Quantification and characterization of acid and pepsin-solubilized collagen from Yellowfin tuna (*Thunnus albacarces*) waste material

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Abstract

Yellowfin tuna is the most popular fish species processed for the export market from Sri Lanka and generates significant amount of fish waste. Waste utilization is one of the key priorities in current research. The aim of the study was to quantify and characterize collagen extracted from Yellowfin tuna waste materials. Acid Soluble Collagen (ASC) and Pepsin Soluble Collagen (PSC) were extracted from Yellowfin tuna fish skin, bones and fins. The collagen yields of skin, bones and fin of Yellowfin tuna were 21.30, 0.89 and 1.22 %, respectively. The extracted collagen samples were characterized using, SDS-PAGE analysis, Fourier Transform Infrared Spectroscopy (FTIR) and amino acid profile analysis. Similar SDS band patterns were recorded for both extracted collagen and human collagen Type-1 which composed of double pattern for α1 (approximately 132 to 139 kDa), α2 (approximately 120 and 124 kDa position) and β chains (approximately 220 to 229 kDa) indicating the higher quality of extracted collagen. FTIR analysis proved that ASC and PSC are integrated and native. Glycine was the most abundant amino acid identified in the Yellowfin tuna collagen. As per the findings of this research collagen can be successfully extracted from Yellowfin tuna waste with a promising yield from the Yellowfin tuna skin (ASC: 11.10% and PSC: 10.20%). Extraction of Collagen from Yellowfin tuna waste is an eco-friendly and economical solution for Yellowfin tuna waste.

Keywords: fish waste utilization, Yellowfin tuna, collagen, SDS-PAGE analysis, Fourier Transform Infrared Spectroscopy (FTIR)

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Introduction

During fish processing, a great deal of wastes including liquids and solids are produced. The wastes are generated when the fish is gutted, headed and further processed. Solid wastes include 50-70 % of raw materials and they consist of different parts, head, intestine, viscera, bone etc., depending on used process (Morrissey *et al*., 2000). In Sri Lanka fish wastes are currently underutilized and therefore, there is a huge potential to develop value added products. Today most of the fish waste is used as raw materials for fish meal production in Sri Lanka. Large quantities of Yellowfin tuna are commercially processed in fish processing factories in Sri Lanka for *Sashimi* market, a delicate tasting raw fish product popular in several other countries. Processing of Yellowfin tuna generates a significant amount of high-quality fish waste produced under more hygienic conditions due to strict export regulations imposed on them which can be used as a raw material for other valuable products. With increased recent interest in the utilization of industrial byproducts, finding different sources, optimizing extracting conditions and characterization of extracts have recently become important research topics. Collagen is a major class of structural proteins in bone, skin, cartilage and connective tissues (Ogawa *et al.*, 2003; Liu *et al.*, 2007). Collagens comprise different types and in the case of fish, collagen Type I is mainly present in the skin. These molecules present a quaternary structure involving three polypeptides (alpha chains), each twisted in left-handed helix and arranged together in right-handed super-helix structure; this is stabilized by the existence of inter chain crosslinks (Bailey *et al.*, 1998). Fish collagen Type I is typically constituted by two alpha chains, α1 and α2, of similar molecular weight of about 100 KDa (Kimura and Ohno, 1987; Gomez-Guillen *et al.*, 2002). The skin, fins, bones and scales of fish arising from processing can be considered as collagen sources. Researchers have studied collagen removal from fish wastes (Swatschek *et al.*, 2002; Sadowska *et al.*, 2003). In its purified form, collagen is a bio material. Generally, collagen has a wide range of industrial applications in food, cosmetic, biomedical, pharmaceutical, leather and film industries (Kittiphattanabawon *et al.*, 2005). The search for new sources of collagen has resulted in studies of functional properties of marine source of collagens: e.g. skins of salt and fresh water fish (Ikoma *et al.*, 2003), shark skins (Yoshimura *et al.*, 2000), brown stripe red snapper skin (Jongjareonrak *et al.*, 2005), skins and bones of bigeye snapper (Kittiphattanabawon *et al.*, 2005), squid skin (Kolodziejska *et al.*, 1999), skins of young and adult Nile perch (Muyonga *et al.*, 2004) and outer skins of the paper nautilus (Nagai and Suzuki, 2000). The collagen from the fish waste is unlikely to be associated with infections such as Bovine Spongiform Encephalopathy (BSE), Transmissible Spongiform Encephalopathy (TSE) and Foot and Mouth Disease (FMD). Therefore, fish waste also may be an effective alternative source for collagen production (Jongjareonrak *et al.*, 2005). Use of waste as a source of collagen can beneficially impact

