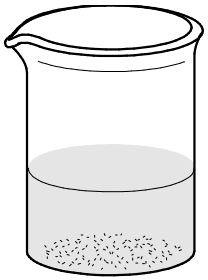
Graphical Abstract

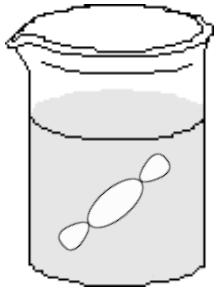
Collagen Extraction

Clown featherback skin



Salt precipitation

Extraction



Dialysis

Filtration

Freeze-drying

Characteristics of collagen

Acid Soluble Collagen/
Pepsin Soluble Collagen

- 82.08% total collagen recovery
- 27.64% yield of ASC
- 44.63% yield of PSC
- 330-333 residues/1000 residues of glycine
- 201-202 residues/1000 residues of imino acid
- type I, comprising $(\alpha 1)_2\alpha 2$-heterotrimer
- T_{max} = 35.23-36.28 °C
- pI = 5.54-5.68

Characteristics of Collagen from the Skin of Clown Featherback (*Chitala ornata*)

To be submitted to International Journal of Food Science and Technology

Phanat Kittiphattanabawon,[a,b*] Soottawat Benjakul,[c]
Sittichoke Sinthusamran[c] and Hideki Kishimura[d]

[a]Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung 93110, Thailand

[b]Department of Agro-Industry Technology and Management, Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok, Prachinburi, 25230, Thailand

[c]Department of Food Technology, Faculty of Agro-Industry Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

[d]Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

* To whom correspondence should be addressed. Tel/Fax: 66-7469-3996
e-mail: kphanat@gmail.com

Summary

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the skin of clown featherback (*Chitala ornata*) were isolated and characterised. Yields of ASC and PSC were 27.64 and 44.63% (dry weight basis) with total collagen recovery of 82.08%. Both collagens contained glycine as the major amino acid with relatively high content of proline, hydroxyproline and glutamic acid/glutamine. Nevertheless, they had the low content of cysteine, histidine and tryrosine. The collagen was characterised as type I, comprising $(\alpha 1)_2\alpha 2$-heterotrimer. Pepsin-aided process did not affect triple-helical structure of PSC as determined by FTIR spectra. Thermal transition temperature of ASC (36.28 °C) was slightly higher than that of PSC (35.23 °C). However, no differences in isoelectirc point (5.54-5.68) between ASC and PSC was observed. Therefore, collagen from the skin of clown featherback could be successfully extracted for further applications.

Keywords: collagen; ASC; PSC; clown featherback; *Chitala ornata*; type I collagen

Introduction

Clown featherback (*Chitala ornata*) is freshwater fish, which is commonly found in Thailand. It has been generally used for fish ball and fish cake production since its muscle is white and has good gel forming ability. During the dressing, skins (17-22% of total weight) are removed. The skins are rich in collagen (Foegeding *et al.*, 1996, Wong 1989) and some proteins have been processed as the crispy fried fish skin. However, the market value is still low. The production of high-valued products could pave the way for gaining the higher benefit or revenue, e.g. production of collagen.

Collagen is a major structural protein in the connective tissue of animal skin and bone (Foegeding *et al.*, 1996). It is constructed from tropocollagen, a rod-shaped protein consisting of three polypeptides unit (called α chains) intertwined to form a triple-helical structure. The three chains are held together primarily by hydrogen bonding between adjacent –CO and –NH group (Wong 1989, Foegeding *et al.*, 1996). Additionally, collagen fibers can be strengthened and are stabilised primarily by covalent cross-linkages (Belitz *et al.*, 2009). Intermolecular crosslinks are confined to the end-overlap region involving a lysine aldehyde in the telopeptide of one chain and a hydroxylysine of an adjacent chain (Foegeding *et al.*, 1996). Normally, collagen production could be divided as acid solubilse collagen (ASC) and pepsin solubilise collagen (PSC), based on extraction process. The non-crosslinking components were easily extracted by acid solution referred to as " ASC". However, the crosslinking components stabilised by covalent bonds at telopeptide region could not be solubilsed by acid solution, leading to lower yield. Pepsin has been applied since it is able to cleave peptides specifically in telopeptide region of collagen, leading to increased

extraction efficiency (Nalinanon *et al.,* 2007). The collagen with the increased extraction yield is termed “PSC”.

Generally, collagen has been produced from bovine and porcine hides and bones. Currently, the increasing attention of alternative sources for replacement of mammalian collagen has been paid, especially fish processing by-products, due to the requirement of halal product from Muslims and the consumer’s safety concern for bovine spongiform encephalopathy and bird flu, etc (Aewsiri *et al.,* 2009, Jongjareonrak *et al.,* 2006, Regenstein and Zhou 2007). However, fish collagens have lower thermal stability than do mammalian counterparts because the formers contain a lower imino acid content than do the latters (Foegeding *et al.,* 1996). Collagen from fish skin has been extracted from many fish species, such as brownbanded bamboo shark (Kittiphattanabawon *et al.,* 2010a), bigeye snapper (Benjakul *et al.,* 2010), threadfin bream (Nalinanon *et al.,* 2011), cobia (Zeng *et al.,* 2012), sea bass (Sinthusamran *et al.,* 2013) and grass carp (Chen *et al.,* 2015). However, there is no information regarding the collagen from the skin of clown featherback, a freshwater fish widely used for fish ball production in Thailand. Therefore, the objective of this investigation was to isolate and characterise the collagen from the skin of clown featherback, a by-product from the processing plants.

Materials and Methods

Chemicals

All chemicals were of analytical grade. Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Type I collagen from calf skin and pepsin from porcine stomach mucosa (EC 3.4.23.1) were obtained from

Sigma Chemical Co. (St. Louis, MO, USA). High-molecular-weight markers and TOYOPEARL® CM-650M were purchased from GE Healthcare UK Limited (Buckinghamshire, UK) and Tosoh Corporation (Tokyo, Japan), respectively.

Preparation of clown featherback skin

Skin of clown featherback (*Chitala ornata*) with the size of 0.7-1.5 kg was obtained from a local fish ball and fish cake processing plant at Talaadthai in Pathumthani province, Thailand. The fresh skin packed in polyethylene bags (1 kg/bag) was placed in ice at a ratio of skin to ice of 1:2 (w/w) using a polystyrene box as a container. The skin was transported to the Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok, Prachinburi province by a car within 4 h. Upon arrival, the skin was washed with cold tap water (≤10 °C). The residual meat on the skin was removed by knife and washed with cold tap water. The clean skin was cut into small pieces (approximately 1.0 x 1.0 cm^2) using a pair of scissors. The prepared skin was placed in polyethylene bags (50-100 g/bag) and stored at -20 °C until used but not longer than 3 months. The moisture content of prepared skin was 65.72% as determined by AOAC method (AOAC 2000). Prior to collagen extraction, the frozen skin was thawed with running water until the core temperature of the skin reached 8–10 °C.

Extraction of collagen from clown featherback skin

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from the prepared skin following the method of Kittiphattanabawon *et al*. (2010a). All procedures were carried out at 4 °C.

Firstly, the prepared skin was mixed with 0.1 M NaOH at a solid/alkali solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred for 6 h continuously using an overhead stirrer (model W20.n, IKA®-Werke GmbH & CO.KG, Stanfen, Germany) at a speed of 250 rpm and the alkali solution was changed every 2 h. Then, the pretreated skin was washed with cold tap water until pH of wash water becames neutral or faintly basic.

To extract collagen, the pretreated skin was soaked in 0.5 M acetic acid with a solid to solvent ratio of 1:15 (w/v) for 48 h with a continuous stirring, followed by filtration with two layers of cheesecloth. The collagen in filtrate was precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M Tris(hydroxymethyl) aminomethane, pH 7.5. The resultant precipitate was collected by centrifugation at 20000 × g at 4 °C for 60 min using a refrigerated centrifuge (model Avanti® J-E, Beckman Coulter, Inc., Palo Alto, CA, USA). The pellet was dissolved in a minimum volume of 0.5 M acetic acid. The solution was then dialysed against 25 volumes of 0.1 M acetic acid for 12 h, followed by the same volume of distilled water for 48 h. Then, the resulting dialysate was freeze dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The obtained collagen from acid solubilisation process was referred to as “acid soluble collagen, ASC”. The undissolved residue obtained after acid extraction was used for pepsin soluble collagen extraction. The residue was soaked in 0.5 M acetic acid with a solid to solvent ratio of 1:15 (w/v). Porcine pepsin (20 unit/g of residue) in which proteolytic activity was determined by the method of Nalinanon, Benjakul, Visessanguan and Kishimura (2008) was added. The mixtures were continuously stirred at 4 °C for 48 h, followed by filtration using two layers of cheesecloth. The filtrate was collected and subjected to precipitation and dialysis in the same manner with those used for ASC as

previously described. The obtained collagen from pepsin solubilisation process was referred to as “pepsin soluble collagen, PSC”.

Yield and recovery of collagen

Yield and recovery of ASC and PSC were calculated based on dry basis of starting raw material

$$\% \text{ Yield} = \frac{\text{Weight of lyophilised collagen (g)}}{\text{Weight of dry skin (g)}} \times 100$$

$$\% \text{ Recovery} = \frac{\text{Hyp content in collagen (mg/g collagen) x weight of collagen obtained (g)}}{\text{Hyp content in skin (mg/g skin) x weight of skin (g)}} \times 100$$

The hydroxyproline (Hyp) content in the skin and collagens were determined according to the method of Bergman and Loxley (1963).

Amino acid analysis

ASC and PSC were hydrolysed under reduced pressure in 4.0 M methane sulphonic acid containing 0.2% (v/v) 3-2(2-aminoethyl)indole at 115 °C for 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 mL was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970). The samples were dissolved in 5% SDS solution. The mixtures were then heated in boiling water for 1 min, followed by centrifugation at 8500 × g for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, Germany) to remove undissolved debris. Solubilised

samples were mixed at 1:1 (v/v) ratio with sample buffer (0.5 M tris-HCl, pH 6.8 containing 4% SDS and 20% glycerol in the presence or absence of 10% (v/v) βME). Samples were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 20 mA/gel. After electrophoresis, gels were fixed with a mixture of 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, followed by staining with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 1 h. Finally, they were destained with 30% (v/v) methanol and 10% (v/v) acetic acid for 1 h and destained again with the same solution for 30 min. High-molecular-weight protein markers (GE Healthcare UK Limited, Buckinghamshire**,** UK) were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as standard collagen. The intensity of bands was quantified with the public domain digital analysis software, ImageJ (ImageJ 1.42q, National Institutes of Health, Bethesda, MD, USA).

TOYOPEARL® CM-650M column chromatography

TOYOPEARL® CM-650M column chromatography was carried out according to the method of Kittiphattanabawon *et al*. (2010a). Collagen samples (30 mg) were dissolved in 3 mL of starting buffer (20 mM sodium acetate buffer, pH 4.8) and boiled for 1 min. The mixtures were centrifuged at 8500 × g at room temperature (25-26 °C) for 10 min. The supernatants were applied onto a TOYOPEARL® CM-650M column (1.8 x 20 cm) previously equilibrated with 10 volumes of the starting buffer at a flow rate of 3 mL/min. After loading, the unbound proteins were washed by the same buffer until A_{230} was less than 0.05, which located the elution time about 60-70 min. Elution was achieved with a linear gradient of 0-0.3 M NaCl in the same buffer at a flow rate of 2 mL/min with a total volume of 400 mL (elution time of 200 min). The

eluant was monitored at 230 nm and fractions (4 mL each) were collected. The selected fractions were subjected to SDS-PAGE using 7.5% separating gel and 4% stacking gel as previously described.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of collagens were obtained using a Bruker model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate (DLATGS) detector. The Horizontal Attenuated Total Reflectance Accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000-650 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean and empty cell at 25 °C. Analysis of spectral data was carried out using a OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

Differential scanning calorimetry (DSC)

The collagens were rehydrated by adding the deionised water to dried samples at a solid to water ratio of 1:40 (w/v). The mixtures were allowed to stand for 2 days at 4 °C prior to analysis. Differential scanning calorimetry (DSC) was performed using a differential scanning calorimeter model DSC 7 (Perkin Elmer, Norwalk, CT, USA). Calibration was run using Indium thermogram. The sample (5-10 mg) was accurately weighed into aluminum pans and sealed. The sample was scanned at 1 °C/min over the range of 25-50 °C using iced water as the cooling medium. An empty

pan was used as the reference. The maximum transition temperature (T_{max}) was estimated from the thermogram. Total denaturation enthalpy (ΔH) was estimated by measuring the area of DSC thermogram.

Zeta potential analysis

Collagens were dissolved in 0.5 M acetic acid to obtain a final concentration of 0.05% (w/v). The mixtures were continuously stirred at 4 °C using a magnetic stirrer model BIG SQUID (IKA®-Werke GmbH & CO.KG, Stanfen, Germany) until the samples were completely solubilised.

Zeta (ζ) potential of collagen solutions was measured by Zeta potential analyser model ZetaPALs (Brookhaven Instruments Co., Holtsville, NY, USA). Solutions (20 mL) were transferred to autotitrator model BI-ZTU (Brookhaven Instruments Co., Holtsville, NY, USA), in which the pHs of solutions were adjusted to 2 to 12 using either 1.0 M nitric acid or 1.0 M KOH. The obtained zeta potential of solution at all pHs determined was recorded.

Statistical analysis

The experiments were carried out in triplicate using three different lots of samples. The difference between means were tested by T-test (Steel and Torrie 1980). The data were presented as means ± standard deviation.

Results and discussion

Yield and recovery of collagen

Yields of ASC and PSC from the skin of clown featherback were 27.6 and 44.6% (dry weight basis), corresponding to 9.5 and 15.3% (wet weight basis),

239 respectively. ASC and PSC extracted from the skin of black carp showed the yield of
240 15.5 and 26.5% (dry weigh basis), respectively (Jia *et al.,* 2012). The differences in
241 yields obtained between fish species might be due to the differences in the skin
242 matrices and the distribution of alignment of component in the skin. The extractable
243 yield of collagen (ASC and PSC) from clown featherback skin (24.8%) was higher
244 than that from brownbanded bamboo shark skin (18.2%), blacktip shark cartilage
245 (3.6%), yellowfin tuna swim bladder (13.17%), bighead carp scale (1.1%), striped
246 catfish skin (12.8%) and bighead carp swim bladders (14.6%) (Kaewdang *et al.*, 2014,
247 Kittiphattanabawon *et al.*, 2010a, Kittiphattanabawon *et al.*, 2010b, Liu *et al.*, 2012,
248 Singh *et al.,* 2011). It might be caused by low cross-linking of collagen fibrils in the
249 skin of clown featherback. The recovery of total collagen (ASC and PSC) was
250 82.08%. The result suggested that approximately 18% was insoluble collagen (ISC),
251 which could not be extracted by pepsin-aided process. ISC was most likely associated
252 with strong crosslinked component of collagen molecules, in which their telopeptides
253 could not be cleaved by pepsin. It was implied that the skin of clown featherback
254 contained low amount of crosslink components, leading to high collagen recovery and
255 yield. However, the ASC, PSC and ISC contents might be variable depending on the
256 extraction time.
257
258 **Amino acid composition**
259 Amino acid composition of ASC and PSC from the skin of clown featherback
260 is shown in Table 1. Glycine was found as the major amino acid (330-333
261 residues/1000 residues), followed by alanine (120-121 residues/1000 residues),
262 proline (117-118 residues/1000 residues), hydroxyproline (83-85 residues/1000
263 residues) and glutamic acid/glutamine (69-75 residues/1000 residues). Relative low

contents of cysteine (1 residue/1000 residues), histidine (4-5 residues/1000 residues) and tyrosine (2-3 residues/1000 residues) were observed. Their amino acid compositions were generally in agreement with those of fish skin collagen from other fish species (Wang *et al.,* 2014, Sinthusamran *et al.,* 2013, Singh *et al.,* 2011). When comparing amino acid compositions, it was found that ASC had higher glutamic acid/glutamine and threonine. On the other hand, PSC showed the higher aspartic acid/asparagine, lysine and serine than ASC. This was more likely governed by the removal of some part of teleopeptide by pepsin. Glycine represents nearly one-third of the total residues, and it is distributed uniformly at every third position throughout most of the collagen molecule. The repetitive occurrence of glycine is absent in the first 14 amino acid residues from the N-terminus and the first 10 amino acid residues from the C-terminus, in which these end portions are termed “telopeptides” (Foegeding *et al.,* 1996). Regenstein and Zhou (2007) reported that the presence of glycine at every three residues is a critical requirement for the collagen super-helix structure. Moreover, the imino acid content (proline and hydroxyproline) are important for the structural integrity of collagen. The presence of proline stabilises the helix structure by preventing rotation of the N-C bond. Hydroxyproline also stabilises the collagen molecule (Foegeding *et al.,* 1996). The imino acid content of both collagens (201-202 residues/1000 residues) was slightly difference with that of tropical fish, such as striped catfish skin (201-202 residues/1000 residues) and higher than that of collagen from cold water fish, such as cod skin (154 residues/1000 residues) and bighead carp skin (165 residues/1000 residues) (Liu *et al.,* 2012, Duan *et al.,* 2009, Singh *et al.,* 2011). Nevertheless, it was slightly lower than that of mammal (calf and porcine skin, 216-220 residues/1000 residues) (Li *et al.,* 2013). It

was noted that the imino acid content correlates with the water temperature of their normal habitat (Foegeding *et al.,* 1996).

Protein patterns

Protein patterns of ASC and PSC under reducing and non-reducing conditions are shown in Fig. 1. No differences in protein patterns of ASC and PSC under both conditions was found, suggesting that both collagens contained no disulphide bonds. This was coincidental with the negligible cysteine content (Table 1). Both collagens contained α1-, α2-, β- and γ-chains. The molecular weights of those bands of PSC was slightly lower than those of ASC. Additionally, the band intensity of γ-chain of ASC was higher than that of PSC. γ-chain was possibly hydrolysed by pepsin, especially at telopeptide region of tropocollagen. The ratio of band intensity of α1/α2 approximately 2:1. It was suggested that the collagen from clown featherback skin was type I collagen. The collagens from other fish skin, such as Spanish mackerel, ornate threadfin bream, seabass, black carp, bighead carp, stripped catfish and Amur sturgeon have also been reported as type I collagen (Singh *et al.,* 2011, Nalinanon *et al.,* 2011, Wang *et al.,* 2014, Sinthusamran *et al.,* 2013, Li *et al.,* 2013, Jia *et al.,* 2012, Liu *et al.,* 2012). However, α1α2α3-herterotrimer might be present in the sample because the molecular weight of α1 and α3 was quite the same (Nagai *et al.,* 2014).

Subunit compositions

The chromatogram of ASC dissociated by heat treatment and protein pattern of selected fractions are shown in Fig. 2. The fractions were eluted as two major peaks. The first peak (fraction Nos. 41-53) had α1-chain as a major component, whilst

the second peak had α2-, β- and γ-chains as dominant constituents. From the result, it was suggested that collagen from clown featherback was type I collagen with molecular composition of $(\alpha 1)_2\alpha 2$-heterotrimer.

Fourier transform infrared (FTIR) spectra

FTIR spectra of ASC and PSC are shown in Fig. 3. The similar patterns in FTIR spectra between ASC and PSC were observed. The spectra included amide A (3263-3289 cm^{-1}), amide B (2923-2924 cm^{-1}), amide I (1629-1633 cm^{-1}), amide II (1531-1532 cm^{-1}) and amide III (1233-1234 cm^{-1}). These major peaks were similar to those of collagen from other fish skins (Sinthusamran *et al.*, 2013, Wang *et al.*, 2014, Li *et al.*, 2013, Singh *et al.*, 2011). In generally, no differences in all bands in FTIR spectra between ASC and PSC were observed, except in amide A (3263 and 3289 cm^{-1} for ASC and PSC, respectively). Amide A, commonly associated with N–H stretching vibration, occurs in the wavenumber range of 3400-3440 cm^{-1} and when the NH group of a peptide is involved in a H-bond, the position is shifted to lower frequency (Doyle *et al.*, 1975). Foegeding *et al*. (1996) reported that monomer of collagen, tropocollagen, consists of three polypeptide chains (called α chains) wound around each other in a suprahelical fashion by hydrogen bonding. It was also suggested that hydrolysis with pepsin at telopeptide region during collagen extraction might destruct hydrogen bonding between adjacent –CO and –NH group to some extentas indicated by the higher wavenumber of amide A in PSC.

No differences in amide I (1629–1633 cm^{-1}) and II peaks (1531–1532 cm^{-1}) between ASC and PSC was found, suggesting the similarity in intra-molecular alignment of triple helix between both collagens (Payne and Veis 1988). When determining the ratio of amplitude of amide III and 1454 cm^{-1} band for both ASC

(1.00) and PSC (1.01), the ratios were close to 1. The results indicated that the collagen were in triple-helical structure (Plepis *et al.,* 1996). Therefore, the secondary structure of collagen were not affected by pepsin-aided process.

Thermal transition

DSC thermograms of ASC and PSC are shown in Fig. 4. Thermal transition temperature (T_{max}) of ASC (36.28 °C) was slightly higher than that of PSC (35.23 °C). When comparing T_{max} of both collagens with those from other freshwater fish skin, collagen from clown featherback had a lower T_{max} than collagen from striped catfish (39.31-39.66 °C) (Singh *et al.,* 2011), but showed higher T_{max} than Amur sturgeon (32.46-32.78 °C) (Wang *et al.,* 2014). The difference in T_{max} amongst fish species might be correlated with their imino acid compositions and/or degree of crosslinking. The stability of collagen was proportional to the total content of pyrrolidine imino acids. Proline and hydroxyproline rich zones of the molecules are most likely involved in the formation of junction zones stabilised by hydrogen bonding (Johnston-Banks 1990). For enthalpy (ΔH) of ASC and PSC, the former (0.99 J/g) had a much higher enthalpy than the latter (0.56 J/g). It might be due to the lower in degree of lysine hydroxylation (Table 1, 48.57 and 21.21% for ASC and PSC, respectively). Hydroxylated lysine contributes to the formation and stabilisation of cross-links, giving rise to complex, non-hydrolysable bonds (Belitz *et al.,* 2009, Kittiphattanabawon *et al.,* 2005). Furthermore, the cleavage of telopeptide region by pepsin or removal of some of those peptides might facilitate the denaturation of PSC induced by heat (Kittiphattanabawon *et al.,* 2010a).

Zeta potential

The zeta potentials of ASC and PSC at pH ranging from 2 to 12 are shown in Fig. 5. Zeta potential analysis was used for monitoring the change of surface charge of sample at different pHs. When the surface charge of sample became zero, such a pH could be assumed to be isoelectric point (pI) of sample. Positive charge of ASC and PSC continuously decreased as pH increased. At pH 5.54 and 5.68, the surface charges of ASC and PSC were zero, respectively. Those pHs were assumed to be the pI of both collagens. At the pH above this point, the surface charge turned to be negative. The pIs of collagen from different fish skin, such as ornate threadfin bream, brownbanded bamboo shark, blacktip shark and seabass as analysed by zeta potential analysis have been reported to be faintly acidic to neutral (6.21-7.02) (Kittiphattanabawon *et al.*, 2010a, Sinthusamran *et al.*, 2013, Kittiphattanabawon *et al.*, 2010c, Nalinanon *et al.*, 2011). Kaewdang *et al*. (2014) reported that the differences in pI between collagens from various fish species might be caused by the difference in their amino acid compositions and distribution of amino acid residues, particularly on the surface domains. Some difference in amino acid compositions, especially acidic and basic amino acids (Table 1), might determine the slight difference in pI between ASC and PSC.

Conclusions

Collagen from clown featherback skin could be extracted by acid solublisation process and/or pepsin-aided process. Use of pepsin treatment could enhance collagen yield and recovery without the effect on triple-helical structure. Collagen obtained from both processes had the similar characteristics. Based on collagen recovery and

its characteristics, the clown featherback could be used as an alternative source for collagen extraction.

Acknowledgment

We would like to thanks Department of Food Technology, Faculty of Agro-industry, Prince of Songkla University, Songkhla, Thailand for any instrument support. This work was supported by The Thailand Research Fund and Thaksin University for contract No. TRG5780036 to Dr. Phanat Kittiphattanabawon. The TRF Distinguished Research Professor Grant was also acknowledged.

References

Aewsiri, T., Benjakul, S. & Visessanguan, W. (2009). Functional properties of gelatin from cuttlefish (*Sepia pharaonis*) skin as affected by bleaching using hydrogen peroxide. *Food Chemistry,* **115,** 243-249.

AOAC (2000). *Official Methods of Analysis.* Arlington, Association of Official Ananlytical Chemists Inc.

Belitz, H. D., Grosch, W. & Schieberle, P. (2009). *Food Chemistry*. Leipzig, Germany, Springer-Verlag Berlin Heidelberg.

Benjakul, S., Nalinanon, S. & Shahidi, F. (2012). Fish collagen. In: *Food Biochemistry and Food Processing* (edited by SIMPSON, B. K., PALIYATH, G., NOLLET, L. M. L., BENJAKUL, S. & TOLDRá, F.). Pp. 365-387. Iowa: John Wiley & Sons, Inc.

Benjakul, S., Thiansilakul, Y., Visessanguan, W., Roytrakul, S., Kishimura, H., Prodpran, T. & Meesane, J. (2010). Extraction and characterisation of pepsin-solubilised collagens from the skin of bigeye snapper (*Priacanthus tayenus* and *Priacanthus macracanthus*). *Journal of the Science of Food and Agriculture,* **90,** 132-138.

Bergman, I. & Loxley, R. (1963). Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Analytical Chemistry,* **35,** 1961-1965.

Chen, Y., Ye, R. & Wang, Y. (2015). Acid-soluble and pepsin-soluble collagens from grass carp (*Ctenopharyngodon idella*) skin: A comparative study on physicochemical properties. *International Journal of Food Science and Technology,* **50,** 186-193.

421 Doyle, B. B., Blout, E. R. & Bendit, E. G. (1975). Infrared spectroscopy of collagen
422 and collagen like polypeptides. *Biopolymers,* **14,** 937-957.
423 Duan, R., Zhang, J., Du, X., Yao, X. & Konno, K. (2009). Properties of collagen from
424 skin, scale and bone of carp (*Cyprinus carpio*). *Food Chemistry,* **112,** 702-706.
425 Foegeding, E. A., Lanier, T. C. & Hultin, H. O. (1996). Characteristics of edible
426 muscle tissues. In: *Food Chemistry* (edited by FENNEMA, O. R.). Pp. 879-
427 942. New York: Marcel Dekker, Inc.
428 Jia, Y., Wang, H., Li, Y., Wang, M. & Zhou, J. (2012). Biochemical properties of skin
429 collagens isolated from black carp (*Mylopharyngodon piceus*). *Food Science*
430 *and Biotechnology,* **21,** 1585-1592.
431 Johnston-Banks, F. A. (1990). Gelatin. In: *Food Gels* (edited by HARRIS, P.). Pp.
432 233-289. London: Elsevier Applied Science.
433 Jongjareonrak, A., Benjakul, S., Visessanguan, W. & Tanaka, M. (2006). Skin gelatin
434 from bigeye snapper and brownstripe red snapper: Chemical compositions and
435 effect of microbial transglutaminase on gel properties. *Food Hydrocolloids,*
436 **20,** 1216-1222.
437 Kaewdang, O., Benjakul, S., Kaewmanee, T. & Kishimura, H. (2014). Characteristics
438 of collagens from the swim bladders of yellowfin tuna (*Thunnus albacares*).
439 *Food Chemistry,* **155,** 264-270.
440 Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Kishimura, H. & Shahidi, F.
441 (2010a). Isolation and characterisation of collagen from the skin of
442 brownbanded bamboo shark (*Chiloscyllium punctatum*). *Food Chemistry,* **119,**
443 1519-1526.

444 Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T. & Tanaka, M.
445 (2005). Characterisation of acid-soluble collagen from skin and bone of bigeye
446 snapper (*Priacanthus tayenus*). *Food Chemistry,* **89,** 363-372.
447 Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. & Shahidi, F. (2010b).
448 Isolation and characterization of collagen from the cartilages of brownbanded
449 bamboo shark (*Chiloscyllium punctatum*) and blacktip shark (*Carcharhinus*
450 *limbatus*). *LWT - Food Science and Technology,* **43,** 792-800.
451 Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. & Shahidi, F. (2010c).
452 Isolation and properties of acid- and pepsin-soluble collagen from the skin of
453 blacktip shark (*Carcharhinus limbatus*). *European Food Research and*
454 *Technology,* **230,** 475-483.
455 Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the
456 head of bacteriophage T4. *Nature,* **227,** 680-685.
457 Li, Z. R., Wang, B., Chi, C. F., Zhang, Q. H., Gong, Y. D., Tang, J. J., Luo, H. Y. &
458 Ding, G. F. (2013). Isolation and characterization of acid soluble collagens and
459 pepsin soluble collagens from the skin and bone of Spanish mackerel
460 (*Scomberomorous niphonius*). *Food Hydrocolloids,* **31,** 103-113.
461 Liu, D., Liang, L., Regenstein, J. M. & Zhou, P. (2012). Extraction and
462 characterisation of pepsin-solubilised collagen from fins, scales, skins, bones
463 and swim bladders of bighead carp (*Hypophthalmichthys nobilis*). *Food*
464 *Chemistry,* **133,** 1441-1448.
465 Nagai, T., Tanoue, Y., Kai, N. & Suzuki, N. (2014). Characterization of collagen from
466 emu (*Dromaius novaehollandiae*) skins. *Journal of Food Science and*
467 *Technology*.

468 Nalinanon, S., Benjakul, S., Kishimura, H. & Osako, K. (2011). Type I collagen from
469 the skin of ornate threadfin bream (*Nemipterus hexodon*): Characteristics and
470 effect of pepsin hydrolysis. *Food Chemistry,* **125,** 500-507.
471 Nalinanon, S., Benjakul, S., Visessanguan, W. & Kishimura, H. (2007). Use of pepsin
472 for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*).
473 *Food Chemistry,* **104,** 593-601.
474 Nalinanon, S., Benjakul, S., Visessanguan, W. & Kishimura, H. (2008). Tuna pepsin:
475 Characteristics and its use for collagen extraction from the skin of threadfin
476 bream (*Nemipterus* spp.). *Journal of Food Science,* **73,** C413-C419.
477 Payne, K. J. & Veis, A. (1988). Fourier transform IR spectroscopy of collagen and
478 gelatin solutions: Deconvolution of the amide I band for conformational
479 studies. *Biopolymers,* **27,** 1749-1760.
480 Plepis, A. M. D. G., Goissis, G. & Das-Gupta, D. K. (1996). Dielectric and
481 pyroelectric characterization of anionic and native collagen. *Polymer*
482 *Engineering and Science,* **36,** 2932-2938.
483 Regenstein, J. M. & Zhou, P. (2007). Collagen and gelatin from marine by-products.
484 In: *Maximising the Value of Marine By-Products* (edited by SHAHIDI, F.).
485 Pp. 279-303. Cambridge: Woodhead Publishing Limited.
486 Singh, P., Benjakul, S., Maqsood, S. & Kishimura, H. (2011). Isolation and
487 characterisation of collagen extracted from the skin of striped catfish
488 (*Pangasianodon hypophthalmus*). *Food Chemistry,* **124,** 97-105.
489 Sinthusamran, S., Benjakul, S. & Kishimura, H. (2013). Comparative study on
490 molecular characteristics of acid soluble collagens from skin and swim bladder
491 of seabass (*Lates calcarifer*). *Food Chemistry,* **138,** 2435-2441.

492 Steel, R. G. D. & Torrie, J. H. (1980). *Principles and Procedures of Statistics: A*
493 *Biometrical Approach.* New York, McGraw-Hill.
494 Wang, L., Liang, Q., Chen, T., Wang, Z., Xu, J. & Ma, H. (2014). Characterization of
495 collagen from the skin of Amur sturgeon (*Acipenser schrenckii*). *Food*
496 *Hydrocolloids*, **38,** 104-109.
497 Wong, D. W. S. (1989). *Mechanism and Theory in Food Chemistry.* New York, Van
498 Nostrand Reinhold Company Inc.
499 Zeng, S., Yin, J., Yang, S., Zhang, C., Yang, P. & Wu, W. (2012). Structure and
500 characteristics of acid and pepsin-solubilized collagens from the skin of cobia
501 (*Rachycentron canadum*). *Food Chemistry,* **135,** 1975-1984.

Table 1
Amino acid compositions of ASC and PSC from the skin of clown featherback (residues/1000 residues).

Amino acid	ASC	PSC
Alanine	120	121
Arginine	54	53
Asparagine /Aspartic acid	39	47
Cysteine	1	1
Glutamine/Glutamic acid	75	69
Glycine	333	330
Histidine	5	4
Isoleucine	13	11
Leucine	23	23
Lysine	18	26
Hydroxylysine	17	7
Methionine	12	12
Phenylalanine	16	13
Hydroxyproline	83	85
Proline	118	117
Serine	20	35
Threonine	32	23
Tyrosine	2	3
Valine	22	20
Total	**1000**	**1000**
Imino acid	201	202

Legends to Figures

Fig. 1. Protein patterns of ASC and PSC from the skin of clown featherback under non-reducing and reducing conditions. M and I denote high molecular weight protein markers, and type I collagen from calf skin, respectively.

Fig. 2. Chromatogram of ASC from the skin of clown featherback on the TOYOPEARL® CM-650M ion-exchange column. The fractions indicated by numbers were examined by SDS-PAGE using 7.5% separating gel and 4% stacking gel. M and C denote high molecular weight protein markers and collagen, respectively.

Fig. 3. FTIR spectra of ASC and PSC from the skin of clown featherback.

Fig. 4. DSC thermogram of ASC and PSC from the skin of clown featherback.

Fig. 5. Zeta (ζ) potential of ASC and PSC from the skin of clown featherback at different pHs. Bars represent the standard deviation (n=3).

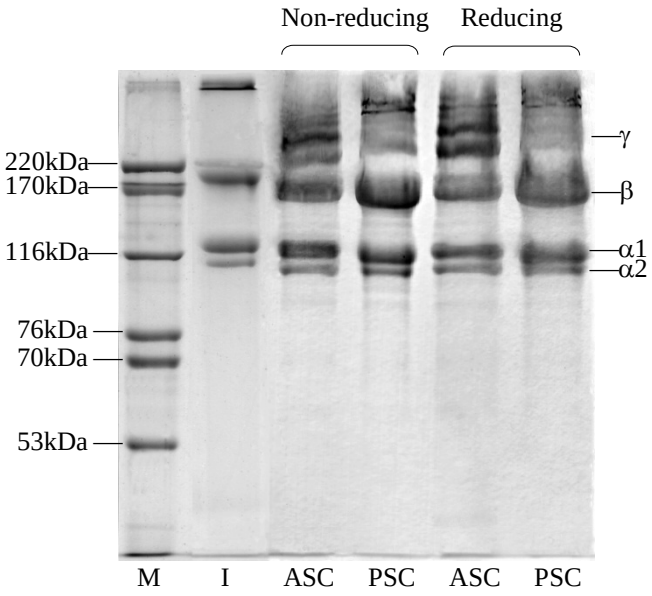

Non-reducing
Reducing
220kDa
170kDa
116kDa
76kDa
70kDa
53kDa
γ
β
α1
α2
M
I
ASC
PSC
ASC
PSC

Fig. 1

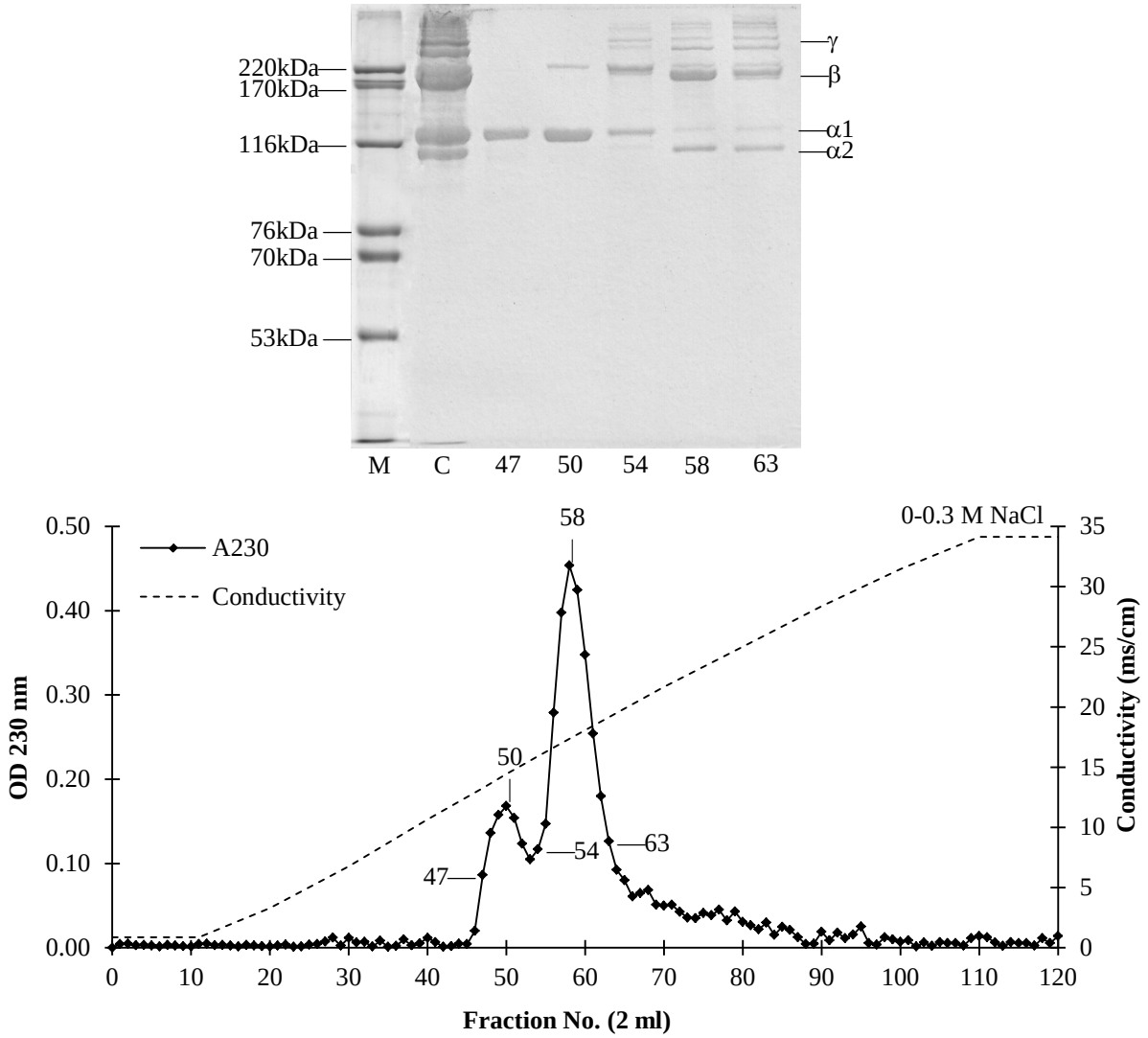

220kDa
170kDa
116kDa
76kDa
70kDa
53kDa
γ
β
α1
α2
M
C
47
50
54
58
63
A230
Conductivity
0-0.3 M NaCl
OD 230 nm
Conductivity (ms/cm)
Fraction No. (2 ml)

Fig. 2

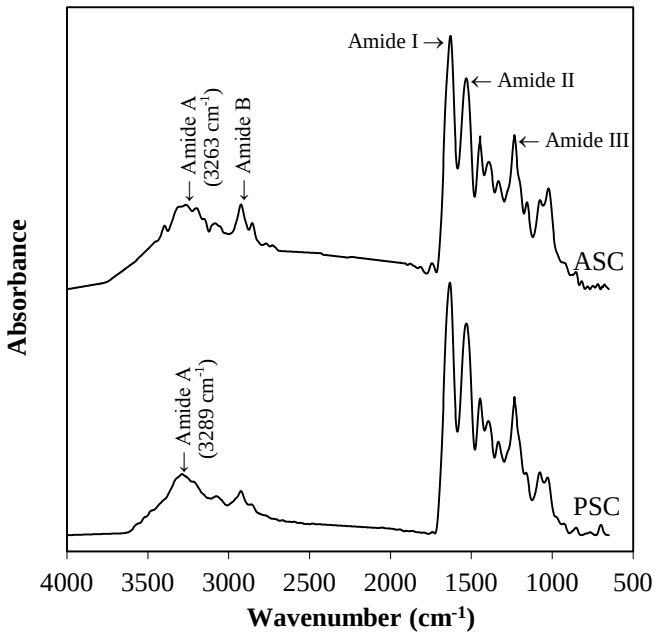

Amide I →
← Amide II
← Amide III
← Amide A (3263 cm-1)
← Amide B
ASC
← Amide A (3289 cm-1)
PSC
Absorbance
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)

Fig. 3

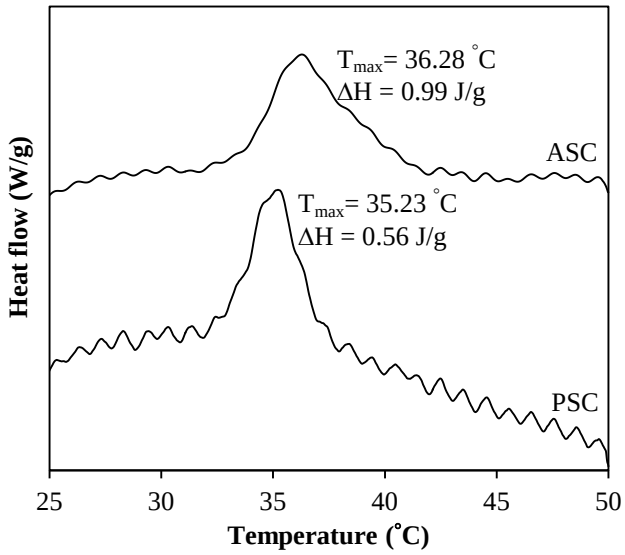

T$_{max}$= 36.28 °C
ΔH = 0.99 J/g
ASC
T$_{max}$= 35.23 °C
ΔH = 0.56 J/g
PSC
Heat flow (W/g)
25
30
35
40
45
50
Temperature (°C)

Fig. 4

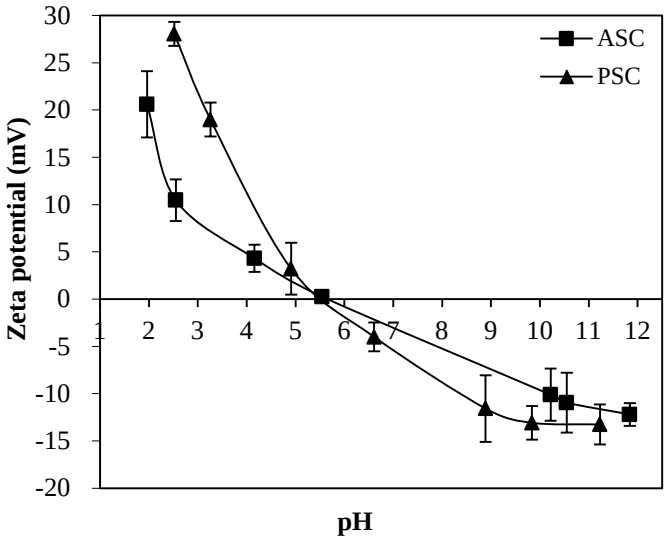

30
25
20
15
10
5
0
-5
-10
-15
-20
1
2
3
4
5
6
7
8
9
10
11
12
Zeta potential (mV)
pH
ASC
PSC

Fig. 5