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Isolation and Characterization of Collagens extracted from the Skin of Pufferfish, *Lagocephalus wheeleri*.

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ABSTRACT

Raw materials obtained from fish have received significant attention in recent years as an alternative source of collagen as the outbreak of bovine spongiform encephalopathy and foot and mouth disease have led to decline supply of collagen from mammalian sources. Collagen has a wide range of application in pharmaceutical, biomedical and food industries. Acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) from *Lagocephalus wheeleri* skin were isolated and characterized. The yields of ASC and PSC were 6% and 14% (based on wet weight basis), respectively. According to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns, both ASC and PSC were characterized to type I collagen. The results of Fourier transform infrared (FTIR) spectroscopy of both ASC and PSC were found to be similar. The denaturation temperature (T_d) of ASC and PSC were 32.9 °C and 31.9 °C, respectively which was about 5 °C lower than that of calf skin collagen (37 °C). Both collagen exhibited high solubility in acidic pH and there was decrease in solubility of collagens with increase in NaCl concentrations. Hence, the extraction of collagen from *L. wheeleri* skin could be a favorable way to obtain the value-added products.

Keywords: Collagen; *Lagocephalus wheeleri;* skin; isolation; characterization; microscopic analysis.

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INTRODUCTION

Collagen is the most abundant protein in living tissues making up about 30% of the whole body protein content [1]. Collagen provides mechanical strength to various parts of the body and helps to protect skin from absorption and spreading of pathogenic organisms and environmental toxins as well. Collagen is commonly derived from by-products of land animals and is widely used in cosmetic surgery, anti-wrinkle creams, skin care products, shampoos, wound healing aids, treating burns and in food industry.

Most of the industries are generally using cows and pigs as the source of collagen. However, concerns related to transmission of highly infectious diseases like Bovine Spongiform Encephalopathy, Transmissible Spongiform Encephalopathy and Foot and Mouth Disease from pigs and cattle, have limited the extraction of collagen from these sources for industrial purposes [2]. Collagen from the scales of marine fishes [3], skin of flatfish [4], scales of spotted golden goatfish [5], skin and bone of bigeye snapper [2], skin of *Lagcephalus gloveri* [6] has been isolated and characterized.

Pufferfish, *Lagocephalus wheeleri* belongs to family Tetraodontidae is a non-toxic species [7]. These fishes are not consumed by many of the predatory fishes due to their inflation behavior and are inedible [8]. Increase in population of these fishes in coastal areas can bring adverse ecological impacts. Due to these reasons, the extraction of collagen from skin (non-toxic organ) of these fishes can potentially be used as an alternative source of collagen for use in various applications.

MATERIALS AND METHODS

Fish skin preparation

Lagocephalus wheeleri Abe, specimens were collected from Machilipatnam coast, Andhra Pradesh in the eastcoast of India in the month of November 2013. They were transported to laboratory and stored at -20 °C until used. The skin was dissected; separated, descaled and adhering muscles were removed manually. The skin was washed in chilled distilled water and then cut into small pieces using scissors, kept in polyethylene bags and stored at -20 °C until use. Pepsin (1:10,000 units, from Porcine stomach mucosa) and high molecular weight protein markers (PMWH) were purchased from Genei, Bengaluru India. All other reagents were of analytical grade.

Determination of proximate composition

Skin portions were taken from different parts of the body. Proximate composition was determined after blending. Moisture, ash, protein and lipid contents were analyzed in the skin of *L. wheeleri* according to AOAC methods of (2000) [9].

Extraction of Acid Soluble Collagen (ASC)

All extraction steps were carried out at 4°C with continuous stirring. Acid soluble collagen was extracted according to the method of Senaratne et al. (2006) [6] with slight modifications. *L. wheeleri* skin was cut into small pieces (1 to 2 cm) and to remove non-collagenous protein, skin pieces were treated with 0.1N NaOH at a solid to solvent ratio of 1:10 (w/v) for 3 days. The alkali solution was changed every day. After 3 days, skin pieces were washed in ample amount of chilled distilled water till the washed water become neutral. Then 10 % butyl alcohol was added at solid to solvent ratio of 1:10 (w/v) to skin pieces for 24 hr. Butyl alcohol is used for de-fatting purpose. After 24 hr, skin was washed in ample amount of cold distilled water. Acetic acid solution (0.5 M) was prepared and extraction was carried out for 3 days. The viscous solution was centrifuged at 12,000xg for 1 hr at 4 °C and supernatant was collected. All the pellets were stored together in a beaker at 4 °C and were used for the extraction of pepsin soluble collagen. The supernatant was salted out by adding NaCl to obtain a final concentration of 2.5 M in the presence of Tris-HCl (pH 7.5). The obtained precipitate was separated by centrifugation at 12,000xg for 1 hr at 4°C. The resultant pellet was dissolved in minimum amount of 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, then with distilled water and lyophilized.

