J Korean Soc Appl Biol Chem (2013) 56, 441–450
DOI 10.1007/s13765-013-3114-9

ORIGINAL ARTICLE

Isolation and Characterization of Collagen from the Skin of Malaysian catfish (Hybrid *Clarias* sp.)

Peck Loo Kiew · Mat Don Mashitah

Received: 10 May 2013 / Accepted: 24 June 2013 / Published Online: 31 August 2013

Abstract Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were isolated from the skin of hybrid *Clarias* sp. with the yields of 18.11±0.32 and 26.69±0.54% (wet weight basis), respectively. Both collagens were characterized as type I collagen, containing α1 and α2 chains. Presence of high molecular weight crosslinks were observed in the gel electrophoresis of both collagens. Fourier transform infrared spectra of both collagens were almost similar, suggesting that pepsin hydrolysis did not disrupt the triple helical structure. The amino acid analysis showed glycine was the most abundant, with 207/1000 and 223/1000 residues present in ASC and PSC, respectively. The amounts of imino acids were 185/1000 residues for both. Thermal denaturation temperatures were determined to be 31.5 and 31.0°C, respectively. Both collagens exhibited high solubility in acidic pH (1–5) and below 4% (w/v) NaCl concentration.

Keywords acid soluble collagen · characteristics · *Clarias* sp · pepsin soluble collagen · skin

Introduction

The term "collagen" is no longer a new word in our daily lives. Bansal (2001) described that collagen was actually derived from the Greek word "kola" for glue and was initially known as "that constituent of connective tissue, which yields gelatin upon boiling". Not only widely known as the most abundant protein in mammals, collagen is also being recognized as the major structural protein, which form molecular cables to strengthen the tendons and the resilient sheets that support the skins and internal organs of mammals and fishes (Bansal, 2001; Quereshi et al., 2010). Recently, numerous reports pointed out that the global demand for collagen has been increasing over the years. It is gradually emerging as a popular biomaterial in both bio related and non bio related industries, following its unique characteristics such as high tensile strength, low antigenicity, bioresorbability, and good compability (Cliche et al., 2003). Rapid expansion and wide utilization of collagen is currently observed in pharmaceutical applications particularly in the cartilage reconstruction, production of wound dressings, skin substitutes for burn patients, vitreous implants, and carriers in drug delivery (Wang et al., 2008). In addition, it is also regarded as an effective edible condiment for skin care and is extensively utilized as food additive or production of edible casings in meat processing industries as well (Singh et al., 2011). Therefore, collagen *per se* is considered as one of the most useful biomaterials nowadays.

Collagen basic structure, known as tropocollagen, is made up of three polypeptide chains, with each twisted in a left-handed helix (α-chain) and coiled around each other to form a right-handed triple helix structure (Mario Hiram et al., 2010; Singh et al., 2011). Each polypeptide chain is characterized by the repeating structure of triplet (Gly-X-Y)n, where glycine residue is the structural prerequisite for the triple helix (Wang et al., 2009). On the other hand, X and Y are often proline (Pro) and hydroxyproline (Hpy) (Senaratne et al., 2006; Palpandi et al., 2010; Singh et al., 2011), respectively. For industrial purposes, the conventional main sources of collagen are limited to land-based animals such as skins and bones of bovine or porcine(Mario Hiram et al., 2010). Even though collagen offers a wide range of useful applications, pessimism and strong concerns still persist among consumers with regard to its usage. This is mainly due to religious sentiments since collagens extracted from bovine sources are prohibited for Sikhs and Hindus, whilst porcine collagen cannot be consumed by Muslims and Jews (Muyonga et al., 2004; Senaratne et al., 2006; Singh et al., 2011). Conventional derivation of collagen from cattle and pigs has also been called into question ever since the

P. L. Kiew · M. D. Mashitah (✉)
School of Chemical Engineering, Universiti Sains Malaysia, 14300 Nibong Tebal, Seberang Perai South, Penang, Malaysia
E-mail: chmashitah@eng.usm.my

emergence of the mad cow disease which is also known as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD) due to concerns and anxieties among concerns and anxieties among consumers on the risks of transferring these diseases to humans (Pati et al., 2010; Żelechowska et al., 2010). Besides that, there is increasing concern among researchers about whether animal tissue-derived collagens are capable of transmitting pathogenic vectors such as prions (Muralidharan et al., 2012). As a consequence, there is a growing interest in developing alternative sources of collagen, especially from aquatic animals which include freshwater, marine fish and mollusk (Singh et al., 2011).

The search for alternative collagen sources has therefore being intensified in the studies of the functional properties of aquatic source collagens: i.e. skins of cobia (Zeng et al., 2012), skins of striped catfish (Singh et al., 2011), skins of brownbanded bamboo shark (Kittiphattanabawon et al., 2010), skins of grass carp (Wang et al., 2009), skins of brown backed toadfish (Senaratne et al., 2006), skin and bone of bigeye snapper (Kittiphattanabawon et al., 2005), and skins of young and adult Nile perch (Muyonga et al., 2004). Even though a number of researches have addressed the properties of fish skin collagens, See et al. (2010) mentioned that they actually differed and varied between fish species. To our best knowledge, the isolation and characterization studies of fish collagen from the Malaysian freshwater fishes, particularly those in the tropical regions are still in its infancy, with very little information available in the literature. Cultured fish is an important fish and protein source in Malaysia, especially in inland areas where marine fishes are not easily available and affordable (Yaacob and Ali, 1994). The main freshwater species cultured locally in Malaysia are red tilapia (26,175.33 tons), catfish (21,891.55 tons), black tilapia (5,848.98 tons) and pangasius catfish (5,748.44 tons) (See et al., 2010). However, promotion of aquaculture practices is greatly restricted mainly due to the poor economic return from investments. Even though advanced aquaculture techniques such as intensive pond and cage farming are well-developed, expanded investment is not preferable since the profit margin is not high. One of the main causes for this situation is lower commercial values of cultured freshwater fishes in contrast to those deep sea species since they are only demanded for daily consumption. Therefore, extraction of collagen from the skins of these freshwater fishes stands as an interesting attempt in converting them into raw material for other applications, not restricted only to food industry, thus boosting up their commercial values (Kiew and Mashitah, 2012).

Locally known as *Keli* in Malaysia, cultured hybrid catfish of *Clarias* sp. (*Clarias gariepinus* × *C. macrocephalus*) is one of the most popular freshwater fishes accepted by consumers contributing by its abundances and cheaper price as compared to other cultured fishes. As reported by Sivakumar et al. (2000), catfish is a good source of protein with considerable amount of collagen existed in the muscles and skins. However, no information on the composition and physicochemical properties of collagen from the skin of this species has been reported so far. Therefore, the objectives of the present study are to extract and characterize the collagen, both acid soluble collagen (ASC) and pepsin soluble collagen (PSC), from the skin of this hybrid catfish (*Claris* sp.).

Materials and Methods

Materials. Cultured *Keli* (hybrid of *C. gariepinus* × *C. macrocephalus*) were purchased from a local wet market in Parit Buntar, Perak. Upon arrival at the laboratory, the fishes were killed, dissected, deboned and the skins were cleaned of adhering tissues before being cut into small pieces (1 cm × 1 cm). The skin were then washed with distilled water and kept frozen at −20°C prior to collagen extraction.

Chemicals. Commercial pepsin from porcine gastric mucosa, sodium hydroxide, and acetic acid were purchased from Merck (Malaysia). 2-mercapethanol was purchased from Sigma Chemical Co. (USA). Laemmli sample buffer and pre-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) standards (6.6–204 kDa) were purchased from Bio-Rad Laboratories, Inc. (Hercules, USA). All other chemicals used were of analytical grade.

Extraction of acid soluble collagen (ASC). All procedures were performed as previously described by Wang et al. (2009) and Kittiphattanabawon et al. (2005), with slight modifications. The extraction process was carried out at 4°C. To remove non-collagenous proteins, the skins were mixed with 0.1 M NaOH at a sample to alkali ratio of 1:20 (w/v). The mixture was stirred for 6 h. The NaOH solution was changed every 2 h. The sample was then washed thoroughly with excessive distilled water until the pH was neutral or slightly basic. Deproteinized skins were defatted with 10% butyl alcohol with a sample to alcohol ratio of 1:20 (w/v) for 24 h. The alcohol solution was changed every 8 h. Defatted skins were then washed with cold water and subjected to collagen extraction using a queous acetic acid. The skins were strongly stirred in 30 volumes (v/w) of 0.7 M acetic acid for 24 h to extract the acid soluble collagen. The viscous collagenous material was separated from the insoluble components by high speed centrifugation at 20,000×g for 40 min. The soluble collagen solution was obtained from the supernatant. The collagen was precipitated by adding NaCl to a final concentration of 0.8 M. Resulting sediment was collected by centrifugation at 20,000×g for 30 min. To further purify the collagen, it was re-dissolved in minimal amount of acetic acid, dialyzed against 0.1 M acetic acid, followed by distilled water and lyophilized. The freeze-dried product was designated as ASC.

Extraction of pepsin soluble collagen (PSC). The PSC was obtained using similar method as ASC except that the defatted skins were continuously stirred in 30 volumes (v/w) of 0.7 M acetic acid containing 1.5% (w/w) pepsin for 24 h.

Collagen yield measurement. The yield of both ASC and PSC from the skin of *Clarias sp*. was calculated using Eq. (1):

$$\text{Yield of coolagen (\%)} = \frac{\text{Weight of coolagen (g)}}{\text{Weight of skin (g)}} \times 100\% \quad (1)$$

The extraction of collagen was performed three times, and the yield value was the average of the triplicate measurement.

SDS-PAGE. SDS-PAGE was performed according to the methods of Laemmli (1970) using discontinuous Tris-glycine buffer system with 4% stacking gel and 12% resolving gel. The collagen samples were dissolved in 0.05% (v/v) acetic acid. Then, the dissolved collagen samples were added to Laemmli sample buffer (Bio-Rad Laboratories USA) containing 5% of 2-mercaptoethanol at the ratio of 1:1. The mixtures were kept at boiling water for about 5 min before loading to the SDS-PAGE. A total of 20 μg of each collagen sample was loaded on the SDS-PAGE. After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie blue R-250 in 30% (v/v) methanol and 10% (v/v) acetic acid and destained with 40% (v/v) methanol and 10% (v/v) acetic acid. Pre-stained SDS-PAGE broad range standards were used to estimate the molecular weight of both of the acid and pepsin soluble collagen. The image of the gel was captured using FUJIFILM Luminescent Image Analyzer LAS-3000 (Fujifilm, Japan). The electrophoresis pattern of the collagen samples were analyzed using Multi Gauge v3.0 software (Fujifilm, Japan).

Amino acid analysis. The freeze dried ASC and PSC were hydrolyzed in inert atmosphere with 6 M HCl containing 1% phenol at 110°C for 24 h. The hydrolysates were then dried under vacuum. This was followed by derivatization, drying, and dilution with sample diluents. The amino acids derivative samples were analyzed using high performance liquid chromatography (HPLC) (1260 Infinity, Agilent Technologies, Malaysia) and compared against the standard amino acids which were analyzed prior to these. The peak area of each amino acid in the chromatogram was calculated and compared with that of the standard and reported as number of residue per thousand amino acids content.

Fourier transform infrared spectroscopy (FTIR). FTIR spectra were obtained from discs containing 2 mg collagen in approximately 100 mg potassium bromide (KBr). Infrared spectra were obtained in the range between 4000 and 500 cm^{-1} using an infrared spectrophotometer (Shimadzu Scientific Instruments' IR-Prestige-21, Thermo Fisher Scientific, Malaysia).

Determination of collagen denaturation temperature. The denaturation of collagen in solution was performed according to the method described by Pati et al. (2010) with slight modifications. A Brookfield viscometer (Brookfield DV-III, Mecomb Malaysia, Malaysia) was filled with 0.3% (m/v) collagen solution in 0.05 M acetic acid. The viscometer was then immersed in a water bath held at 4°C and left to stand for 15 min, in order to allow the collagen solution to equilibrate to the water bath temperature. The temperature was raised stepwise up to 50°C and maintained at each temperature for 10 min. Collagen solution viscosities were measure at temperature intervals of about 2°C from 4°C up to 50°C. Fractional viscosities were computed for each temperature as follows:

$$\text{Fractional viscosity} = \frac{\text{measured viscosity} - \text{minimum viscosity}}{\text{maximum viscosity} - \text{minimum viscosity}} \quad (2)$$

Thermal denaturation curves were then obtained by plotting the fractional viscosities against temperature for ASC and PSC from the skin of *Clarias* sp. The denaturation temperature was taken to be the temperature at which fractional viscosity was 0.5.

Collagen solubility. The solubility of ASC and PSC were determined by the method of Singh et al. (2011) and Kittiphattanabawon et al. (2005). The collagens were dissolved in 0.05 M acetic acid to obtain a final concentration of 3 mg/mL, and the mixtures were stirred at 4°C until collagens were completely solubilized.