on fish waste management. Therefore, if high-quality collagen can be extracted from Yellowfin tuna byproducts, it would be an economically important source of disease-free collagen and substantially reduce the amount of pollution from waste products. Because of all these reasons many researchers have attempted to extract collagen from fish materials using different methods including acid extraction (ASC), alkali extraction and enzyme (Pepsin) extraction (PSC) (Hukimi and Sarbon, 2018; Hamdan and Sarbon, 2019). However, the acid extraction and enzyme extraction methods are identified as the most common collagen extraction methods. PSC is commonly applied in combination with 0.5 M of acetic acid (Wu *et al*., 2014; Kaewdang *et al*., 2014). The objectives of this study were quantification and characterization of acid soluble and pepsin soluble collagen from different waste material of Yellowfin tuna including skin, bones and fins.

Materials and Methods

Chemical reagents

All chemical reagents, human collagen and molecular marker (V849A) used in this study were purchased from Sigma-Aldrich (USA), BDH (UK) and Promega (USA) respectively through local suppliers.

Raw materials

Waste (skins, bones and fins) of Yellowfin tuna fish was collected from Jay Sea Foods Processing (Pvt.) Ltd, Negombo, Sri Lanka. Skins, bones and fins were transported to the Aquatic Product Development Laboratory of National Aquatic Resources Research and Development Agency (NARA), Sri Lanka in chilled condition. Then, cleaned properly and stored at -20 ^{0}C until extraction.

Preparation of collagen

Extraction of Acid-Soluble Collagen (ASC) from skin

Collagen was extracted using the methods of (Nagai and Suzuki, 2000) with slight modifications. All the preparations were performed at 4 ^{0}C. The skins were first cut into small pieces of about 0.5 cm in length. Then deproteinized with 0.1 M NaOH at a sample: alkaline ratio of 1:8 (w/v) to remove non-collagenous proteins. The mixture was stirred (Velp Scientifica DLS overhead stirrer) and the waste was washed with distilled water until neutral pH was achieved. The deproteinized skins were then defatted with 10% butyl alcohol at sample: alcohol ratio of 1:10 (w/v) overnight and washed with distilled water (to remove alcohol) thoroughly before extraction with 0.5 M acetic acid (to solubilize the fibril collagen) for 1 day. Skins were removed after that and the remaining solution was subjected to salting-out procedure by adding NaCl to a final concentration of 2.5 M in the presence of 0.05 M tris (hydroxymethyl) aminomethane, pH 7.0 to precipitate the solubilized collagen. The obtained precipitate was collected as pellets by

centrifugation at 4 ^{0}C, (Digicen 21R cooling centrifuge) at 5,000 rpm for 30 minutes. Pellets were dissolved in 0.5 M acetic acid and dialyzed against 0.1 M acetic acid in order to remove salts and acetic acid and acid-soluble collagen was lyophilized.

Extraction of ASC from fish bones

Fish bones were first cut into small pieces and soaked in distilled water for five hours. Next, the bones were immersed in 0.1 M NaOH at sample to alkali ratio of 1:5 (w/v) to remove non-collagenous proteins for 24 hours. Bones were decalcified with 0.5 M ethylene-diamine tetra acetic acid (EDTA) (pH 7.4) for 5 days before defatting with 10% butyl alcohol. Solution was subjected to salting-out and precipitate was collected as pellets by centrifugation. It was dissolved in 0.5 M acetic acid and dialyzed against 0.1 M acetic acid for one day. Finally, Acid-Soluble Collagen (ASC) was extracted and lyophilized.

Extraction of ASC from fish fins

The fins were first cut into small pieces of about 0.5 cm in length and immersed in 0.1 M NaOH to remove non-collagenous proteins. Next, washed thoroughly with distilled water and insoluble matter in fins was extracted with 0.5 M acetic acid for three days. After centrifugation collagen in the acid-soluble fraction was salted-out by adding NaCl to a final concentration of 0.8 M, followed by a further precipitation of the collagen by addition of NaCl (final concentration of 2.5 M) at neutral pH (in 0.05 M Tris-HCl, pH 7.5). The resultant precipitate was obtained by centrifugation and dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid and distilled water then lyophilized (ASC).

Extraction of Pepsin-Soluble Collagen (PSC)

PSC was extracted from ASC according to the method of (Hwang *et al*., 2007) with slight modifications. The residue after extracting ASC was thoroughly washed with distilled water and stirred for digestion in 10 volumes of 0.5 M acetic acid containing porcine pepsin with substrate ratio of 1:20 (w/w) for two days. It was centrifuged at 5,000 rpm for 30 minutes. The pellets were then washed with distilled water at a 1:2 (w/v) ratio. The supernatant from pepsin treatment and the filtrate from rinsing were combined and subjected to salting-out and dialysis by the same procedure as explained for the purification procedures of ASC. The freeze-dried product was designated as PSC.

Analysis of extracted collagen samples

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and each sample was separated according to Weber and Osborn (1969) for characterization. The vertical slab gel was used for SDS-PAGE (7.5% polyacrylamide

gel concentration) to analyze the extracted collagen. The samples were dissolved in 0.02 M sodium phosphate containing 1 % Sodium Dodecyl Sulphate and 3.5 M urea as a denaturing agent (pH 7.2). The mixtures were then centrifuged at 5,000 rpm for 5 minutes at room temperature to remove undissolved debris. Solubilized samples containing 20 μg μl^{-1} of protein were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris– HCl, pH 6.8, containing 4 % SDS, 20 % glycerol). Samples were loaded on to the gel and a constant current of 15 mA/gel was passed through for 75 min. Broad range molecular weight markers (V849A10–225 kDa) (Promega, USA) were used to estimate the molecular size of proteins. Type I human collagen was used as positive control. The gels were visualized by staining with Colloidal Coomassie Brilliant Blue G-250.

Fourier Transform Infrared Spectroscopy (FTIR)

Extracted and freeze-dried collagen samples (ASC and PSC) were subjected for FTIR spectroscopy and it was carried out at Sri Lanka Institute of Nano Technology (SLINTEC). Bruker Vertex 80 FTIR Spectrophotometer equipped with components to cover the spectral ranges of far, mid and near IR. The FTIR had transmittance, diffuse reflectance and Attenuated Total Reflectance (ATR) modes.

Amino acid analysis

A five-gram aliquot of collagen was dissolved in 6 mL of conc. HCl, and digested in the microwave accelerated reaction system (CEM-MARS-XP-1500+). Microwave digested and neutralized ASC samples of skin, bones and fins were submitted to Industrial Technology Institute (ITI) of Sri Lanka for amino acid analysis. Samples were reconstituted with double distilled water up to 25.0 mL and filtered through 0.45 μm membrane prior to HPLC analysis. Agilent 1,260 infinity HPLC equipped with a quaternary gradient pump, diode array detector and thermos stated column compartment was used. Chromatographic separation was achieved with a ZORBAX Eclipse AAA (4.6 mm x 150 mm, 5 μm) guard column. A combination of standard methods and research methods were used to optimize the chromatographic conditions for the analysis.

Results

Yield of extracted collagen

The collagen extraction from Yellowfin tuna skin resulted high amounts in both ASC and PSC. The value of ASC was about 11.10% based on the lyophilized dry weight, while PSC yielded 10.20% on the same basis.

SDS- PAGE analysis

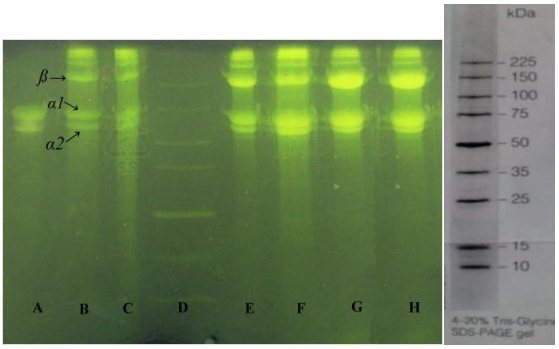


Figure 1. SDS-PAGE profiles of collagen, extracted from Yellowfin tuna (skin, bone and fin)