Extraction of Pepsin Soluble Collagen (PSC)

Undissolved matter from acid soluble collagen, collected as pellet was further extracted in 0.5 M acetic acid containing 1% pepsin (w/w) at a ratio of 1:10 (w/v) for 48 hr at 4°C following the method of Matmaroh et al. (2011) [5]. After extraction, centrifugation was performed at 12,000xg for 1 hr and supernatant was collected. Salting out was performed same like ASC. Resultant precipitate was separated by centrifugation, dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, distilled water and lyophilized and stored. Collagen extracted by the means of pepsin was referred to as pepsin soluble collagen. Both ASC and PSC were subjected to following analysis.

Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of ASC and PSC was performed according to the method of Laemmli (1970) [10] with slight modification, using 7.5% separating gel containing 0.1% SDS at pH 8.8 and 5% stacking gel. About 5 mg of ASC and PSC were dissolved in 1 ml of sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 20% (v/v) glycerol) with 10% 2-mercaptoethanol. Calf skin acid solubilized type I collagen (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was used as standard collagen and was prepared same as sample preparation and 15 µl of all samples were loaded per well. High-molecular weight marker (Genei, Bengaluru) mixed with sample buffer was loaded as 8 µl/well at alongside the *L. wheeleri* collagen sample. Electrophoresis was performed at a constant current of 20 mA/gel using Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond CA, USA). After electrophoresis, gel was stained using 0.1% Coomassie Brilliant Blue R 250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid.

Peptide mapping

Peptide mapping of ASC and PSC was performed according to the method of Saito et al. (2002) [11] with some modifications. The collagen samples (0.5 mg) were dissolved in 0.1 ml of 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5 % SDS (w/v). The mixture was then heated at 100 °C for 10 min. After this, the mixture was cooled and the digestion was initiated by adding 10 µl of the same buffer containing 5 µg of *Staphylococcus aureus* V8 protease (Endoproteinase Glu-C, Sigma, P2922-100UN) and the reaction mixture was incubated at 37 °C for 25 min. The reaction was terminated by adding the reaction mixture to boiling water for 3 min. Electrophoretic-patterns of the peptides were observed using 10 % separating gel. Peptide mapping of calf skin collagen was also performed in the same manner.

Determination of denaturation temperature of ASC and PSC

The denaturation temperature of both ASC and PSC was determined by the method of Kittiphattanabawon et al. (2005) [2]. The lyophilized collagen samples were dissolved in 0.1 M acetic acid with a sample and solution ratio of 1:40 (w/v). The mixtures were kept at 4°C for 2 days. Calorimetric measurements were performed using a differential scanning calorimeter, DSC (NETZSCH, DSC 204). The collagen samples were accurately weighed in aluminum pans and sealed. The samples were scanned over a range of 20-50 °C at a heating rate of 1 °C / min using liquid N_2 as cooling medium. An empty sealed pan was used as the reference. The peak of DSC thermogram was used to determine the denaturation temperature.

Fourier transforms infrared (FTIR) spectroscopy

FTIR spectra of both ASC and PSC from *L. wheeleri* skin were recorded using Fourier transforms infrared spectroscopy (IRAffinity-1) according to method described by Muyonga et al. (2004) [1]. The spectra were acquired over the range of 4,000 to 500 cm^{-1} at a data acquisition rate of 4 cm^{-1}.

Effect of pH and NaCl on collagen solubility

The effect of pH and NaCl on the solubility of collagen was determined following the method of Montero et al. (1991) [12]. Collagen samples were dissolved in 0.5 M acetic acid with gentle stirring at 4 °C to obtain final concentration of 3 and 6 mg/ml.

To determine the effect of pH on collagen solubility, 8 ml of collagen solution (3 mg/ml) in the centrifuge tube were adjusted with either 6 N HCl or 6 N NaOH across the pH range (1-10) and the final volume was made up to 10 ml using distilled water previously adjusted to the same pH as the collagen solution. These mixtures were stirred gently at 4 °C for 30 min and then centrifuged at 20,000xg at 4 °C for 30 min. Protein content in the supernatant was determined by the method of Lowry et al. (1951) using bovine serum albumin as the protein standard. Relative solubility was calculated in comparison with that obtained at the pH exhibiting highest solubility.

To determine the effect of NaCl concentration on collagen solubility, 5 ml of collagen solution (6 mg/ml) was mixed with 5 ml of NaCl in 0.5 M acetic acid at various concentrations (0%, 2%, 4%, 6%, 8%, 10%, (w/v)). The mixtures were stirred gently at 4 °C for 30 min and then centrifuged at 20,000xg at 4 °C for 30 min. Protein content and relative solubility in the supernatant were measured and calculated as described by the methods above.

Microscopic analyses

Micro-architecture of isolated collagens was analyzed by scanning electron microscope (ZEISS, (EV018) USA). The collagen samples were cut using a punch and mounted on a standard SEM sample holder and fixed. The surface of collagen samples were sputtered with gold and introduced into specimen chamber of SEM and examined for surface morphology. The SEM observations were made at 15 kV accelerating voltage and secondary electron imaging was employed to scan the microscopic images of the samples.

RESULTS AND DISCUSSIONS

Proximate composition of *Lagocephalus wheeleri* skin

According to proximate composition analysis, *L. wheeleri* contained very high moisture content which was found to be 75.75%. This value was similar to the results obtained from *Lagocephalus gloveri* skin (73.4%) [6] and Nile perch skin (68.4-72.7%) [1] but it was found to be greater than moisture content obtained for balloonfish skin (62.23%) [13] and bigeye snapper skin (64.08%) [2]. The crude protein, crude lipid, and ash content of the *L. wheeleri* skin on a dry weight basis were found to be 90.61%, 2.55%, and 6.42%, respectively. The crude protein, crude lipid, and ash content of *L. gloveri* skin were found to be 90.3%, 1.3%, and 8.4%, respectively on dry weight basis [6]. The crude protein, fat and ash contents of Nile perch skin were 21.6%, 6.8%, and 6.0%, respectively [1]. The crude protein content of *L. wheeleri* skin was found to be similar to *L. gloveri* skin but higher than Nile perch skin. The crude lipid of *L. wheeleri* skin was higher than *L. gloveri* skin but the ash contents were found to be lower than that of *L. gloveri* skin.

Yield of Acid Soluble Collagen (ASC) and Pepsin Soluble Collagen (PSC) from *L. wheeleri* skin

Yield of ASC and PSC from the skin of *L. wheeleri* were 6% and 14% (based on the wet weight of skin), respectively. This indicated that the use of 1% pepsin to extract the residual matter from ASC extraction increased the yield of collagen. This result can be explained on the fact that the collagen molecules in the skin of *L. wheeleri* were most probably linked by covalent bonds through the condensation of aldehyde groups of the telopeptide region as well as the intermolecular cross-linking which is responsible for decrease in solubility of collagen [14]. Thus, pepsin showed the efficacy in increasing the yield of collagen. Similar results were observed for collagen from brownbanded bamboo shark (ASC: 9.38%, PSC: 8.86%) [15], striped catfish (ASC: 5.1%, PSC: 7.7%) [16] on wet weight basis.

Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) pattern

The protein patterns of ASC and PSC samples were analyzed by SDS-PAGE using 7.5% resolving gel as shown in Figure 1. Both ASC and PSC consisted of 2 α bands (α1 and α2) with their molecular weight of about 100 kDa and one β band of molecular weight about 205 kDa which was found to be similar to the electrophoretic pattern of typical type I collagen. The similar electrophoresis pattern of ASCs and PSCs are observed as PSCs are obtained by cleaving the cross-links at the telopeptide region of collagen molecule without damaging the integrity of the triple helix [17]. Collagen is a type of fibrous protein and is found in connective tissue. It has three left handed helices which form a right handed super helix. The protein patterns

of ASC and PSC were similar to the patterns observed for several other fish species [18]. Type I collagen has also been reported from the skin of *L. gloveri* [6], ocellate pufferfish [19], Nile perch [1] and bigeye snapper [2].

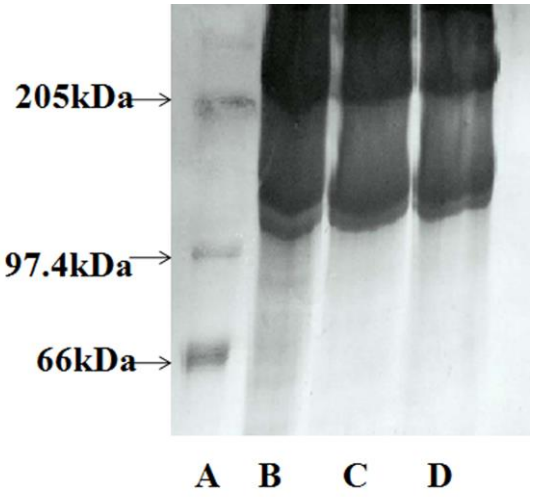

Figure 1: SDS-PAGE patterns of (A) marker protein, (B) Calf skin collagen, (C) acid-soluble collagen (ASC) and (D) pepsin-soluble collagen (PSC) from *Lagocephalus wheeleri* skin.

Peptide mapping

The electrophoretic pattern of V8 protease digested *Lagocephalus wheeleri* collagen suggested that the ASC and PSC gave similar peptide maps; however, these patterns were different from standard type-I calf skin collagen (Figure 2). V8 protease hydrolysed the α-chain and high molecular weight crosslinked molecules of ASC and PSC from *Lagocephalus wheeleri* skin in to small molecular weight peptides ranging from 29kDa to205kDa. However the α-components, the α1 and α2 of calf skin collagen were hydrolysed to some extent. This result suggested that α and molecular cross-linked components of calf skin collagen were more resistant to hydrolysis by V8 protease whereas ASC and PSC from *Lagocephalus wheeleri* skin were more susceptible to digestion. It is reported that V8 protease exhibits a high degree of specificity for glutamic acid and aspartic acid residues of peptides and proteins [20]. So ASC and PSC from *L. wheeleri* skin might contain more glutamic acid and aspartic acid residues than calf skin collagen. It is reported that peptide maps of collagen differ among other sources and species [21].

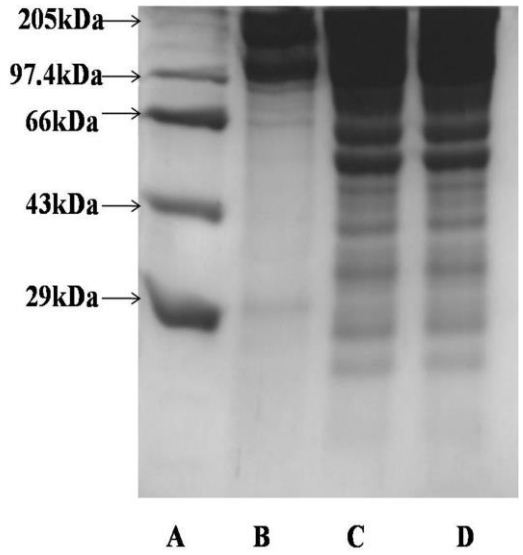

Figure 2: Peptide maps of ASC and PSC digested by V8 protease. (A) marker protein, (B) Calf skin collagen, (C) acid-soluble collagen (ASC) and (D) pepsin-soluble collagen (PSC)

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Denaturation temperature of collagen

Denaturation temperature is an important factor in determining the thermal stability of collagen. Differential scanning calorimetry (DSC) gives information about the thermal transition of protein by measuring heat flow between sample and reference zone. The denaturation temperature (T_d) is determined by the maximum transition point (the endothermic peak) of the thermal denaturation curves. DSC thermograms of ASC and PSC from the skin of *L. wheeleri* are shown in Figure 3a and 3b, respectively. The ASC and PSC showed transitional curves with maximum temperature (T_d) of 32.9 °C and 31.9 °C, respectively. The T_d of ASC and PSC of *L. wheeleri* skin were found to be lower around 5 °C than that of calf skin collagen (37 °C) [17]. This difference can be correlated with environmental conditions and body temperature [22]. The stability of helical structure of collagen molecule depends on the imino acid content of collagen [23]. The T_d of *Lagocephalus gloveri* was found to be *28*°C [1], ocellate pufferfish, 28°C [19], skin of tiger puffer, 28.4°C, red stingray, 32.2°C [24].

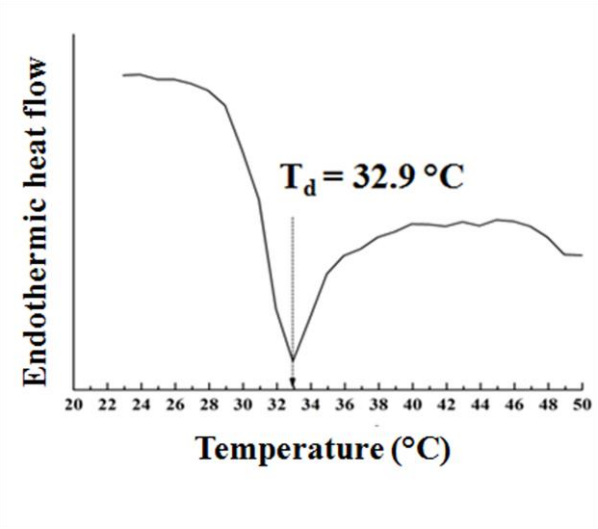

Figure 3a: Thermal denaturation temperature of acid-soluble collagen (ASC) from *Lagocephalus wheeleri* skin.

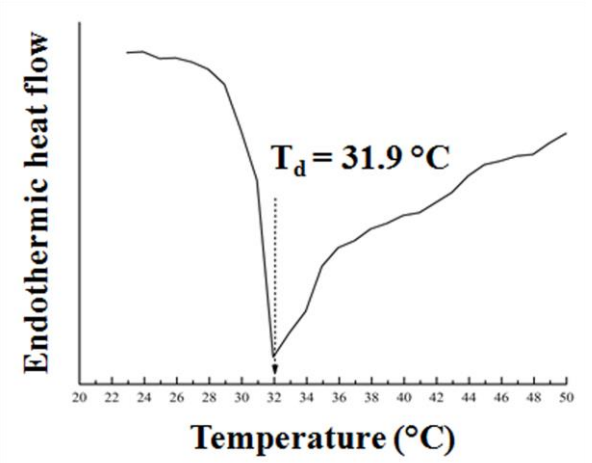

Figure 3b: Thermal denaturation temperature of pepsin-soluble collagen (PSC) from *Lagocephalus wheeleri* skin.

FTIR spectroscopy

FTIR spectra of both ASC and PSC from the skin of *L. wheeleri* are demonstrated in Figure 4 and table 1. The ASC and PSC had almost similar spectra with slight differences were observed. Both ASC and PSC exhibited characteristic peaks of amide I, II, III as well as amide A and B. Amide A band positions of ASC and PSC were found at wave-number 3417.86 and 3419.79 cm^{-1}, respectively. Amide A band is generally associated

with N-H stretching vibration and it occurs in the wave-number range 3,400-3,440 cm^{-1} [25]. Amide B band is associated with asymmetrical stretch of CH_2 [1]. Amide B band position of ASC and PSC was observed at 2926.01 and 2927.02 cm^{-1}, respectively. The amide I band of ASC and PSC was found at wave-number 1620.21 and 1620.23 cm^{-1}, respectively. The amide I band is mainly associated with the stretching vibration of carbonyl group (C=O), occurring in the range of 1600-1700 cm^{-1} [26] and is an important factor in determining the secondary structure of protein. Amide II of ASC and PSC was found at 1535.34 and 1536.34 cm^{-1}, respectively. Amide II band is associated with NH bending and CN stretching vibration [27]. Amide III band having absorption between 1236 and 1452 1 cm^{-1} suggests the presence of helical structure as well as consists of components from C-N stretching and N-H in plane bending from amide linkages [28]. The band position of Amide III for ASC and PSC was observed at 1402.25 and 1403.45 cm^{-1}, respectively. In studies of flatfish skin collagen by Heu et al. (2010) [4], amide bands (amide A, amide I, amide II and amide III) of flatfish skin 3,312, 1,651, 1,549, and 1,248 cm^{-1}, respectively and there was no difference in amide region between ASC and PSC were found. From FTIR spectra study of collagen from *L. wheeleri* skin, a slight change in the structure of PSC was obtained. It can be due to removal of telopeptide region by the aid of pepsin but the triple helical structure was still predominant.

Table 1: Peak assignments of the FTIR spectra of collagens extracted from *Lagocephalus wheeleri* skin

Region	Peak wavenumbers (cm^{-1})		Assignments
	ASC	PSC	
Amide A	3417.86	3419.79	NH stretching vibration
Amide B	2926.01	2927.02	Asymmetrical stretch of CH_2
Amide I	1620.21	1620.23	Stretching vibration of carbonyl group (C=O)
Amide II	1535.34	1536.34	NH bending and CN stretching vibration
Amide III	1402.25	1403.45	NH bend coupled with CN stretch

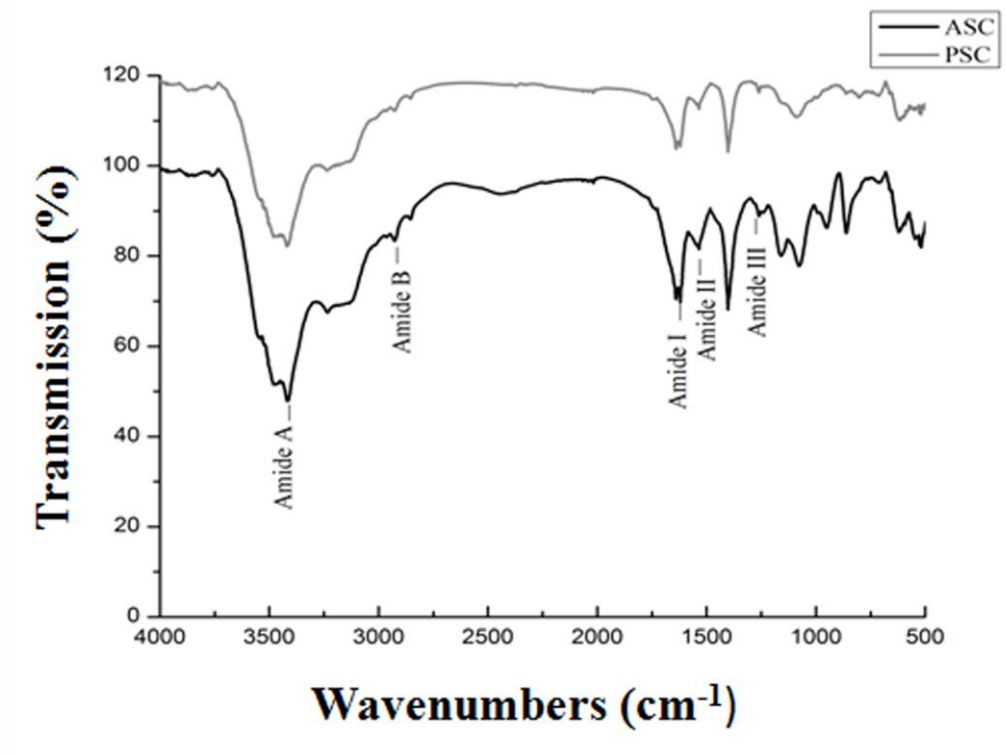

Figure 4: Fourier transform infrared spectra (FTIR) of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) from *Lagocephalus wheeleri* skin.

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Effect of pH and NaCl concentration on collagen solubility

The effect of pH on the solubility of ASC and PSC in 0.5 M acetic acid is as shown in Figure 5a. The highest solubility of both ASC and PSC was found at pH 3. Similar result was reported for collagen for ASC from grey mullet, lizard fish and yellow-back sea bream [3]. At above pH 3, solubility started decreasing. Low solubility was observed at pH 7-10. A protein has a net negative or positive charge, when pH values are above and below pI [29]. These results indicate that the pH of ASC and PSC reached the p*I* in range (7-10), which results in protein precipitation and the net charge on protein becomes zero [30]. However, at pHs lower than the pI, the repulsion forces between charged residues of protein molecules are greater than the attracting forces, which results in protein solubilisation [31].

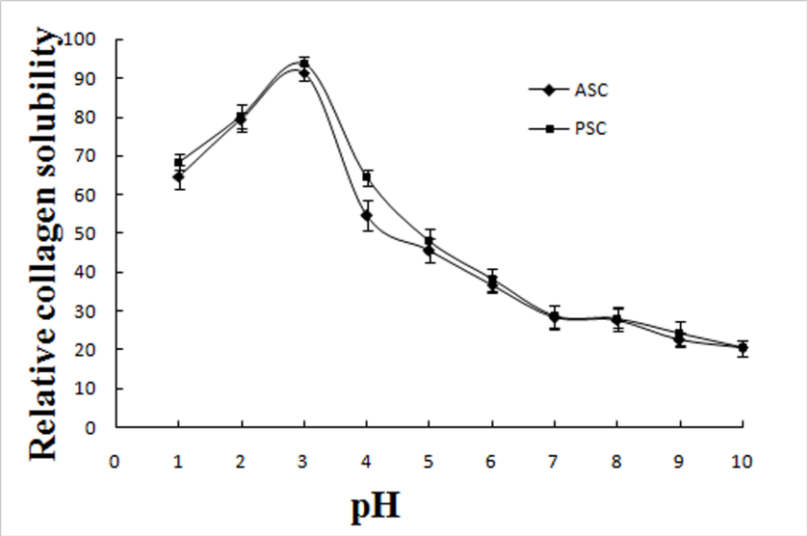

Figure 5a: Effect of pH on solubility of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC)

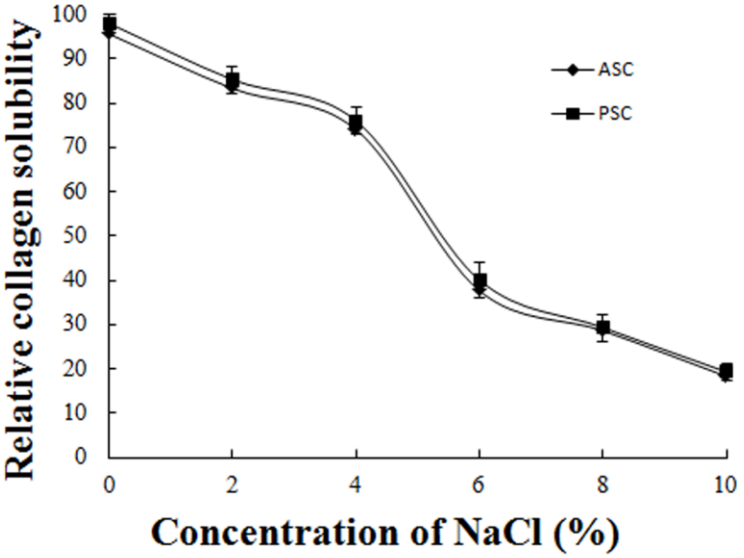

Figure 5b: Relative solubility of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) at various NaCl concentrations.

Solubility of ASC and PSC at different NaCl concentration is shown in Figure 5b. Both ASC and PSC showed similar solubility behavior. There was decrease in solubility observed for ASC and PSC in 0.5 M acetic

acid with increase in NaCl concentration. There was a sharp decrease in solubility above 4% NaCl concentration. The decrease in solubility of collagens can be explained on the basis of 'salting-out' effect, which occurs at relatively high NaCl concentrations [32]. The result was in accordance with that of Heu et al. (2010) [4].

Microscopic analysis of collagen

Scanning electron microscopy images of ASC and PSC from *L. wheeleri* skin are shown in Figure 6a and 6b, respectively. The fibrillar structures of collagen samples were observed. The irregular, wavy collagen fibers were found to be arranged singly or in small groups. These collagen fibrils formed bundles which varied in width and thickness and intertwined with each other. The cross-linked fiber networks can be mediated by hydrogen bond, hydrophobic interaction, electrostatic bond, entropic and dispersion forces [33]. The regular porous structure of collagen was clearly visible and the collagen surface was found to be rough and uneven.

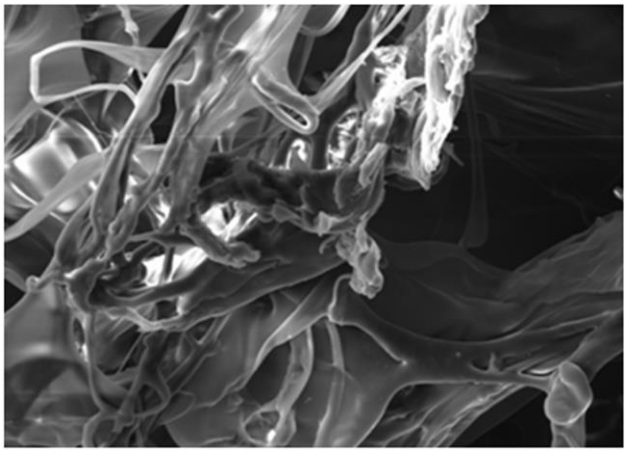

Figure 6a: Scanning electron microscopy images of ASC from *Lagocephalus wheeleri* skin.

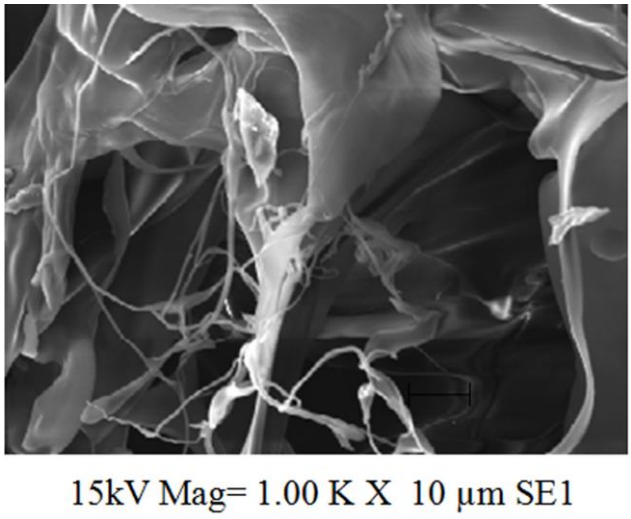

Figure 6b: Scanning electron microscopy images of PSC from *Lagocephalus wheeleri* skin.

CONCLUSION

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) could be isolated from the skin of *Lagocephalus wheeleri.* Both the collagens extracted in the study were identified as type I collagen. Pepsin digestion could increase the yield of collagen. The denaturation temperatures of the collagens (ASC and PSC) were around 32.9 °C and 31.9 °C, respectively. Collagens showed high solubility at acidic pH and the solubility

markedly decreased with increase in concentration of NaCl. Scanning electron microscopy showed the fibrillar structure of collagens. Collagens isolated from *Lagocephalus wheeleri* skin have the potential to be an alternative source of collagen in various fields.

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REFERENCES

[1] Muyonga JH, Cole CGB, Duodu KG. Food Chem 2004; 86: 332-523.
[2] Kittiphattanabawon P, Benjakul S, Visesssangun W, Nagai T, Tanaka M. Food Chem 2005; 89: 363-372.
[3] Thuy LTM, Okazaki E, Osako K. Food Chem. 2014; 149: 264-270.
[4] Heu MS, Lee JH, Kim HJ, Jee SJ, Lee JS, Jeon YJ, Shahidi F. Kim JS. Food Sci Biotechnol 2010; 19: 27-33.
[5] Matmaroh K, Benjakul S, Prodpran T, Encarnacio A, Kishimura H. Food Chem 2011; 129: 1179-1186.
[6] Senaratne LS, Park PJ, Kim SK. Bioresource Technol 2006; 97: 191-197.
[7] Hwang DF, Kao CY, Yang HC, Jeng SS, Noguchi T, Hashimoto K. Nippon Suisan Gakkaishi 1992; 58: 1541-1547.
[8] Mohamed KS, Sathnandan TV, Kripa V, Zacharia PU. Curr Sci 2013; 104: 426-429.
[9] AOAC. (2000). Official methods of analysis. Arlington: Association of Official Analytical Chemists Inc.
[10] Laemmli UK. Nature 1970;227: 680-685.
[11] Saito M, Kunisaki N, Urano N, Kimura S. J Food Sci 2002; 67: 1319-1322.
[12] Montero P, Jimenez-Colmenero F, Borderias J. J Agric Food Chem 1991; 38: 604-609.
[13] Huang YR, Shiau CY, Chen HH, Huang BC. Food Hydrocolloids 2011; 25: 1507-1513.
[14] Zhang M, Liu WT, Li GY. Food Chem 2009; 115: 826-831.
[15] Kittiphattanabawon P, Benjakul S, Visesssangun W, Nagai T, Kishimura H, Shahidi F. Food Chem 2010;119: 1519-1526.
[16] Singh P, Benjakul S, Maqsood S, Kishimura H. Food Chem 2011; 124: 97-105.
[17] Ogawa M, Moody MW, Portier RJ, Bell J, Schexnayder M, Losso JN. J Agric Food Chem 2003; 51: 8088-8092.
[18] Gómez-Guillén MC, Turnay J, Fernández-Díaz MD, Ulmo N, Lizarbe MA, Montero P. Food Hydrocolloids 2002; 16: 25-34.
[19] Nagai T, Araki Y, Suzuki N. Food Chem 2002; 78: 173-177.
[20] Vercaigne-Marko D, Kosciarz E, Nedjar-Arroume N, Guillochon D.Biotechnol and Applied Biochem 2000; 31:127–134.
[21] Mizuta S, Yamasa Y, Miyagi T, Yoshinaka R. J of Food Sci 1999; 64:991–995.
[22] Rigby BJ. Nature 1968; 219: 166-167.
[23] Wong DWS. 1989. Mechanism and Theory in Food Chemistry. Van Nostrand Reinhold, New York.
[24] Bae I, Osatomi K, Yoshida A, Osako K, Yamaguchi A, Hara K. Food Chem 2008; 108: 49–54.
[25] Sai KP, Babu M. Biochem Mol Biol 2001; 128: 81-90.
[26] Payne KJ, Veis A. Biopolymers 1988; 27: 1749-1760.
[27] Barth A, Zscherp C. Quarterly Reviews of Biophy 2002; 35: 369-430.
[28] Guzzi PAMD, Goissis G, Gupta DK. Polymer Engineering and Sci 1996; 36: 2932-2938.
[29] Vojdani F. 1996. Solubility. In G. M. Hall (Ed). Methods of testing protein functionality. 1st ed: 11-60.
[30] Jongjareonrak A, Benjakul S, Visessanguan W, Nagai T, Tanaka M. Food Chem 2005; 93: 475-484.
[31] Damodaran S. In O. R. Fennema (Ed.), New York: Marcel Dekker. Food Chem 1996; 321-429.
[32] Asghar A, Henrickson RL. J of Food Quality 1982; 5: 271-284.
[33] Nemethy G, Steinberg IZ, Scheraga HA. Biopoly 1963; 1: 43-69.