Effect of pH on collagen solubility. Collagen solution (3 mg/mL; 8 mL) was transferred to a 50 mL centrifuge tube and the pH was adjusted with either 6 M NaOH or 6 M HCl to obtain the final pH ranging from 1 to 10. The volume of solutions was made up to 10 mL by distilled water which previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 20,000×*g* 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 standard. Relative solubility was computed in comparison with that obtained at the pH rendering the highest solubility.

Effect of NaCl on collagen solubility. Collagen solution (6 mg/mL; 5 mL) was mixed with 5 mL of various concentrations (0, 2, 4, 6, 8, 10, and 12%) of NaCl in 0.05 M acetic acid. The mixture was stirred continuously at 4°C for 30 min, followed by centrifugation at 20,000×*g* for 30 min at 4°C. Protein content in the supernatant was measured and the relative solubility was calculated as described previously.

Statistical analysis. All methods of collagen extraction and analysis were replicated three times. Mean values with standard deviations (SD) were reported.

Results and Discussion

Isolation of ASC and PSC from the skin of hybrid *Clarias* sp. Yields of 18.11±0.32 and 26.69±0.54% (based on the wet weight of the skin) were obtained for ASC and PSC, respectively. The results suggested that collagen in the skin was not completely extracted with 0.7 M acetic acid, as shown by the lower yield of ASC obtained. This was in agreement with the findings of few researchers who reported that the skins of cobia, striped catfish, balloon fish, and brownbanded bamboo shark were not entirely soluble in 0.5 M acetic acid, but dissolved completely when being extracted in 0.5 M acetic acid with the presence of pepsin (Kittiphattanabawon et al., 2010; Huanget al., 2011; Singh et al., 2011; Zeng et al., 2012). Difference in the yield of ASC and PSC in the present study could be explained by the fact that collagen molecules in Malaysian catfish were cross-linked by covalent bonds through the condensation of aldehyde groups at the

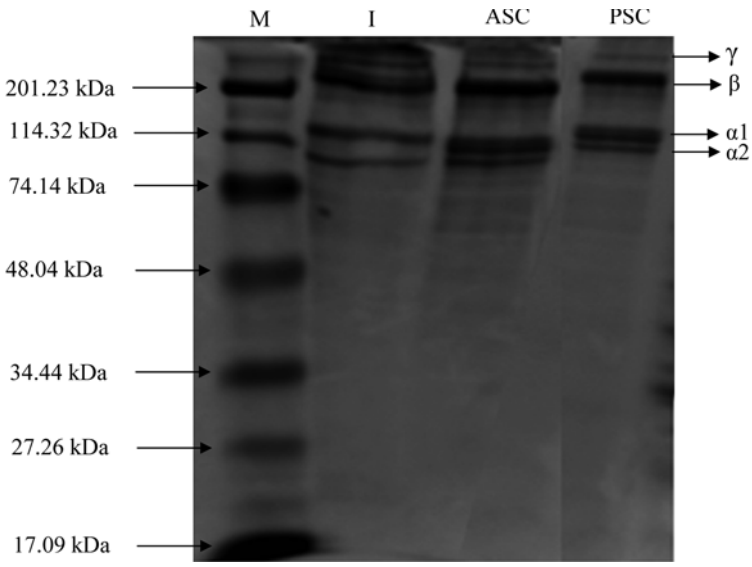

Fig. 1 SDS-PAGE of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the skin of hybrid *Clarias* sp. under reducing condition. M and I denote high molecular weight protein markers and type I collagen from calf skin.

telopeptide region (non-helical ends) (Kittiphattanabawon et al., 2010; Singh et al., 2011). In addition, Zeng et al. (2012) and Jongjareonrak et al.(2005) added that the presence of inter-molecular cross-linking at the telopeptide region was also another main factor that resulted in limited solubility of collagen in acetic acid. Therefore, with the help of pepsin digestion, cross-linked molecules at the non-helical region were cleaved allowing inter-molecular crosslinks to be hydrolyzed, thereby resulting in further extraction (Singh et al., 2011). Pepsin was able to cleave specifically at the telopeptide region without damaging the integrity of the triple helix (Jongjareonrak et al., 2005). Hence, pepsin could be used as an option to increase the yield of extraction of collagen from the skin of Malaysian catfish.

Compared with the results from literature, the yield of ASC and PSC (on wet basis) isolated from the skin of *Clarias* sp. were much higher than that from striped catfish (5.1 and 7.7%, respectively) (Singh et al., 2011), brownbanded bamboo shark (9.38 and 8.86%, respectively) (Kittiphattanabawon et al., 2010), bigeye snapper (6.4 and 1.1%, respectively) (Kittiphattanabawon et al., 2005), and those from brownstripe red snapper (9 and 4.7%, respectively) (Jongjareonrak et al., 2005). On the contrary, the approximate collagen content in terms of dry weight basis in *Clarias* sp. skin tissue was estimated to be 50.7±0.32% and 75.37±0.54% of ASC and PSC, respectively. It was found that the yield of ASC in this study was higher than that from the skin of tilapia (39.4% on dry basis) (Zeng et al., 2009) and in agreement with that from Japanese sea bass (51.4% on dry basis), chub mackerel (49.8% on dry basis) and bullhead shark (50.1% on dry basis) (Nagai and Suzuki, 2000). According to Zeng et al. (2012), discrepancies in the construction of collagens among different fish species and the variation in the extraction method performed could be the factors which resulted in the difference of the collagen yields.

SDS-PAGE. The electrophoresis patterns of ASC and PSC from the skin of Malaysian catfish are shown in Fig. 1. Results showed that both ASC and PSC consisted of at least two α chains (α1 and α2). Both collagens contained inter- and intra- molecular cross-linked components of β (dimers) and γ (trimers) (Zeng et al., 2012). This is in accordance with those of collagens from most other fish species previously reported such as striped catfish (Singh et al., 2011), Nile perch (Muyonga et al., 2004), brown banded bamboo shark (Kittiphattanabawon et al., 2010), largefin longbarbel catfish (Zhang et al., 2009b), bigeye snapper (Kittiphattanabawon et al., 2005) and brown backed toadfish (Senaratne et al., 2006). The electrophoretic pattern of Malaysian hybrid *Clarias* sp. skin was also similar to that of calf skin collagen. Apart from that, α1 and α2 chains of both ASC and PSC were found at a ratio of approximately 2:1. This is a typical feature of type I collagen, which is the major collagen in dermal tissue (Muyonga et al., 2004). Nevertheless, it was found that the proportion of high molecular weight components was greater in ASC as compared to PSC, as evidenced by higher band intensity of β and γ chains as well as more cross-linked components than the latter. Therefore, the results of the present study implied that the intra- and inter-molecular crosslinks of collagen were richer in ASC than in PSC. As suggested by Singh et al. (2011) and Matmaroh et al. (2011), pepsin was able to cleave peptides at the telopeptide region, in which β and γ chains were cleaved into α-components, resulting in increased band intensity of the α-chains in PSC.

Amino acid analysis. Table 1 shows the amino acid composition of the ASC and PSC extracted from the skin of Malaysian catfish. The composition was expressed as amino acid residues per 1000 total amino acid residues. Since collagen is triple helical in nature with the characteristic amino acid of $(Gly\text{-}Pro\text{-}Hyp)_n$ (Singh et al., 2011), glycine (Gly) was the most abundant compound with the amount of 207 and 223 units of the total amino acids present in ASC and PSC, respectively. Even though most of the characterization

Table 1 Amino acid composition of fish skin collagen of hybrid *Clarias* sp. (amino acid residues per 1000 total amino acid residues)

Amino acid		Acid soluble collagen (ASC)	Pepsin soluble collagen (PSC)
Aspartic acid	Asp	60	57
Serine	Ser	46	50
Glutamic acid	Glu	105	95
Glycine	Gly	207	223
Histidine	his	2	2
Arginine	Arg	86	102
Threonine	Thr	21	23
Alanine	Ala	90	95
Hydroxyproline	Hyp	55	59
Proline	Pro	130	126
Cystine	Cys	0	0
Tyrosine	Tyr	13	6
Valine	Val	36	33
Methionine	Met	19	20
Lysine	Lys	41	40
Isoleucine	Ile	28	15
Leucine	leu	31	30
Phenylalanine	Phe	30	24
Total		1000	1000

studies of collagen reported glycine content of approximately 30% of the total amino acids (Kittiphattanabawon et al., 2005; Senaratne et al., 2006; Huang et al., 2011), finding in the present study is almost similar to the characteristics of collagen isolated from the skins of Nile perch as reported by Muyonga et al. (2004) where the glycine content was in the range of 21–22%, which was slightly lower than 1/3 of total amino acids. In general, glycine occurs uniformly, at every third residue throughout most of the collagen molecules (Zeng et al., 2012), except for the first 14 amino acid residues from the N-terminus and the first 10 amino acid residues from the C-terminus of the molecules (Senaratne et al., 2006). Both collagens were also rich in proline, glutamic acid, alanine, and hydroxyproline. In addition, relatively low content of methionine, isoleucine, tyrosine, and histidine was consistent with the amino acid compositions of other aquatic collagens reported in literature. Absence of cysteine in collagen obtained from this hybrid species also emphasized the presence of type I collagen. The amount of imino acid (proline+hydroxyproline) of ASC and PSC was found to be 185/1000 residues for both collagens. It was observed that the imino acid residues in ASC from Malaysian catfish skin was higher than that in balloon fish skin (179/1000 residues) (Huang et al., 2011) and cod skin (179/1000 residues) (Duan et al., 2009) but was slightly lower than carp skin (179/1000 residues) (Duan et al., 2009), brownbanded bamboo shark skin (204/1000 residues) (Kittiphattanabawon et al., 2010), and striped catfish skin (206/1000 residues) (Singh et al., 2011). As for the PSC, the imino acid content was higher than that from balloon fish skin (174) (Huang et al., 2011) but lower than that from brownstripe red snapper skin (221) (Jongjareonrak et al., 2005), striped catfish, cobia, tilapia, and yellowfin tuna, which contained imino acids ranging from 190 to 216/1000 residues (Woo et al., 2008; Huang et al., 2011; Singh et al., 2011; Zeng et al., 2012). Fish collagens were normally made up of lower imino acid contents as compared to mammalian collagens (Kittiphattanabawon et al., 2005) where porcine dermis collagen and calf skin collagen were reported to contain 220/1000 residues and 215/1000 residues of imino acid, respectively (Kittiphattanabawon et al., 2005; Kittiphattanabawon et al., 2010). Variation in the imino acid content, especially hydroxyproline, between animals was correlated with the difference in their habitats, particularly temperature of the living environments (Kittiphattanabawon et al., 2010; Huang et al., 2011). Bae et al. (2008) reported that the collagens isolated from fish species living in warm environment have higher amount of hydroxyproline and exhibit higher thermal stability than those living in cold environment. This was due to the fact that hydroxyproline stabilized the triple helix structure of collagen molecules (Senaratne et al., 2006).

FTIR. The infrared spectra of ASC and PSC as well as the major peaks with their corresponding assignments are shown in Fig. 2 and Table 2. FTIR spectra obtained in the present study were similar to those of collagens from other fish species (Muyonga et al., 2004; Singh et al., 2011). The amide A band of ASC and PSC was found at wavenumbers of 3348 and 3336 cm^{-1}, respectively. According to Abe and Krimm (1972), amide A band is associated with the N-H stretching frequency. A free N-H stretching vibration is expected to occur in the range of 3400–3440 cm^{-1}, but Doyle et al. (1975) mentioned that when the NH group of a peptide is involved in hydrogen bond, the position might be shifting to a lower frequency, usually around 3300 cm^{-1}. Therefore, a shift towards lower wavenumbers of amide A could be described as the indication of water-mediated hydrogen bonding in these collagens, probably with the carbonyl group of the peptide chain (Yakimets et al., 2007; Zeng et al., 2012). Amide B band of both collagens was observed at 2364 and 2951 cm^{-1}, in agreement with that reported by other researchers (Kittiphattanabawon et al., 2010; Pati et al., 2010; Singh et al., 2011). The amide I band, with characteristic frequencies in the range of 1600–1700 cm^{-1}, was mainly associated with the stretching vibrations of carbonyl groups along polypeptide backbone (Singh et al., 2011). In addition, it was also a sensitive marker of peptide secondary structure (Pati et al., 2010). Amide I of PSC was found at lower wavenumber (1654 cm^{-1}) compared to ASC (1655 cm^{-1}). Similar pattern was observed for Amide II bands. Amide I, II and III bands were known to be correlated to the degree of molecular order and associated with the triple helical structure of collagen, resulting from the C=O stretching, N-H bending, and C-H stretching (Muyonga et al., 2004; Zeng et al., 2012). Amide I and II bands of PSC shifted to lower wavenumber than the ASC, suggested that the latter has more or stronger hydrogen bonds. This was in accordance with the peak location of Amide A band for both collagens. Based on the location of Amide I and II peaks, it appeared that PSC from Malaysian catfish skin had a lower degree of molecular order,

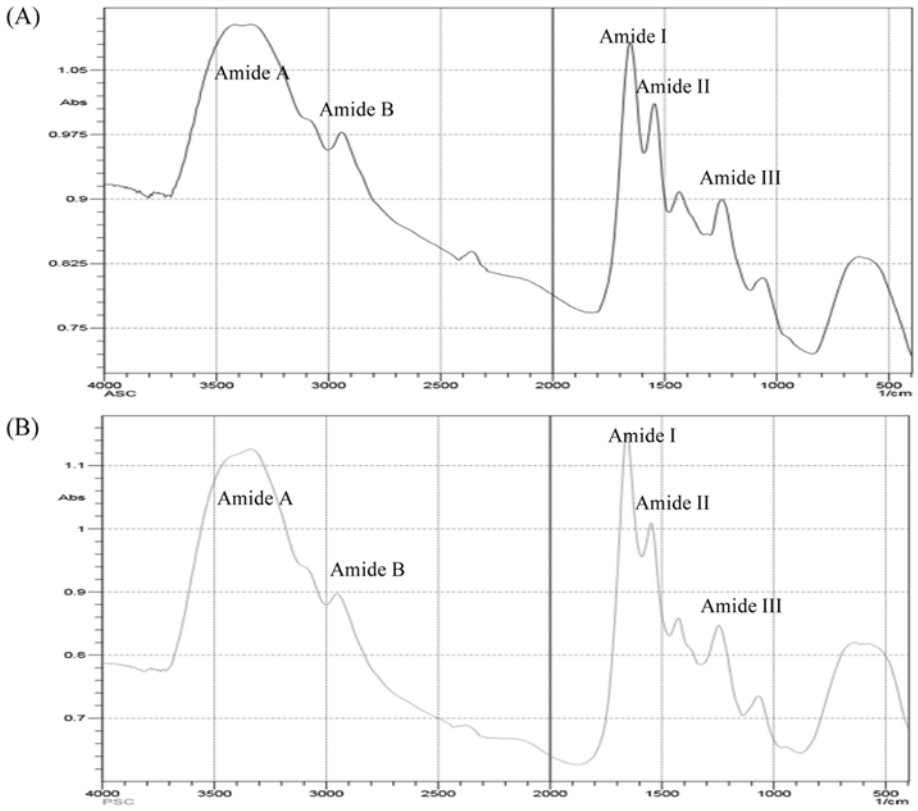

Fig. 2 Fourier transform infrared spectra of type I collagen from skin of *Clarias* sp. (A) Acid soluble collagen (ASC) and (B) Pepsin soluble collagen (PSC).

Table 2 Fourier transform infrared spectra peak locations and assignment for type I collagen from *Clarias* sp. skin.

Region	Peak wavenumber (cm^{-1})		Assignment	References
	ASC	PSC		
Amide A	3348	3336	NH stretching	(Sai and Babu, 2001)
Amide B	2943	2951	CH_2 asymmetrical stretching	(Abe and Krimm, 1972)
-	2364	2380	CH_2 symmetrical stretching	(Abe and Krimm, 1972)
Amide I	1655	1654	C=O stretching, hydrogen bonding coupled with COO-	(Payne and Veis, 1988)
Amide II	1547	1547	NH bending coupled with CN stretching	(Jackson et al.,1995)
-	1472	1435	CH_2 bending	(Jackson et al., 1995)
-	1315	-	CH_2 wag of proline	(Jackson et al., 1995)
Amide III	1246	1246	NH bending coupled with CN stretching	(Jackson et al., 1995)
-	1065	1068	C-O stretching	(Jackson et al., 1995)
-	-	953	C-O stretching	(Jackson et al., 1995)
-	632	640	Skeletal stretching	(Muyonga et al., 2004)

since a shift of these peaks to lower wavenumbers is associated with a decrease in the molecular order (Muyonga et al., 2004; Zeng et al., 2012). Thus, it could be deduced that pepsin disrupted non helical region of the telopeptide, resulted in lower intermolecular crosslinks in PSC. This was in agreement with the electrophoresis analysis of the ASC and PSC. Apart from that, the intensity ratio between Amide III band and 1454 cm^{-1} band has been used to indicate the stability of the triple helical structure of collagen (Plepis et al., 2004; Matmaroh et al., 2011). In this study, the ratio was found to be 1.17 for both ASC and PSC. A ratio of approximately 1.0 indicated the presence of helical structure (Plepis et al., 2004; Singh et al., 2011). Due to the same intensity ratio obtained for both collagens, pepsin hydrolysis obviously had no pronounced effect on the triple-helical structure of PSC and it was confirmed that the structure was maintained for both ASC and PSC isolated from the skin of Malaysian catfish.

Determination of collagen denaturation temperature. Fig. 3 shows the changes in fractional viscosity, with increasing

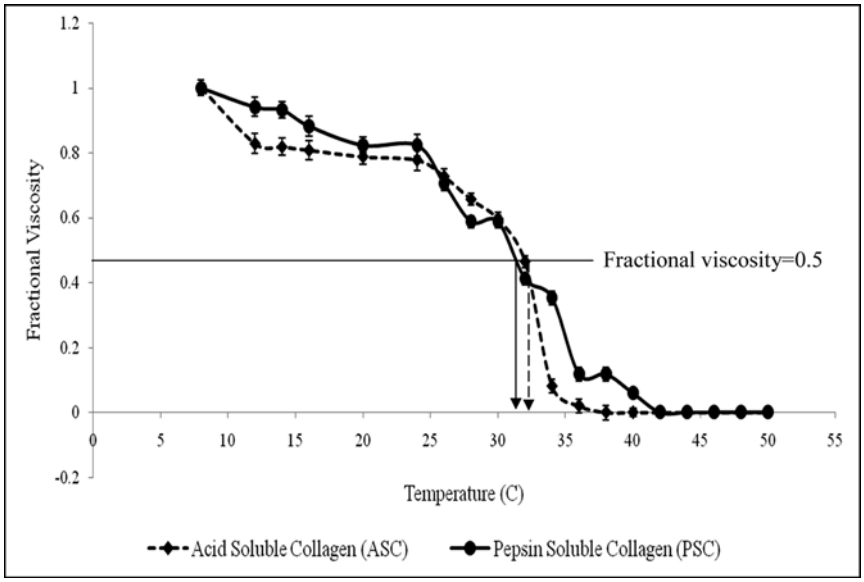

Fig. 3 Change in fractional viscosity with temperature of skin collagen from the hybrid *Clarias* sp.

Table 3 Variation of denaturation temperature of collagen of different species with their imino acid contents and habitats

Habitat	Species	Source	Imino acid content	Denaturation temperature (°C)	References
Land animals	Calf	Skin	215	40.8	(Kittiphattanabawon et al., 2010)
	Pig	Skin	220	37	(Kittiphattanabawon et al., 2005)
Freshwater	Catfish (*Clarias gariepinus* × *C. macrocephalus*)	Skin	185	31.5	Present study
	Rohu (*Labeo rohita*)	Scale	201	36.5	(Pati et al., 2010)
	Catla (*Catla catla*)	Scale	214	36.5	(Pati et al., 2010)
	Silver carp (*Hypophthalmichthys molitrix*)	Skin	192	29	(Zhang et al., 2009a)
	Nile tilapia (*Oreochromis niloticus*)	Skin	210	32	(Zeng et al., 2009)
	Carp (*Cyprinus carpio*)	Skin	190	28	(Duan et al., 2009)
		Scale	192	28	
		Bone	192	28	
Sea	Ornate threadfin bream (*Nemipterus hexodon*)	Skin	188	33.35	(Nalinanon et al., 2011)
	Deep-sea redsh (*Sebastes mentella*)	Skin	160	15.7	(Wang et al., 2007)
	Brown backed toadfish (Lagocephalus gloveri)	Skin	170	28	(Senaratne et al., 2006)
	Walleye pollock (*Theragra chalcogramma*)	Skin	184	24.6	(Yan et al., 2008)

temperature, for both ASC and PSC from the skin of Malaysian catfish. Both collagens exhibited a rapid loss of viscosity with heating. This could be attributed to the denaturation of collagen (Muyonga et al., 2004). Collagen denatures at temperatures above 40°C, forming a mixture of random-coil single, double and triple strands (Kittiphattanabawon et al., 2005). The thermal denaturation temperature (T_d) was determined to be approximately 31.5 and 31°C for ASC and PSC, respectively. This suggested that denaturation temperatures of collagen from Malaysian hybrid *Claris* sp. skin were in agreement with those collagens isolated from tropical and sub-tropical species (living in warm environment), such as black drum (ASC 34.2°C, PSC 35.8°C), sheepshead (ASC 34.0°C, PSC 34.3°C), balloon fish (ASC 29.01°C, PSC 30.01°C), and carp (ASC 28.0°C) (Zeng et al., 2012). The T_d observed in this study was however found to be higher than those from temperate (cold water) fish species. It was previously reported that collagen denaturation temperatures for cod was 15°C, Alaska Pollack was 16.8°C, Japanese seabass was 30°C, skip jack tuna was 29.7°C, and chum salmon was 19.4°C (Muyonga et al., 2004). As mentioned in most studies in the literature, the amount of imino acid (proline and hydroxyproline) was directly associated with the thermal stability of collagen through hydrogen bonds (Senaratne et al., 2006). Zeng et al. (2012) explained that proline and hydroxyproline imposed restrictions on the conformation of a polypeptide chain and helped to strengthen the triple helical structure of collagen. Therefore, the higher the imino acid content, more stable was the helical structure of the collagen, resulting in higher denaturation temperature (Senaratne et al., 2006). In

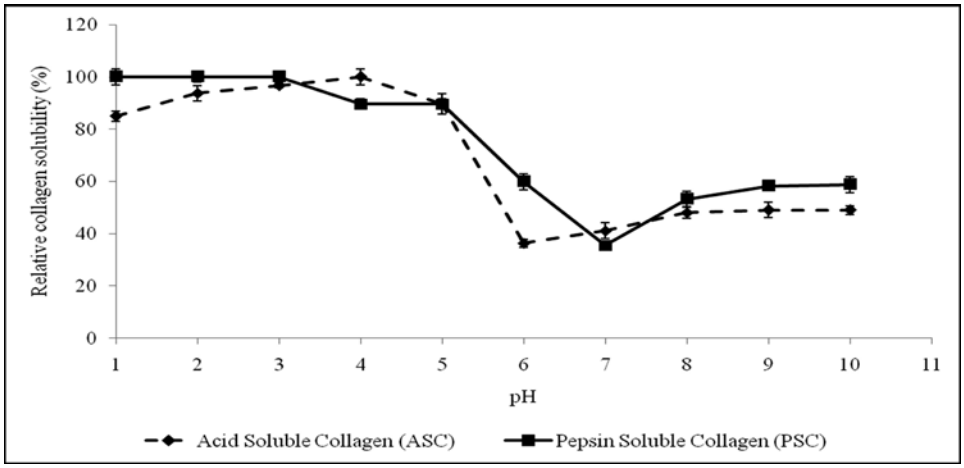

Fig. 4 Relative solubility of ASC and PSC at different pH. The vertical bars represent the standard deviations (n=3).

addition, the difference in T_d amongst collagens from different animals and species was also contributed by both body and habitat temperatures (Pati et al., 2010; Singh et al., 2011). It was stated earlier that species living in colder environments had lower amount of imino acid. The water temperature from where the Malaysian catfish was caught ranged 28–34ºC; thus explaining the higher T_d of ASC and PSC from this study than those from cold water and more or less similar to those from warm water environment. Variation in denaturation temperature of collagen from different species with their imino acid contents and habitats are presented in Table 3.

Effect of pH on collagen solubility. The solubility of ASC and PSC from the skin of Malaysian catfish as influenced by different pH was comparatively studied. The effect of pH on the solubility of both collagens in acetic acid is presented in Fig. 4. Both ASC and PSC showed high solubility in very acidic pH ranging from 1–5, with the relative solubility higher than 80%. At pH above 5, a significant decrease in the solubility was observed for both collagens. The solubilities of ASC and PSC reached the minimum when the pH was increased to 6 and 7, respectively. Low solubility was also found in the neutral and slightly alkaline pH range (Matmaroh et al., 2011). The results in the present study were supported by the fact that collagen has isoelectric points ranging from pH 6 to 9 (Huang et al., 2011). It was known that when a protein dissolved in buffer at its isoelectric point, total net charges of the protein molecules were zero. Subsequently, the hydrophobic-hydrophobic interaction increased, leading to precipitation and aggregation of the protein, resulting in low solubility in solution (Huang et al., 2011; Singh et al., 2011). On the other hand, when pH was lower or higher than the isoelectric point, the net positively or negatively charged residues of proteins increased, resulting in increased solubility by the repulsive forces between the chains (Kittiphattanabawon et al., 2005; Huang et al., 2011; Zeng et al., 2012). Similar result was reported for collagen from bigeye snapper (Nalinanon et al., 2007), tiger puffer, red stingray, sea chub (Bae et al., 2008), and balloon fish (Huang et al., 2011). In addition, the PSC in the present study showed higher solubility than the ASC at all of the tested pHs except at pH 4 and 7. This leads to the deduction that the PSC may contain a lower degree of crosslink or weaker bonds compared to the ASC (Jongjareonrak et al., 2005). Huang et al. (2011), on the other hand described that part of peptide bonds were hydrolyzed in addition to the peptide bonds hydrolysis in telopeptide region of the collagen molecules, leading to higher solubility of PSC than ASC.

Effect of NaCl on collagen solubility. Solubility of ASC and PSC at various NaCl concentrations was measured in acetic acid solution (Fig. 5). The solubility of Malaysian catfish skin collagens gradually decreased with increasing NaCl concentrations until reaching 4% (w/v), after which a drastic decrease was observed for both collagens at 6% (w/v) NaCl and above. Generally, it had been reported in literature that the solubility of collagen from striped catfish, cobia, bigeye snapper, yellowfin tuna, balloon fish, tiger puffer, dusky spinefoot, and red stingray in acetic acid solution decreased with increasing NaCl concentration (Kittiphattanabawon et al., 2005; Bae et al., 2008; Huang et al., 2011; Singh et al., 2011; Woo et al., 2008; Zeng et al., 2012). This could be related to the 'salting out' phenomena, which occurred at relatively high NaCl concentration (Singh et al., 2011). According to Matmaroh et al. (2011), at low concentrations of NaCl, salt ions bound weakly to the charged groups on protein surface, without affecting the hydration shell on those domains. Therefore, it led to the high solubility of ASC and PSC in this study. Nevertheless, as the concentration of NaCl was increased, increment in ionic strength was induced, thus enhanced the hydrophobic-hydrophobic interactions between the protein chains. This led to greater competition for water of ionic salts, thereby causing the protein to precipitate and resulting in low solubility (Nalinanon et al., 2007; Bae et al, 2008; Huang et al., 2011). Similar behaviors were found for ASC and PSC, yet PSC exhibited greater solubility than ASC at all tested concentrations of NaCl. This finding was in accordance with that of Matmarohet al. (2011) and Zenget al. (2012). Huang et al. (2011) and Singhet al. (2011) correlated this

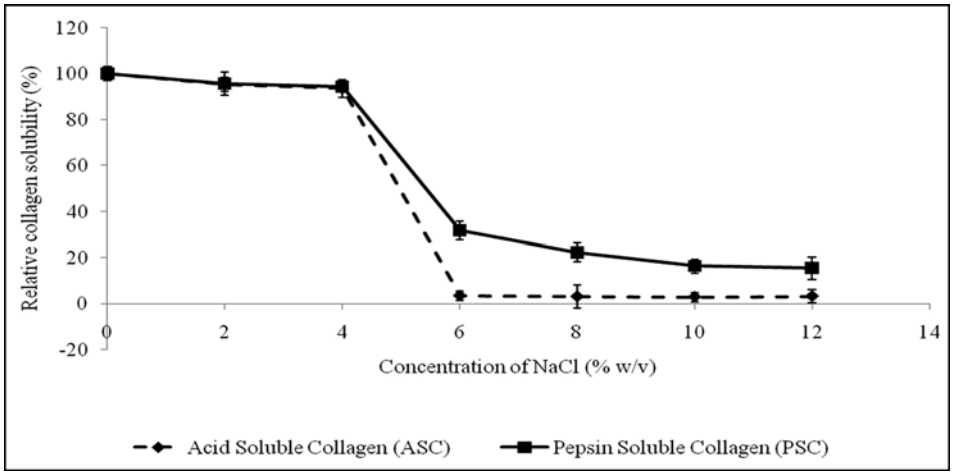

Fig. 5 Relative solubility of ASC and PSC at different NaCl concentrations. The vertical bars represent the standard deviations (n=3).

phenomenon to the partial hydrolysis of high molecular weight crosslinked molecules, which enhanced the solubility of PSC as compared to ASC. In addition, PSC with different amino acid compositions might be less susceptible to 'salting out' effect in comparison with ASC.

Acknowledgements The authors gratefully acknowledge the USM Fellowship provided by Universiti Sains Malaysia (USM) and Exploratory Research Grant Scheme (ERGS) with grant number: 203/PJKIMIA/ 6730068 from the Ministry of Higher Education (MOHE) Malaysia to support this research. Authors would also like to extend their gratitude to Madam Hajah Haslawati Baharuddin and Tuan Haji Rosly bin Hassan of the Malaysia Freshwater Fisheries Research Center (FFRC) for valuable advices in species identification of the cultured catfish.

References

Abe Y and Krimm S (1972) Normal vibrations of crystalline polyglycine I. *Biopolymers* **11,** 1817–39.

Bae I, Osatomi K, Yoshida A, Osako K, Yamaguchi A, and Hara K (2008) Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes. *Food Chem* **108,** 49–54.

Bansal M (2001) The madras triple helix: origins and current status.*Resonance October* 38–47.

Cliché S, Amiot J, Avezard C, and Gariepy C (2003) Extraction and characterization of collagen with or without telopeptides from chicken skin. *Poultry Sci* **82,** 503–9.

Doyle BB, Bendit EG, and Blout ER (1975) Infrared spectroscopy of collagen and collagen-like polypeptides. *Biopolymers* **14,** 937–57.

Duan R, Zhang J, Du X, Yao X, and Konno K (2009) Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). *Food Chem* **112,** 702–6.

Huang YR, Shiau CY, Chen HH, and Huang BC (2011) Isolation and characterization of acid and pepsin-solubilized collagens from the skin of balloon fish (*Diodon holocanthus*). *Food Hydrocolloids* **25,** 1507–13.

Jackson M, Choo LP, Watson PH, Halliday WC, and Mantsch HH (1995) Beware of connective tissue proteins: Assignment and implications of collagen absorptions in infrared spectra of human tissues. *BBA –Mol. Basis Dis* **1270,** 1–6.

Jongjareonrak A, Benjakul S, Visessanguan W, Nagai T, and Tanaka M (2005) Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of Brownstripe red snapper (*Lutjanus vitta*). *Food Chem* **93,** 475–84.

Kiew PL and Mashitah MD (2012) Isolation of collagen from the skin of *Keli*: Potential alternative for mammalian type I collagen. International Conference on Environment 2012. Penang, Malaysia, 562–8.

Kittiphattanabawon P, Benjakul S, Visessanguan W, Kishimura H, and Shahidi F (2010) Isolation and characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*). *Food Chem* **119,** 1519–26.

Kittiphattanabawon P, Benjakul S, Visessanguan W, Nagai T, and Tanaka M (2005) Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*) *Food Chem* **89,** 363–72.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–5.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with Folin phenol reagent. *J Biol Chem* **193,** 265–75.

Mario Hiram UM, Luis AMJ, Maribel PJ, Hisila SO, Ofelia RS, Luis CLJ et al. (2010) Jumbo squid (*Dosidicus gigas*) mantle collagen: extraction, characterization, and potential application in the preparation of chitosan-collagen biofilms. *Bioresource Technol* **101,** 4212–9.

Matmaroh K, Benjakul S, Prodpran T, Encarnacion AB, and Kishimura H (2011) Characteristics of acid soluble collagen and pepsin soluble collagen from scale of spotted golden goatfish (*Parupeneus heptacanthus*). *Food Chem* **129,** 1179–86.

Muralidharan N, Soottawat B, Thummanoon P, Ponusa S, and Hideki K (2012) Characteristics and functional properties of gelatin from splendid squid (*Loligo formosana*) skin as affected by extraction temperatures. *Food Hydrocolloids* **29,** 389–97.

Muyonga JH, Cole CGB, and Duodu KG (2004) Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). *Food Chem* **85,** 81–9.

Nagai T and Suzuki N (2000) Isolation of collagen from fish waste material — skin, bone and fins. *Food Chem* **68,** 277–81.

Nalinanon S, Benjakul S, Kishimura H, and Osako K (2011) Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*): Characteristics and effect of pepsin hydrolysis. *Food Chem* **125,** 500–7.

Nalinanon S, Benjakul S, Visessanguan W, and Kishimura H (2007) Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). *Food Chem* **104,** 593–601.

Palpandi C, Ramasamy P, and Rajinikanth T (2010) Extraction of collagen from mangrove archeaogastropod *Nerita (Dostia) crepidularia* Lamarck, 1822. *Am-Eur J Sci Res* **5,** 23–30.

Pati F, Adhikari B, and Dhara S (2010) Isolation and characterization of fish scale collagen of higher thermal stability. *Bioresource Technol* **101,** 3737–42.

Payne KJ and Veis A (1988) Fourier transform ir spectroscopy of collagen and gelatin solutions: Deconvolution of the amide I band for conformational studies. *Biopolymers* **27,** 1749–60.

Plepis AMDG, Goissis G, and Das-Gupta DK (2004) Dielectric and pyroelectric characterization of anionic and native collagen. *Polym Eng Sci* **36,** 2932–8.

Quereshi S, Mhaske A, Raut D, Singh R, Mani A, and Patel J (2010) Extraction and partial characterization of collagen from different animal skins. *Rec Res Sci Technol* **2,** 28–31.

Sai KP and Babu M (2001) Studies on *Rana tigerina* skin collagen. *Comp Biochem Phys B* **128,** 81–90.

See SF, Hong PK, Ng KL, Wan Aida WM, and Babji AS (2010) Physicochemical properties of gelatins extracted from skins of different freshwater fish species. *Int Food Res J* **17,** 809–16.

Senaratne LS, Park PJ, and Kim SK (2006) Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin. *Bioresource Technol* **97,** 191–7.

Singh P, Benjakul S, Maqsood S, and Kishimura H (2011) Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*). *Food Chem* **124,** 97–105.

Sivakumar P, Arichandran R, Suguna L, Mariappan M, and Chandrakasan G (2000) The composition and characteristics of skin and muscle collagens from a freshwater catsh grown in biologically treated tannery effluent water. *J Fish Biol* **56,** 999–1012.

Wang L, An X, Xin Z, Zhao L, and Hu Q (2007) Isolation and characterization of collagen from the skin of deep-sea redfish (*Sebastes mentella*). *J Food Sci* **72,** 450–5.

Wang L, Yang B, and Du X(2009) Extraction of acid-soluble collagen from grass carp (*Ctenopharyngodon Idella*) skin. *J Food Process Eng* **32,** 743–51.

Wang L, Yang B, Du X, Yang Y, and Liu J (2008) Optimization of conditions for extraction of acid-soluble collagen from grass carp (*Ctenopharyngodon idella*) by response surface methodology. *Innov Food Sci Emerg Technol* **9,** 604–7.

Woo JW, Yu SJ, Cho SM, Lee YB, and Kim SB (2008) Extraction optimization and properties of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin. *Food Hydrocolloids* **22,** 879–87.

Yaacob WAA and Ali A (1994) Portable canvas tanks for culture of hybrid catfish (*Clarias gariepinus* × *Clarias macrocephalus*) by small scale farmers in Malaysia. Naga, *The ICLARM Quarterly* **17,** 25–7.

Yakimets I, Paes SS, Wellner N, Smith AC, Wilson RH, and Mitchell JR (2007) Effect of water content on the structural reorganization and elastic properties of biopolymer films: A comparative study. *Biomacromolecules* **8,** 1710–22.

Yan M, Li B, Zhao X, Ren G, Zhuang Y, Hou H et al. (2008) Characterization of acid-soluble collagen from the skin of walleye pollock (*Theragra chalcogramma*). *Food Chem* **107,** 1581–6.

Żelechowska E, Sadowska M, and Turk M (2010) Isolation and some properties of collagen from the backbone of Baltic cod (*Gadus morhua*). *Food Hydrocolloids* **24,** 325–9.

Zeng SK, Zhang CH, Lin HL, Yang P, Hong PZ, and Jiang Z (2009) Isolation and characterisation of acid-solubilised collagen from the skin of Nile tilapia (*Oreochromis niloticus*). *Food Chem* **116**, 879–83.

Zeng S, Yin J, Yang S, Zhang C, Yang P, and Wu W (2012) Structure and characteristics of acid and pepsin-solubilized collagens from the skin of cobia (*Rachycentron canadum*). *Food Chem* **135**, 1975–84.

Zhang J, Duan R, Tian Y, and Konno K (2009a) Characterisation of acid-soluble collagen from skin of silver carp (*Hypophthalmichthys molitrix*). *Food Chem* **116,** 318–22.

Zhang M, Liu W, and Li GL (2009b) Isolation and characterisation of collagens from the skin of largefin longbarbel catfish (*Mystus macropterus*). *Food Chem* **115,** 826–31.