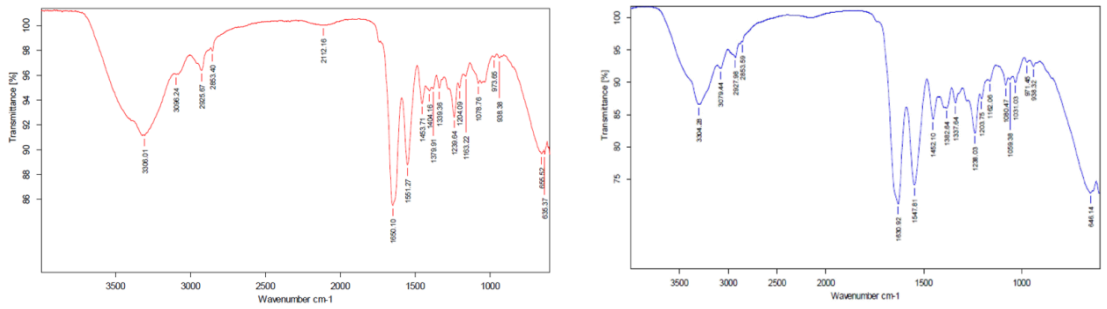
A -Human collagen, B- Skin ASC, C- Skin PSC, D- Molecular weight marker, E- Fin ASC, F-Fin PSC, G- Bone ASC, H- Bone PSC

FTIR Spectroscopy

The infrared spectra of ASC, PSC and the major peaks with their corresponding assignments are shown in Table 1 and Figures from 2 to 4.

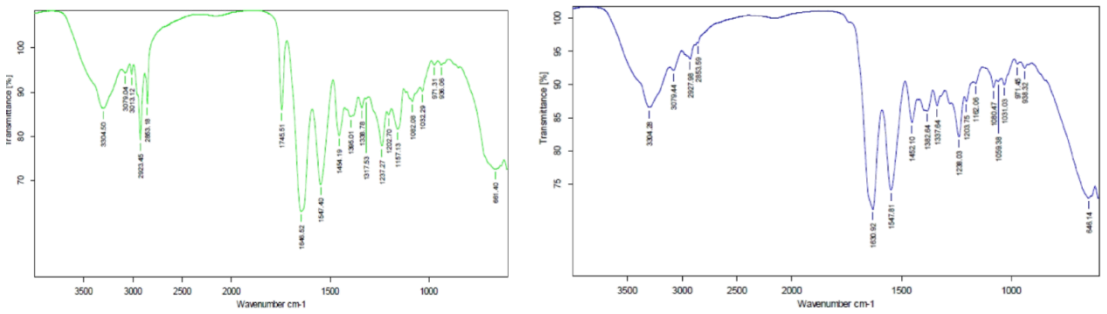
Table 1. FTIR spectra peak locations for Type 1 collagen from yellowfin tuna waste

Type of waste	Amide A[1] cm^{-1}	Amide I[2] cm^{-1}	Amide II[3] cm^{-1}	Amide III[4] cm^{-1}
Tuna skin ASC	3306	1650	1551	1239
Tuna skin PSC	3304	1630	1547	1238
Tuna fin ASC	3298	1635	1545	1239
Tuna fin PSC	3303	1631	1549	1239
Tuna bone ASC	3304	1646	1547	1237
Tuna bone PSC	3304	1630	1547	1238
Assignment	N-H stretching	C=O stretching, hydrogen bonding coupled with COO-	N-H bending coupled with CN stretching	C-H stretching



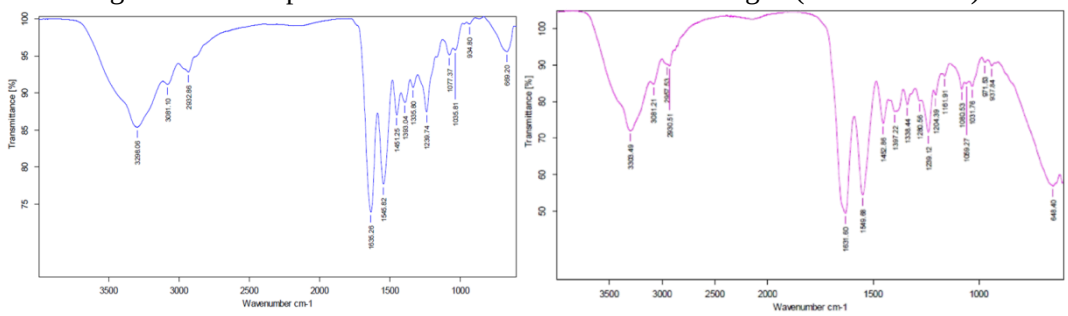
ASC PSC

Figure 2. FTIR Spectrums of Yellow fin tuna skin collagen (ASC and PSC)



