TITLE : Characteristics of collagen from rohu (*Labeo rohita*) skin

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ABSTRACT

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were isolated from rohu skin with the yield of 64.2 and 6.8% (dry weight basis), respectively. Both collagens had glycine as the major amino acid with imino acid content of 196-202 residues/1000 residues and were characterised as type I collagen with molecular composition of $(\alpha1)_2\alpha2$-heterotrimer. FTIR spectra of both collagens were similar with no shift in wavenumber of all amide bands. The T_{max} value of ASC and PSC was 36.40 and 35.48 °C, respectively. The zero surface net charge of ASC and PSC was found at pH 5.9 and 5.3, respectively.

Keywords: Collagen, ASC, PSC, rohu, *Labeo rohita*, type I collagen

INTRODUCTION

Collagen is fibrous protein found in connective tissue, including skin, bone and tendon. It was generally used in food, pharmaceutical and biomedical applications (Benjakul, et al., 2012, Regenstein and Zhou, 2007). Skin and bone were the major source for collagen extraction, however there are religious constraints and consumer's safety concern for bovine spongiform encephalopathy (BSE) (Kittiphattanabawon, et al., 2010). As a consequence, more increasing interest and attempt have been paid to the alternative collagen, especially fish skin collagen (Chen, et al., 2015, Kittiphattanabawon, et al., 2010, Sinthusamran, et al., 2013, Wang, et al., 2014). Rohu is a freshwater fish, which is a commonly aquaculture in Asia, especially India, Bangladesh, Nepal, Myanmar, Laos and Thailand, with a total amount of 1.6 metric tonne per year (FAO, 2014). The production of rohu is almost for consuming and processing. Sini *et al.* (2008) reported that rohu has good chemical and physical characteristics for fish sausage which was comparable to that of commercial meat sausages. As a consequence, a lot of skin, which is approximately of 30% estimated from rohu production in local processing plant, was discarded as waste with low market value. Therefore, utilisation of the skin for production of high value-added product, especially collagen, is an alternative choice for increasing revenue for the producer and decreasing the cost of disposal or waste management. Moreover, less information regarding the collagen from the skin of rohu, a freshwater fish wildly cultured in Asia, has been reported. The objective of this study was to isolate and characterise collagen from the skin of rohu (*Labeo rohita*).

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 Rohu Skin

Skin of rohu (*Labeo rohita*) with the size of 1-1.5 kg was obtained from a local fish 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 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 64.78% 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 Rohu Skin

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from the prepared skin following the method of Kittiphattanabawon et al. (2010). 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 became 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

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

$$\% \text{ Recovery} = \frac{\text{Hyp content in collagen (mg/g collagen) x weight of collagen obtained (g)}}{\text{Hyp content in skin (mg/g skin) x weight of skin (g)}} \times 100 \quad (1)$$

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

Characteristics of Collagen

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.

TOYOPEARL® CM-650M Column Chromatography

TOYOPEARL® CM-650M column chromatography was carried out according to the method of Kittiphattanabawon et al. (2010). This technique, which is a cation exchange chromatography, was used for identification of collagen type. 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. 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. 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 to identify the subunit composition of collagen.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra of collagens were obtained using a Bruker model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany). 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 an OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany). Also, the ratio of amplitude of amide III and 1454 cm^{-1} band was calculated to evaluate the changes of triple helical structure of collagen.

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 DSC thermogram. Total denaturation enthalpy (ΔH) was estimated by measuring the area of the 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). The 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 was tested by T-test (Steel and Torrie, 1980). The data were presented as means ± standard deviation.

RESULTS AND DISCUSSION

Yield and Recovery of Collagen

ASC and PSC extracted from rohu skin showed the yield of 64.2 and 6.8% (dry weight basis), respectively. The yield of ASC was 9.44 fold higher than that of PSC, whilst that of ASC from the skin of clown featherback and black carp (27.6 and 15.5%, respectively) showed much lower than that of their PSC (44.6 and 26.5%, respectively) (Jia, et al., 2012, Kittiphattanabawon, et al., 2015). The result indicated that collagen from rohu skin might have lower intermolecular cross-links compared with that from the skin of clown featherback and black carp, leading to the ease of collagen solubilisation during acid extraction. Total recovery of collagen from rohu skin was 82.5%. Similar result in total recovery of collagen was observed when compared with that from clown featherback skin collagen (82.1%) (Kittiphattanabawon, et al., 2015). It was implied that only approximately 18% could not be extracted. However, the amount of unextractable collagen could be decreased by increasing amount of pepsin used and taking longer time extraction during pepsin-aided process (Nalinanon, et al., 2007).

Characteristics of Collagen

Amino acid composition

Amino acid composition of ASC and PSC from the skin of rohu is shown in Table 1. Both collagens had glycine as their major amino acid (317-330 residues/1000 residues) and had high amount of alanine (119-121 residues/1000 residues), proline (116-117 residues/1000 residues) and hydroxyproline (80-85 residues/1000 residues). Very low amount of cysteine (1 residue/1000 residues), tyrosine (3-4 residues/1000 residues), histidine (4 residues/1000 residues) and hydroxylysine (6-7 residues/1000 residues) was found. Their amino acid composition was generally in agreement with those of fish collagen from the skin of other freshwater fish species (Jia, et al., 2012, Kittiphattanabawon, et al., 2015, Wang, et al., 2014, Wang, et al., 2014). Generally, collagen contained glycine, imino acid (proline+hydroxyproline) and alanine about 33, 20 and 11% of the total amino acid residues, respectively (Balian and Bowes, 1977, Foegeding, et al., 1996, Pearson and Young, 1989). Imino acid content of both collagens was about 196-202 residues/1000 residues, which is similar, lower and higher to that of collagen from clown featherback skin (201-202 residues/1000 residues), cod skin (154 residues/1000 residues) and calf skin (215 residues/1000 residues), respectively (Duan, et al., 2009, Kittiphattanabawon, et al., 2015). The difference in imino acid content amongst animals was associated with the difference in the living environments of their sources, particularly habitat temperature (Regenstein and Zhou, 2007). Hydroxyproline plays an important role in stabilization of the helix structure by preventing rotation of the N–C bond, which correlated with thermal stability of collagen (Foegeding, et al., 1996).

Protein Patterns and Subunit Compositions

Protein patterns of ASC and PSC from the skin of rohu under reducing and non-reducing conditions are shown in Figure 1. Both collagens consisted of α1-, α2- and β-chains as major component and some of crosslinking components (γ-chain and MW higher than γ-chain). ASC showed quite similar in protein pattern to PSC. The result was in accordance with collagen from the skin of other fish species (Chen, et al., 2015, Nalinanon, et al., 2010, Wang, et al., 2014). No difference in protein pattern of both collagens determined under reducing and non-reducing conditions. The result suggested that no disulphide bond was found in the collagens as accordance in their amino acid composition (Table 1). The intensity of α1-chain was found approximately 2-fold higher than that of α2-chain. As shown in the chromatogram (Figure 2), the fractions of ASC were eluted as 2 major peaks (fraction numbers 49-54 and 54-65). The α1-chain was found in the first peak, whilst α2-, β- and γ-chains and crosslinking components were found in the second peak. According to the protein pattern and chromatogram, the results indicated that collagen from rohu skin was type I collagen with molecular composition of $(\alpha1)_2\alpha2$-heterotrimer. The similar results were found in collagen extracted from the skin of clown featherback, squid, Amur sturgeon and grass carp (Chen, et al., 2015, Kittiphattanabawon, et al., 2015, Veeruraj, et al., 2015, Wang, et al., 2014).

Fourier Transform Infrared (FTIR) Spectra

FTIR spectra of ASC and PSC are shown in Figure 3. The major peaks of the spectra were in amide band region, including amide I (1632-1635 cm^{-1}), amide II (1533-1535 cm^{-1}), amide III (1233-1235 cm^{-1}), amide A (3274-3276 cm^{-1}) and amide B (2919-2920 cm^{-1}). The amide I and amide II bands are associated with C=O

stretching vibration or hydrogen bond coupled with COO^- and N–H bending vibration coupled with C–N stretching vibration, respectively (Krimm and Bandekar, 1986, Payne and Veis, 1988). The amide III is associated with N–H deformation and C–N stretching vibration (Muyonga, et al., 2004). The amide A band is associated with the N–H stretching vibration and the existence of hydrogen bonds, whilst the amide B is related to asymmetrical stretch of CH_2 stretching vibration (Abe and Krimm, 1972, Doyle, et al., 1975). No shift in wavenumber of all amide bands was observed for both collagens (P>0.05). A shift of amide I and II peaks to lower wavenumber is associated with a decrease in the molecular order (Payne and Veis, 1988). The result indicated that the pepsin did not affect to the structure of collagen. It was reconfirmed by the ratio of amplitude of amide III and 1454 cm^{-1} band, which revealed the triple-helical structure of collagen, between ASC (0.99) and PSC (0.99) was not different. