and collapse of cavitation bubbles [12]. Currently, ultrasonication has been successfully implemented to enhance fat removal from hydrolyzed collagen powder [13]. Nevertheless, no information concerning the use of ultrasonication for fat removal of fish skin, especially high-fat skin, such as salmon skin, has been documented. In addition, ASC has been fortified commercially in some acidic drinks, especially fruit juice, such as pomegranate juice, etc. Nevertheless, no information on the digestibility of ASC, as affected by the defatting process, exists. This study aimed to elucidate the impact of the defatting process of salmon skin, without and with the aid of ultrasonication, on molecular properties and digestibility in a simulated gastrointestinal tract.

2. Materials and Methods

2.1. Chemicals

Acetic acid and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), N,N,N',N'-tetramethylethylenediamine, and Coomassie Blue R-250 were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Protein molecular weight (MW) markers were procured from Sigma Chemicals (St Louis, MO, USA). All other chemicals used in the present study were of analytical grade.

2.2. Preparation of Salmon Skin

Skins of sockeye salmon (Oncorhynchus nerka) used for this research were bought from Nissui (Thailand) Co., Ltd., Songkhla, Thailand, which were discarded from fish processing. The skins were stored on ice (1:2, w/w), in which a polystyrene box was used as a container. When the skins reached the laboratory after 1 h of transportation, the manual removal of the remaining meat was carried out. Prepared skins were washed using cold water (≤10 °C), followed by storage at −20 °C in polyethylene bags (less than 1 month).

Before use, frozen skins were cut into small pieces (1.5 × 1.5 cm²) using an electric sawing machine. The prepared samples were then subjected to the non-collagenous protein removal process, as tailored by Nilsuwan, Chantakun, Chotphruethipong, and Benjakul [13]. Skins were mixed with 0.05 M NaOH (1:10, w/v) and stirred gently at 150 rpm for 1 h, followed by draining. Alkali pretreatment was performed in the same manner for a total of three times. Alkali-treated skins were washed with water until they were neutralized. The prepared skins were treated with 0.05 M citric acid (1:10, w/v) with gentle agitation for 15 min and allowed to stand for 45 min. Swollen skins were washed with water until neutral pH was reached. All the operations were carried out at refrigerated temperature (4–8 °C). Pretreated skins (PS) were further used for defatting.

2.3. Effect of Defatting Process of Salmon Skin

2.3.1. Conventional Method

PS was mixed with 30% (v/v) isopropanol at a skin/solvent ratio of 1:10 (w/v). The mixture was stirred at 150 rpm for 60 min with a magnetic stirrer at 4–8 °C. Subsequently, the skin was collected and washed with distilled water at a skin/solvent ratio of 1:10 (w/v) 3 times. The skin was drained on the screen and used for collagen extraction.

2.3.2. Ultrasonic-Assisted Method

PS was mixed with 30% (v/v) isopropanol at a skin/solvent ratio of 1:10 (w/v). The mixture was further mixed with the aid of ultrasonic processor, using a 13 mm probe, at
an amplitude of 70% with pulse mode (on/off at 10 s) for 10 min. The mixture was then stirred at 150 rpm for a total of 60 min with a magnetic stirrer at 4–8 °C. Finally, the skin was collected, washed, and drained as previously described prior to extraction of collagen.

2.4. Collagen Extraction from Defatted Salmon Skins

ASC was extracted at 4 °C following the method of Ali et al. [14], with slight modifications as illustrated in Figure 1. Defatted PS (50 g) was mixed with 0.5 M acetic acid using a defatted PS/acid solution ratio of 1:40 (w/v) and stirred continuously for 48 h using the overhead stirrer. After being filtered using double-layered cheesecloth, the filtrate was subjected to precipitation using 0.05 M tris(hydroxymethyl) aminomethane and 2.6 M NaCl. The precipitate was collected by centrifugation at 10,000 × g for 60 min at 4 °C using a high-speed refrigerated centrifuge (model CR22N Hitachi, Tokyo, Japan). Thereafter, the pellet was resuspended in 0.5 M acetic acid (1:10, w/v), dialysis was carried out in 20 volumes of 0.1 M acetic acid for 24 h, followed by 20 volumes of distilled water for another two days. The distilled water was changed every day. Dialysate was lyophilized using a freeze-dryer (model CoolSafe 55 ScanLaf A/S, Lynge, Denmark). The obtained ASC powders from defatted swollen skins, with the conventional method and the ultrasonic-assisted method, were named as ‘C-ASC’ and ‘U-ASC’, respectively. Both C-ASC and U-ASC were analyzed.

![Figure 1](#) Scheme for extraction of acid-soluble collagen from salmon skin defatted using 30% isopropanol without (C-ASC) and with the aid of ultrasonication (U-ASC).

2.5. Analyses

2.5.1. Extraction Yield

The yield was computed using the following equation:

\[
\text{Yield} \% = \frac{\text{Dry weight of collagen powder (g)}}{\text{Dry weight of skin used for extraction (g)}} \times 100
\]
2.5.2. Fat Content

C-ASC and U-ASC powders were determined for fat content as per the method of AOAC [15]. Soxhlet apparatus (Soxterm–Gerhardt, Bonn, Germany) was utilized and petroleum ether was used as the solvent.

2.5.3. Hydroxyproline Content

Hydroxyproline (Hyp) content in the ASC powders was determined by the spectrophotometric method, as detailed by Bergman and Loxley [16].

2.5.4. Color

Color parameters consisting of $L^*$ (lightness/darkness), $a^*$ (redness/greenness), and $b^*$ (yellowness/blueness) were determined using a Hunter Lab Colorimeter [17]. $\Delta E^*$ (color difference) was also examined using the following equation.

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

where $\Delta L^*$, $\Delta a^*$, and $\Delta b^*$ are the differences between the color parameter of the samples and those of white standard ($L^* = 92.80$, $a^* = -1.15$, $b^* = 0.43$).

2.5.5. Differential Scanning Calorimetry (DSC)

Prior to analysis, ASC samples were rehydrated in deionized water (1:40, w/v) at 4 °C for 24 h. The analysis was performed as described by Ali, Benjakul, Prodpran, and Kishimura [14], using a differential scanning calorimeter Perkin–Elmer Model DSC7 (Norwalk, CA, USA). Endothermic peak, demonstrating maximum transition temperature ($T_{\text{max}}$), was scanned over the temperature range of 0–50 °C at the rate of 1 °C min$^{-1}$. Additionally, total denaturation enthalpy ($\Delta H$) was also measured.

2.5.6. Protein Patterns

Protein patterns of ASC samples were analyzed for protein pattern using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [18], as modified by Ali, Benjakul, Prodpran, and Kishimura [14]. Samples (12 µg of protein), as measured by the Biuret method, were loaded onto polyacrylamide gel (4% stacking and 7.5% running gel). After separation with a constant current of 15 mA/plate, staining and destaining of gels were performed. Intensity of protein bands was quantified using public domain digital analysis software (ImageJ 1.42q, Bethesda, MD, USA).

2.5.7. Zeta Potential

Twenty milligrams of ASC samples (0.04% in 50 mM acetic acid, 4 °C) were subjected to an auto-titrator model BI-ZTU (Brookhaven Instruments Co., Holtsville, NY, USA). Desired pHs of the samples (pH 4, 5, 6, 7, and 8) were achieved using 1 M nitric acid or 1 M potassium hydroxide. Zeta potential was measured using a zeta potential analyzer model ZetaPALs (Brookhaven Instruments Co., New York, NY, USA). pH showing zeta potential of 0 was considered as the isoelectric point (pI).

2.5.8. FTIR Spectroscopy

Dehydrated ASC samples were subjected to ATR-FTIR model Equinox 55 (Bruker, Ettlingen, Germany). The method of Petcharat, Benjakul, Karunjanapramut, and Nalinnanon [12] was adopted. Spectra ranging from 400 to 4000 cm$^{-1}$, with a step resolution of 4 cm$^{-1}$, was recorded using OPUS 3.0 software (Bruker, Ettlingen, Germany).

2.5.9. Circular Dichroism (CD)

CD spectra of ASC samples were analyzed following the procedure of Ali, Benjakul, Prodpran, and Kishimura [14]. Samples dissolved in 0.5 M acetic acid, with a concentration of $2.0 \times 10^{-4}$ g/mL, were prepared. The mixture was stirred for 4 h and centrifuged.
at 10,000× g for 5 min at 4 °C. The analysis was performed using a JASCO CD J-815 spectrometer (Jasco Corp, Tokyo, Japan). Scanning was performed at the wavelength range of 190–230 nm, with a bandwidth of 1 nm and a step resolution of 0.2 nm. Nitrogen was used as atmosphere at room temperature. The spectral data obtained for 0.5 M acetic acid was used as blank and was subtracted from that of sample.

2.5.10. Amino Acid Composition

ASC powder was treated with 4.0 M methanesulphonic acid at 110 °C for 22 h, under reduced pressure to prevent the oxidation of tryptophan. The sample was neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot (100 µL) was injected into an amino acid analyzer (JLC-500/V AminoTac™, JEOL USA Inc., Peabody, MA, USA). The amino acid composition was reported as residue per 1000 residues (R/1000R).

2.5.11. Digestibility and Antioxidant Activities of ASC in Gastrointestinal Digestion Model System

Simulated gastrointestinal digestion system was adopted from the method of Garrett et al. [19] with minor modifications. ASC powders were dissolved in 5 mM HCl–KCl buffer (pH 1.5) to obtain a concentration of 0.01 g/mL. Two mL of pepsin dissolved in the same buffer were added to the mixture to obtain a concentration of 0.04 g/mL and incubated at 37 °C for 1 h, with continuous shaking (Memmert Model SV 1422, Schwabach, Germany). Thereafter, the pH of the reaction mixture was raised to 5.3 with 0.9 M sodium bicarbonate. Nine milliliters of freshly prepared pancreatic juice (12 mg/mL bile salts and 2 mg/mL pancreatin in 0.9 M sodium bicarbonate) were added. The mixture was incubated at 37 °C for 3 h with continuous shaking. Final concentrations of bile extract and pancreatin in the reaction mixture were 2.4 and 0.4 mg/mL, respectively. The digestion was terminated by placing the mixture in boiling water for 10 min. Digest was divided into 2 portions. For the first portion, the digest (1 mL) was taken and added with hot 5% SDS (85 °C) at a 1:1 (v/v) ratio and heated at 85 °C for 30 min to solubilize total proteins and peptides. Degree of hydrolysis (DH) of the mixture was determined using TNBS (2, 4, 6-trinitrobenzenesulphonic acid), as tailored by Benjakul and Morrissey [20].

For the second portion, the digest was lyophilized using a freeze-dryer. Antioxidant activities of powders were determined for ABTS radical-scavenging activity [21], DPPH radical-scavenging activity [21], ferric reducing antioxidant power (FRAP) [21], and oxygen radical absorbance capacity (ORAC) [21]. The fluorescence decay curves of both digests (2.0 mg/mL), Trolox (0.6 mg/mL) and 75 mM phosphate buffer (control), were plotted between relative fluorescence intensity (%) and time (min). All activities were expressed as µmol Trolox equivalent (TE)/g sample.

2.6. Statistical Analysis

Completely randomized design (CRD) was used for the entire study. Experiments and analyses were conducted in triplicate (n = 3). Analysis of variance (ANOVA) was performed and the differences among samples were determined using Duncan’s multiple range test at the p < 0.05 level. The analysis was performed with a SPSS package (SPSS for windows, Version 28, SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Extraction Yield

The yield of ASC extracted from the salmon skin that was defatted using 30% isopropanol, without and with the aid of ultrasonication, is shown in Table 1. The yield of the ASC from the salmon skin defatted with the conventional process (C-ASC) was 25.95% (dry weight basis), while the ASC from the skin defatted with the aid of ultrasonication (U-ASC) was 23.18% (dry weight basis). The yields of C-ASC and U-ASC were higher than those of the ASC from clown featherback skin (23.46%) [12] and Nile tilapia skin (21.40%) [22] but were lower than that of ASC from grass carp skin (42.38%) [23]. Moreover, U-ASC had
slightly lower yield than C-ASC. This result might be attributed to the cavitation effect of ultrasonication applied for fat removal. The high shear generated by microbubble collapse effectively weakened the skin matrix [6], thus loosening the skin matrix [12]. As a result, some collagen matter in the skin could be lost during defatting. Therefore, ultrasonication could cause the destruction of skin, thus facilitating defatting as well as the subsequent extraction of collagen. However, it could result in the loss of some collagens, particularly during defatting process.

**Table 1.** Yield and physicochemical properties of acid-soluble collagen from salmon skin defatted with 30% isopropanol without (C-ASC) and with the aid of ultrasonication (U-ASC).

| Parameters                        | C-ASC                  | U-ASC                  |
|-----------------------------------|------------------------|------------------------|
| Yield (%), dry weight basis       | 25.95 ± 0.88 a         | 23.18 ± 1.07 b         |
| Fat content (%), dry weight basis | 2.10 ± 0.07 a          | 1.86 ± 0.12 b          |
| Hydroxyproline content (mg/g dry sample) | 48.28 ± 0.11 b | 49.15 ± 0.42 a         |
| L*                                | 85.53 ± 0.53 a         | 85.37 ± 0.24 a         |
| a*                                | -0.04 ± 0.01 a         | -0.12 ± 0.01 b         |
| b*                                | 4.20 ± 0.06 a          | 1.72 ± 0.07 b          |
| ΔE*                               | 8.20 ± 0.56 a          | 7.62 ± 0.25 a          |
| T_onset (°C)                      | 6.84                   | 5.93                   |
| T_max (°C)                        | 11.3                   | 11.6                   |
| T_endset (°C)                     | 16.58                  | 15.65                  |
| ΔH (/g)                           | 0.25                   | 0.15                   |

Values are reported as mean ± SD (n = 3). Different lowercase letters in the same row denote significant difference (p < 0.05).

### 3.2. Fat Content

Low fat content (1.86–2.10%) was observed for both of the ASC powders from the salmon skin defatted with 30% isopropanol (Table 1). It is worth noting that isopropanol was capable of removing fat effectively. Solvent, having slight polarity, was suitable for the removal of lipids in skin containing membrane lipids, such as phospholipids, etc. [11,24]. Moreover, lower fat content (p < 0.05) was found for U-ASC (1.86%) than that of C-ASC (2.10%). This result indicated that the cavitation effect of the ultrasonication process could reduce the fat content in salmon skin to a higher extent, in which the fat associated with the skin matrix was disrupted and more fat was released into the solvent used [12]. Therefore, the salmon skin defatted with 30% isopropanol with the aid of ultrasonication could potentially decrease the fat in skin more.

### 3.3. Hydroxyproline Content

Theoretically, collagen is the only protein containing hydroxyproline, whereas no hydroxyproline is available in other proteins [25]. Thus, hydroxyproline has been used as the index of collagen present in foods [25]. Hydroxyproline content in ASC was slightly increased as the ultrasonication was implemented. The hydroxyproline contents of C-ASC and U-ASC were 48.28 and 49.15 mg/g dry sample, respectively (Table 1). This result might be related to the higher defatting efficiency with the aid of ultrasonication. Petcharat, Benjakul, Karnjanapratum, and Nalinanon [12] documented that the high power of ultrasonication could loosen the skin matrix to a high degree, resulting in the release of some components from the skin matrix, including fat. As the fat was effectively removed with the aid of ultrasonication, the collagen in the defatted skin became more concentrated, as witnessed by the slight increase in hydroxyproline content. Additionally, the defatted skin could be extracted with acetic acid more easily since the lowered fat distributed in the skin could be a hindrance for collagen solubilization by acid solution. Therefore, the defatting process with the aid of ultrasonication could augment the purity of the resulting collagen from salmon skin, as ascertained by the higher hydroxyproline content in U-ASC.
3.4. Color

Generally, lower $a^*$ and $b^*$-values ($p < 0.05$) were noticeable for U-ASC powder, compared to that of C-ASC powder (Table 1). Via the defatting process using isopropanol, the fat in the ASC powder was eliminated to some degree. The defatting process with the aid of ultrasonication likely facilitated the removal of fat to a higher extent [13]. Fat containing some carotenoids, such as astaxanthin, could contribute to the yellowish orange color [26], as witnessed by high $a^*$- and $b^*$-values in C-ASC, while the lower values were found in U-ASC. Nevertheless, no differences ($p > 0.05$) in $L^*$ and $\Delta E^*$-values between C-ASC and U-ASC powders were observed. Therefore, collagen powders defatted with 30% isopropanol with the aid of ultrasonication, which possessed lower fat contents, had less yellow or red color. In addition, the remaining fat in ASC might undergo oxidation, leading to discoloration, especially via Maillard reaction. Oxidation products rich in carbonyl compounds are sensitive to the Maillard reaction with amino groups in ASC.

3.5. DSC Thermogram

$T_{\text{max}}$ of U-ASC was 11.6 $^\circ$C, while C-ASC had $T_{\text{max}}$ of 11.3 $^\circ$C (Table 1), indicating similar thermal stability of both of the ASC samples. Imino acid content, particularly hydroxyproline, determines the thermal stability of ASC [2]. Higher hydroxyproline content was found for U-ASC, compared to C-ASC (Table 1). Since salmon is a cold-water fish, both C-ASC and U-ASC therefore showed low $T_{\text{max}}$ (11.3–11.6 $^\circ$C). It is known that the thermal stability of collagen is governed by body and environmental temperature [2]. The present study was in line with the low denaturation temperature of collagen from cold-water fish, such as Pacific cod skin (14.5 $^\circ$C) [27], Baltic cod (15.2 $^\circ$C) [8], and chum salmon (19.0 $^\circ$C) [9]. Enthalpy ($\Delta H$ value) corresponded with the ordered phase structure [28]. The aforementioned value could reflect an amount of ordered and compact structure in the collagen, particularly triple-helix structure. Higher $\Delta H$ value was observed for the C-ASC sample (0.25 J/g), whereas U-ASC showed a lower $\Delta H$ value (0.15 J/g). This result suggested that U-ASC had a less ordered structure than that of C-ASC. It might be associated with a slight change in the triple-helix of U-ASC, mediated by cavitation effect during defatting process [6]. Therefore, the defatting process with the aid of ultrasonication had little effect on the molecular properties of ASC, as indicated by a slight difference in thermal stability.

3.6. Protein Pattern

The protein patterns of the ASC extracted from the skin of salmon defatted with 30% isopropanol without (C-ASC) and with the aid of ultrasonication (U-ASC) are shown in Figure 2. Both of the ASC samples comprised $\beta$-chains (dimer of the $\alpha$-chains) and $\alpha$-chains ($\alpha_1$- and $\alpha_2$-chains) as major components. The $\alpha_1$ and $\alpha_2$ bands of both of the samples had similar molecular weight (MW) of $\sim$118 and $\sim$108 kDa, respectively. The $\gamma$-chain (trimer of the $\alpha$-chains) was also detected, suggesting that both of the ASC samples had high molecular weight cross-linkages [29]. The band intensity ratio of $\alpha_1/\alpha_2$-chains was around 2:1, revealing the presence of type I collagen [14]. The collagens from other fish skins, such as golden carp [14], bigeye tuna [30], Pacific cod [27], turbot [31], and clown featherback [12], have also been identified as type I collagen. Additionally, no difference in molecular weight (MW) and intensity of protein bands between C-ASC and U-ASC samples was observed. This is worth noting that the defatting process with the aid of ultrasonication had no marked effect on the collagen chains. Nevertheless, Kim et al. [32] documented that the degradation of seabass skin collagen, as indicated by the appearance of a smear band at low MW (>97 kDa), occurred when higher amplitude (>80%) and longer treatment times (>3 h) of ultrasonication were used. Thus, the defatting process, with proper conditions of ultrasonication (70% amplitude for 10 min), could enhance fat removal with no effect on the alteration of primary protein structure, as indicated by the non-significant alteration of protein patterns.
Zeta potentials of acid-soluble collagen from salmon skin defatted with 30% isopropanol without (C-ASC) and with the aid of ultrasonication (U-ASC). HM denotes high molecular weight protein markers.

3.7. Zeta Potential

The ζ-potential of C-ASC and U-ASC at the pH range of 4–8 is shown in Figure 3. Typically, the ζ-potential has been used to monitor the alteration of the surface net charge of proteins, including collagen at various pHs [33]. In the pH range of 4–6, a similar profile was noted for both of the ASC samples and the net charge was positive. With upsurging pH values, the charge of collagen was reduced. Within pH 7–8, the isoelectric point (pI) of both of the samples were attained, where the zero net charge was found. The pI values of C-ASC and U-ASC were estimated to be 7.17 and 7.40, respectively. The pIs of collagen from other fish skin, as determined by the zeta potential, were reported, such as golden carp (6.54–6.79) [14], clown featherback (Chitala ornata) (5.39–7.76) [12], and bigeye tuna (Thunnus obesus) (5.50–6.40) [30]. When pH was above the pI, the surface charge became negative. A protein generally possessed net negative and positive charge when the pH was above and below its pI, respectively. This results from the deprotonation and protonation of the amino acids, respectively [34]. Nevertheless, the ζ-potential profile and pI value of U-ASC were slightly different, compared to that of C-ASC. The ultrasonication might cause the partial denaturation of collagen molecules, leading to the exposure of some of the amino acids that were embedded inside the triple-helix to some degree. This might cause the slight difference in pI [29].

Figure 2. Protein patterns of acid-soluble collagen from salmon skin defatted using 30% isopropanol without (C-ASC) and with the aid of ultrasonication (U-ASC). HM denotes high molecular weight protein markers.

Figure 3. Zeta potentials of acid-soluble collagen from salmon skin defatted with 30% isopropanol without (C-ASC) and with the aid of ultrasonication (U-ASC). Bars represent the standard deviation (n = 3).
3.8. FTIR Spectra

FTIR spectra of U-ASC in comparison with C-ASC are depicted in Figure 4. Both of the samples showed similar FTIR spectra. The collagen from the fish skins generally had the characteristic peaks, such as amide A, B, I, II, and III [14]. Amide A, which is associated with N–H stretching vibration, was found in the range of 3292–3296 cm\(^{-1}\) [33]. When the N–H group of a peptide is involved in a hydrogen bond, the peak was shifted to lower frequencies [12]. C-ASC and U-ASC had the amide A at 3298 and 3300 cm\(^{-1}\), respectively. The ultrasonication treatment might cause the partial unfolding of proteins, resulting in an increase in the exposure of the free amino group, leading to the shift of amide A to a higher wavenumber [12]. Amide B represents asymmetrical CH\(_2\) stretching [33]. Amide B for C-ASC and U-ASC were found at 2926 and 2924 cm\(^{-1}\), respectively. Additionally, amide I, II, and III bands are typical for collagen, which showed the peaks at 1600–1700, 1500–1600, and 1200–1300 cm\(^{-1}\), respectively [14]. The similar patterns in amide I, II, and III bands between C-ASC and U-ASC were noticeable. Amide I band is associated with stretching vibration of C=O along the polypeptide backbone and it is a sensitive marker of the polypeptide secondary structure [14]. The amide I bands of the C-ASC and U-ASC were found at the wavenumbers of 1637 and 1640 cm\(^{-1}\), indicating a looser molecular order of U-ASC. C-ASC and U-ASC samples possessed the amide II region at 1537 and 1544 cm\(^{-1}\), respectively. The amide II bands corresponded to N–H bending [12]. C-ASC showed a lower wavenumber of amide II than that of U-ASC, suggesting that the C-ASC had higher interaction via hydrogen bonds between the adjacent chains of the collagen triple-helix. Nevertheless, amide III represents the combination of C–N stretching and N–H deformation involved in the intermolecular interactions of collagen [12]. Both of the ASC samples displayed the peak at 1236–1237 cm\(^{-1}\), representing hydrogen bonds stabilizing the native structure. The triple-helical structure of collagens was reconfirmed by the peak ratio between amide III and the 1450 cm\(^{-1}\) bands [33]. Collagen with triple-helical structure has the ratio of 1.0 [35]. C-ASC (1.05) and U-ASC (0.98) had slightly different ratios. This result suggested that the defatting with the aid of ultrasonication affected the triple-helical structure of collagen to small degree. The molecular structure and functional groups of collagens were not markedly influenced by defatting with aid of ultrasonication.
3.9. CD Spectra

The CD spectra of C-ASC and U-ASC are shown in Figure 5. CD spectroscopy has been used in order to determine the secondary structure of proteins. Native collagen has a triple-helical conformation with a negative peak at around 200 nm and a positive peak at 220–230 nm [36]. Similar CD spectra patterns were attained for both of the ASC samples. However, C-ASC and U-ASC showed a difference in the negative peaks, which appeared at 197 and 204 nm, respectively. Additionally, a lower intensity of negative peak was found for U-ASC, compared to that of C-ASC. This result might be related to the partial alteration of the triple-helical matrix by defatting process with the aid of ultrasonication. Both of the samples had a positive peak at 220 nm. Therefore, both of the ASC samples still had a triple-helical structure with a slight change in the compactness of the molecules. The defatting process with the aid of ultrasonication had, thus, little effect on the secondary structure of ASC, as indicated by a slight difference in CD spectra.

![CD Spectra](image)

Figure 5. CD spectra of acid-soluble collagen from salmon skin defatted with 30% isopropanol without (C-ASC) and with the aid of ultrasonication (U-ASC).

3.10. Amino Acid Composition

Generally, amino acid composition is related to physicochemical properties, stability and nutritive value of collagen [37]. Both of the ASC samples had similar amino acid composition (Table 2). Glycine was the dominant amino acid (297–308 R/1000R), followed by proline (135–136 R/1000R), alanine (107–109 R/1000R), glutamic acid/glutamine (68–69 R/1000R), and hydroxyproline (56–57 R/1000R). Glycine is localized at every third position in formation of inter-chain hydrogen bonds in α-chain [38]. The imino acid (proline and hydroxyproline) content is generally correlated with the thermal stability of collagen [25]. Furthermore, the imino acid content of C-ASC and U-ASC was in the range of 191–193 R/1000R, which was similar to that of golden carp collagen (194 R/1000R) but was higher than that found in the collagen from Pacific cod skin (157 R/1000R) [6,27]. This was plausibly governed by the different species, pretreatment, and the processes that were used for collagen extraction. Additionally, the imino acid content of U-ASC (193 R/1000R) was slightly higher than that of C-ASC (191 R/1000R). The higher fat removal with the aid of ultrasonication might favor the concentration of imino acid content in U-ASC, which was consequently related to the slight difference in the physicochemical properties between C-ASC and U-ASC. Overall, the defatting process with the aid of ultrasonication had no profound impact on the amino acid composition of the resulting collagen.
Table 2. Amino acid composition of acid-soluble collagen from salmon skin defatted with 30% isopropanol without (C-ASC) and with the aid of ultrasonication (U-ASC).

| Amino Acids                  | Content (Residues per 1000 Residues) |
|------------------------------|--------------------------------------|
|                              | C-ASC      | U-ASC     |
| Alanine                      | 109        | 107       |
| Arginine                     | 59         | 55        |
| Aspartic acid/Asparagine     | 57         | 56        |
| Cystine                      | 1          | 0         |
| Glutamic acid/Glutamine      | 68         | 69        |
| Glycine                      | 297        | 308       |
| Histidine                    | 17         | 15        |
| Hydroxylsine                 | 7          | 6         |
| Hydroxyproline (Hyp)         | 56         | 57        |
| Isoleucine                   | 11         | 10        |
| Leucine                      | 25         | 24        |
| Lysine                       | 37         | 30        |
| Methionine                   | 12         | 13        |
| Phenylalanine                | 15         | 15        |
| Proline (Pro)                | 135        | 136       |
| Serine                       | 52         | 57        |
| Threonine                    | 22         | 24        |
| Tyrosine                     | 3          | 3         |
| Valine                       | 17         | 16        |
| Total                        | 1000       | 1000      |
| Imino acids (Hyp + Pro)      | 191        | 193       |

3.11. Digestibility and Antioxidant Activities of C-ASC and U-ASC in Gastrointestinal Digestion Model System

3.11.1. Degree of Hydrolysis (DH)

Table 3 shows the DH of C-ASC and U-ASC powder digested in the gastrointestinal digestion model system. Higher DH was observed for the digested U-ASC. This result indicated that the U-ASC, with a slightly looser structure, could favor hydrolysis, thus producing higher peptides, as witnessed by the higher DH. The cleavage of collagen into peptides could provide the functionality and bioactivity of the digested sample. Therefore, the collagen digested in the gastrointestinal digestion model system could serve as the source of active peptides for health benefits.

Table 3. Degree of hydrolysis and antioxidant activities of acid-soluble collagen powders from defatted salmon skins after digestion in a simulated gastrointestinal tract model system.

| Degree of Hydrolysis/Activities | C-ASC       | U-ASC       |
|---------------------------------|-------------|-------------|
| Degree of hydrolysis (%)        | 22.31 ± 0.29 b | 23.19 ± 0.70 a |
| ABTS radical-scavenging activity (µmol Trolox equivalent/g sample) | 442.64 ± 57.46 a | 475.81 ± 22.66 a |
| DPPH radical-scavenging activity (µmol Trolox equivalent/g sample) | 0.27 ± 0.04 a | 0.32 ± 0.03 a |
| Ferric reducing antioxidant power (FRAP) (µmol Trolox equivalent/g sample) | 13.41 ± 0.79 a | 14.61 ± 1.89 a |
| Oxygen radical absorbance capacity (ORAC) (µmol Trolox equivalent/g sample) | 203.95 ± 32.15 a | 204.33 ± 10.83 a |

C-ASC: acid-soluble collagen from salmon skin defatted with 30% isopropanol; U-ASC: acid-soluble collagen from salmon skin defatted with 30% isopropanol with the aid of ultrasonication. Values are reported as mean ± SD (n = 3). Different lowercase letters in the same row denote significant difference (p < 0.05).

3.11.2. Antioxidant Activities

ABTS and DPPH radical-scavenging activities, FRAP, and ORAC of collagen powders digested in the gastrointestinal digestion model system are shown in Table 3. No marked
difference ($p > 0.05$) in the antioxidant activities between the digested C-ASC and U-ASC was observed. ABTS activity of both of the digested samples was 442–475 μmol Trolox equivalent (TE)/g sample. Thus, the digested collagen samples were capable of scavenging the stable radical cation ABTS$^+$ (2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)), a blue-green chromophore with a maximum absorption at 734 nm. The decrease in intensity of radicals is found when antioxidants were present [39]. Both of the digests exhibited low DPPH radical-scapenging activity (0.27–0.32 μmol TE/g sample). DPPH was used as a hydrophobic free radical in order to evaluate the antioxidative activity [40]. The FRAP of both of the digests was in the range of 13.41–14.61 μmol TE/g sample. The FRAP assay was used to measure the reduction in ferric ion (Fe$^{3+}$)-ligand complex to the intensely blue-colored ferrous (Fe$^{2+}$) complex by antioxidants under acidic condition [39].

Furthermore, the digested C-ASC and U-ASC had ORAC values of 203.95 and 204.33 μmol TE/g sample, respectively. The ORAC assay has been used to monitor the inhibition of peroxyl radicals by the antioxidants [39]. Among all of the samples, both of the digests were able to prevent the decay of fluorescence, while the control had a drastic decrease in fluorescein intensity within the first 80 min (Figure 6). However, Trolox showed the highest maintenance of fluorescence, indicating the highest ability in scavenging peroxyl radical. The ORAC results indicated that the ASC samples that were digested in the gastrointestinal digestion model system were able to prevent the reaction between peroxyl radicals and fluorescein by donating a hydrogen atom to the radicals. Therefore, both C-ASC and U-ASC were shown to have antioxidant activities after being digested in the gastrointestinal digestion system. The peptides that were generated exhibited different modes of actions. More likely, the peptides were hydrophilic, rather than hydrophobic, in nature. This was evidenced by the high ability of scavenging hydrophilic ABTS$^+$, rather than DPPH radical, which is hydrophobic. Therefore, the ASC from salmon skin could be a potential functional ingredient for food and pharmaceutical industries, regardless of the use of ultrasonication for the defatting of skin.

![Figure 6](image.png)

**Figure 6.** Fluorescence decay curves of fluorescein of acid-soluble collagen powders from defatted salmon skins after digestion in a simulated gastrointestinal tract model system.

### 4. Conclusions

The ASC with low fat content, extracted from salmon skin that was defatted with 30% isopropanol with the aid of ultrasonication, was classified as type I collagen. The use of ultrasonication resulted in slightly low yield (23.18%), but higher hydroxyproline content of ASC, compared with the conventional process (without ultrasonication). According to the thermal properties, protein pattern, FTIR, and CD spectra, the fat removal with the aid of ultrasonication had no negative effect on the triple-helical structure of the resulting collagen. After gastrointestinal digestion, U-ASC was more prone to hydrolysis than C-ASC.
However, no differences in antioxidant activities between both of the digested samples were observed. Therefore, the developed defatting process could produce collagen with low fat content and digestibility, which could be potentially applied in food or pharmaceutical industries.

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**Institutional Review Board Statement:** In this research, the raw material used is ‘the skin’, which is considered as the waste from fish processing plants. The authors bought them from a company and did not deal with the live fish or any process before or during processing of salmon.

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**References**

1. McCormick, R.J. Collagen. In *Applied Muscle Biology and Meat Science*; Du, M., McCormick, R.J., Eds.; CRC Press: Boca Raton, FL, USA, 2009; pp. 130–148.

2. Li, Y.; Yang, L.; Wu, S.; Chen, J.; Lin, H. Structural, functional, rheological, and biological properties of the swim bladder collagen extracted from grass carp (*Chenopharyngodon idella*). *LWT Food Sci. Technol.* 2022, 153, 112518. [CrossRef]

3. Sun, S.; Gao, Y.; Chen, J.; Liu, R. Identification and release kinetics of peptides from tilapia skin collagen during alcalase hydrolysis. *Food Chem.* 2022, 378, 132089. [CrossRef]

4. Liu, S.; Lau, C.-S.; Liang, K.; Wen, F.; Teoh, S.H. Marine collagen scaffolds in tissue engineering. *Curr. Opin. Biotechnol.* 2022, 74, 92–103. [CrossRef]

5. Rastogi, K.; Vashishtha, R.; Shaloo; Dan, S. Scientific advances and pharmacological applications of marine derived-collagen and chitosan. *Biointerface Res. Appl. Chem.* 2022, 12, 3540–3558. [CrossRef]

6. Ali, A.M.M.; Kishimura, H.; Benjakul, S. Extraction efficiency and characteristics of acid and pepsin soluble collagens from the skin of golden carp (*Probarbus jullieni*) as affected by ultrasonication. *Process Biochem.* 2018, 66, 237–244. [CrossRef]

7. Aryee, A.N.A.; Simpson, B.K.; Phillip, L.E.; Cue, R.I. Effect of temperature and time on the stability of salmon skin oil during storage. *J. Am. Oil Chem. Soc.* 2012, 89, 287–292. [CrossRef]

8. Tylingo, R.; Mania, S.; Panek, A.; Piątek, R.; Pawłowicz, R. Isolation and characterization of acid soluble collagen from the skin of african catfish (*Clarias gariepinus*), salmon (*Salmo salar*) and baltic cod (*Gadus morhua*). *J. Biotechnol. Biomater.* 2016, 6, 2. [CrossRef]

9. Jafari, H.; Lista, A.; Siekapan, M.M.; Ghaffari-Bohlouli, P.; Nie, L.; Alimoradi, H.; Shavandi, A. Fish collagen: Extraction, characterization, and applications for biomaterials engineering. *Polymers* 2020, 12, 2230. [CrossRef]

10. Aryee, A.N.A.; Simpson, B.K. Comparative studies on the yield and quality of solvent-extracted oil from salmon skin. *J. Food Eng.* 2009, 92, 353–358. [CrossRef]

11. Sae-leaw, T.; Benjakul, S.; O’Brien, N.M. Effect of pretreatments and defatting of seabass skins on properties and fishy odor of gelatin. *J. Food Biochem.* 2016, 40, 741–753. [CrossRef]

12. Petcharat, T.; Benjakul, S.; Karnjanapratum, S.; Naliniwon, S. Ultrasound-assisted extraction of collagen from clown featherback (*Chitala ornata*) skin: Yield and molecular characteristics. *J. Sci. Food Agric.* 2021, 101, 648–658. [CrossRef] [PubMed]

13. Nilsuwan, K.; Chantakun, K.; Chotpruheiphong, L.; Benjakul, S. Development of hydrolysis and defatting processes for production of lowered fishy odor hydrolyzed collagen from fatty skin of sockeye salmon (*Oncorhynchus nerka*). *Foods* 2021, 10, 2257. [CrossRef]

14. Ali, A.M.M.; Benjakul, S.; Prodpran, T.; Kishimura, H. Extraction and characterisation of collagen from the skin of golden carp (*Probarbus jullieni*), a processing by-product. *Waste Biomass Valorization* 2018, 9, 783–791.

15. AOAC. *Official Method of Analysis*; Association of Official Chemists: Washington, DC, USA, 2000.

16. Bergman, I.; Loxley, R. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Anal. Chem.* 1963, 35, 1961–1965. [CrossRef]
Fishes

17. Benjakul, S.; Karnjanapratum, S.; Visessanguan, W. Production and characterization of odorless antioxidative hydrolyzed collagen from seabass (Lates calcarifer) skin without descaling. *Waste Biomass Valorization* 2018, 9, 549–559. [CrossRef]

18. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227, 680–685. [CrossRef]

19. Garrett, D.A.; Failla, M.L.; Sarama, R.J. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. *J. Agric. Food Chem.* 1999, 47, 4301–4309. [CrossRef] [PubMed]

20. Benjakul, S.; Morrissey, M.T. Protein hydrolysates from Pacific whiting solid wastes. *J. Agric. Food Chem.* 1997, 45, 3423–3430. [CrossRef]

21. Sinthusamran, S.; Benjakul, S.; Kijroongrojana, K.; Prodpran, T.; Kishimura, H. Protein hydrolysates from Pacific white shrimp cephalothorax manufactured with different processes: Compositions, characteristics and antioxidative activity. *Waste Biomass Valorization* 2020, 11, 1657–1670. [CrossRef]

22. Giraldo-Rios, D.E.; Rios, L.A.; Zapata-Montoya, J.E. Kinetic modeling of the alkaline deproteinization of Nile-tilapia skin for the production of collagen. *Heliyon* 2020, 6, e03854. [CrossRef]

23. Zhu, S.; Yuan, Q.; Yang, M.; You, J.; Yin, T.; Gu, Z.; Hu, Y.; Xiong, S. A quantitative comparable study on multi-hierarchy conformation of acid and pepsin-solubilized collagens from the skin of grass carp (Ctenopharyngodon idella). *Mater. Sci. Eng. C* 2019, 96, 446–457. [CrossRef] [PubMed]

24. Ambati, R.R.; Phang, S.-M.; Ravi, S.; Aswathanarayana, R.G. Astaxanthin: Sources, extraction, stability, biological activities and its commercial applications—A review. *Mar. Drugs* 2014, 12, 128–152. [CrossRef]

25. Sun, J.; Zhang, J.; Zhao, D.; Xue, C.; Liu, Z.; Mao, X. Characterization of turbot (Scophthalmus maximus) skin collagen and fabrication of collagen sponge as a good biocompatible biomedical material. *Process Biochem.* 2017, 63, 229–235. [CrossRef]

26. Ali, A.M.M.; Benjakul, S.; Kishimura, H. Molecular characteristics of acid and pepsin soluble collagens from the scales of golden carp (Probarbus jullieni). *Emir. J. Food Agric.* 2017, 29, 450–457. [CrossRef]

27. Ahmed, R.; Haq, M.; Chun, B.-S. Characterization of marine derived collagen extracted from the by-products of bigeye tuna (Thunnus obesus). *Int. J. Biol. Macromol.* 2019, 135, 668–676. [CrossRef]

28. Kim, H.K.; Kim, Y.H.; Kim, Y.J.; Park, H.J.; Lee, N.H. Effects of ultrasonic treatment on collagen extraction from skins of the sea bass Lateolabrax japonicus. *Fish. Sci.* 2012, 78, 485–490. [CrossRef]

29. Kittiphattanabawon, P.; Sriket, C.; Kishimura, H.; Benjakul, S. Characteristics of acid and pepsin solubilized collagens from Nile tilapia (Oreochromis niloticus) scale. *Emir. J. Food Agric.* 2019, 31, 95–101. [CrossRef]

30. Benjakul, S.; Sinthusamran, S.; Visessanguan, W.; Rohotra, S.; Kishimura, H.; Prodpran, T.; Meesane, J. Extraction and characterization of pepsin-solubilized collagens from the skin of bigeye snapper (Priacanthus tayenus and Priacanthus macracanthus). *J. Sci. Food Agric.* 2010, 90, 132–138. [CrossRef] [PubMed]

31. Plepis, A.M.D.; Goisis, G.; Das-Gupta, D.K. Dielectric and pyroelectric characterization of anionic and native collagen. *Polym. Eng. Sci.* 1996, 36, 2932–2938. [CrossRef]

32. Cruz-López, H.; Rodriguez-Morales, S.; Enriquez-Paredes, L.M.; Villarreal-Gómez, L.J.; Olivera-Castillo, L.; Cortes-Santiago, Y.; López, L.M. Comparison of collagen properties from the skin and swim bladder of Gulf corvina (Cynoscion othonopterus). *Tissue Cell* 2021, 52, 101593. [CrossRef]

33. Foegeding, E.A. Characteristics of edible muscle tissues. In *Food Chemistry;* Marcel Dekker, Inc.: New York, NY, USA, 1996.

34. Song, Z.; Liu, H.; Chen, L.; Chen, L.; Zhou, C.; Hong, P.; Deng, C. Characterization and comparison of collagen extracted from the skin of the Nile tilapia by fermentation and chemical pretreatment. *Food Chem.* 2021, 340, 128139. [CrossRef] [PubMed]

35. Shahidi, F.; Zhong, Y. Measurement of antioxidative activity. *J. Funct. Foods* 2015, 18, 757–781. [CrossRef] [PubMed]

36. Shimada, K.; Fujikawa, K.; Yahara, K.; Nakamura, T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food Chem.* 1992, 40, 945–948. [CrossRef]