ASC PSC

Figure 3. FTIR Spectrums of Yellow fin tuna bone collagen (ASC and PSC)



ASC PSC

Figure 4. FTIR Spectrums of Yellow fin tuna fin collagen (ASC and PSC)

Amino acid analysis

Table 2 shows the amino acid composition of collagen extracted from skin, bones and fins of Yellowfin tuna.

Table 2. Amino acid composition of collagen from skin, bone and fins of Yellowfin tuna (%)

Amino Acid	Skin	Bone	Fin
Aspartic acid	1.0	1.6	1.1
Glutamic acid	2.9	4.2	2.8
Serine	0.1	0.1	0.3
Histidine	0.3	0.3	0.2
Glycine	13.4	11.0	13.0
Threonine	0.2	0.3	0.4
Arginine	2.5	3.0	2.5
Alanine	2.7	3.7	2.8
Tyrosine	0.1	0.1	0.1
Cystine	ND	ND	ND
Valine	0.4	0.4	0.3
Methionine	0.6	1.0	0.7
Phenylalanine	0.8	0.8	0.5
Isoleucine	0.5	0.7	0.3
Leucine	0.9	1.1	0.8
Lysine	1.5	1.8	1.4

ND=Not Detected

Amino acids were profiled from skin, bones and fins of Yellowfin tuna (Table 2). Glycine was the most abundant amino acid found in skin (13.4%), bones (11.0%) and fins (13.0%) of Yellowfin tuna. Glutamic acid, Alanine, Arginine and Lysine were found in moderately higher percentages. Cysteine was not detected in collagen from skin, bones and fins of Yellowfin tuna whereas Tyrosine, Serine, Histidine and Threonine were found in extremely low quantities.

Discussion

The majority of collagen was distributed in the skin compared to fin and bone of Yellowfin tuna analyzed in this study. As compared to other reported collagens, skin demonstrated higher collagen yield although it depends much on the fish species. The extractability of collagen depends on the construction of collagen which consists of two non- helical terminal ends that assist the cross-linked structure of the collagen (Pang *et al.,* 2013). It can be suggested that collagen with more inter molecular cross-links is present to a greater extent in the skin of tuna than in bones and fins. Veeruraj *et al.,* 2013 reported that the yield of PSC from the skin of marine eel fish (*Evenchelys macrura*) was

about 7.10% on dry weight basis. Sotelo *et al.,* 2016 also extracted similar amounts of collagen from skin of *Nezumia aequalis* (20.98 %) and from *Chimaera monstrosa* (20.09%).

All extracted collagen samples were observed with SDS-PAGE together with Type I human collagen and molecular marker (lane A and D in Figure 1). ASC and PSC comprised of both α1 and α2 chains. The SDS-PAGE pattern showed that all collagens had a double pattern for α1 (approximately 132 to 139 kDa) and α2 chains (approximately 120 and 124 kDa) and β chain (approximately 220 to 229 kDa). The density for α1 is twice as much as of α2, (α1, upper; α2, lower). Collagen of skin, bones and fins of fish waste matches the band patterns of the human skin collagen. Based on the α1 and α2 pattern, suggesting the fish collagens might have a composition of (α1) 2α2 heterotrimer, a Type I collagen. Similar results were observed in carp fish (Duan *et al*., 2009), deep-sea redfish (Wang *et al*., 2007), Nile perch (Muyonga *et al*., 2004), black drum (Ogawa *et al*., 2003) and brown stripe red snapper (Foegeding *et al*., 1996; Jongjareonrak *et al*., 2005). Type I collagen has also been extracted from skin, bone, fins, and scales of fresh water and marine fishes, chicken skin and different marine organisms such as squid, octopus, jellyfish, star fish and fish (Nagai *et al*., 2004; Sadowska *et al*., 2003; Swatschek *et al*., 2002). The isolated collagen appears to be Type I, which was previously observed by (Kimura *et al*., 1993; Muyonga *et al*., 2004) who reported that the carp skin, scale, and bone collagen was the Type I based on its electrophoretic mobility. Kimura, (1992) also reported that bony fish skin collagens had a chain composition of two α1 chains and a single α2 chain. These results indicated that ASC and PSC extracted could be characterized as Type I collagen with reference to human collagen.

The FTIR spectrums of ASC and PSC of skin, bone and fins were recorded, and effective peak was obtained (Table 1). FTIR spectra of Yellowfin tuna skin collagen (ASC and PSC) are shown in Figure 2 and bone collagen (ASC and PSC) are shown in Figure 3. Figure 4 shows the FTIR spectrum of Yellowfin tuna fin collagen (ASC and PSC).

The regions of amides I, II and III are known to be directly related with the shape of a polypeptide. Amide A band (3,298 and 3,304 cm^{-1}) is related to N–H stretching vibrations. Amide I band (1,650 and 1,630 cm^{-1}) is associated with stretching vibrations of carbonyl groups in peptides, being the most important factor in investigating the secondary structure of a protein. Amide II (1,551 and 1,547cm^{-1}) is associated with NH bending and CN stretching. Amide III (1,239 and 1,238 cm^{-1}) is related to CN stretching and NH, and is involved with the triple helical structure of collagen (Jakobsen, 1983; Muyonga, 2004; Surewicz and Mantsch, 1988). In terms of IR absorption ratio, collagen from Yellowfin tuna fish skin, fins and bones demonstrated equal ratio between amide

A band (3,298 to 3,306 cm^{-1}), amide I (1,630 to 1,650 cm^{-1}), amide II (1,545 to 1,551 cm^{-1}) and amide III (1,237 to 1,239 cm^{-1}).

FTIR spectroscopy: The infrared spectra of ASC, PSC and the major peaks with their corresponding results confirmed the helical structure of the collagen which is reserved in good conditions. FTIR spectra obtained in present study were similar to those of collagens from other fish species (Muyonga *et al*., 2004; Singh, 2011).

In a study done by Woo *et al*., (2008), regions of amides A, I, II and III for Yellowfin tuna collagens were 3,427 cm^{-1} (amide A); 1,651 cm^{-1} (amide I); 1,544 cm^{-1} (amide II); and 1,240 cm-1 (amide III), respectively, exhibiting peaks in similar wave numbers.

In studies of Nile perch skin collagen by Muyonga *et al*., (2004), amide regions (A, I, II and III) were 3,434, 1,650, 1,542 and 1,235 cm^{-1} respectively and amide regions of adult Nile perch skin collagen were 3,458, 1,654, 1,555 and 1,238 cm^{-1} respectively.

Amino acids were profiled from skin, bones and fins of Yellowfin tuna (Table 2). Glycine was the most abundant amino acid found in skin (13.4%), bones (11.0%) and fins (13.0%) of Yellowfin tuna. Woo *et al*., (2008) also found glycine was the most prevalent amino acid (23.1%) in Yellowfin tuna skin collagen. Glutamic acid, Alanine, Arginine and Lysine were found in moderately higher percentages. Cysteine was not detected in collagen from skin, bones and fins of Yellowfin tuna whereas tyrosine, serine, histidine and threonine were found in extremely low quantities. The results of the present study are also supported by the findings of Zhang *et al*., 2007; Kim and Mendis, 2006; Mahboob *et al*., 2014 who demonstrated that glycine is the major amino acid in each collagen type. Hema *et al*., (2013) also didn't detect and Duan *et al*., (2009) also said that collagen commonly has a low amount of tyrosine. This result is in line with reported results of hake and trout collagen (Montero *et al*., 1990). Amino acid composition of the extracted collagen proved the purity of collagen.

Conclusion

Yellowfin tuna fish skin is a good source of collagen. The results indicated that ASC and PSC extracts could be characterized as Type I collagen with reference to human collagen from the SDS-PAGE pattern. It was further confirmed by FTIR analysis. Thus, extracted collagen could serve as reasonable alternative for mammalian collagen for the potential applications in food, beverage, cosmetic, and biomedical industries.

The results showed that it is possible to use fish waste as an important and alternative collagen source and the tuna skin discarded as waste by the seafood processing plants may become an additional and alternative possible non-conventional source for different industries including pharmaceutical industry.

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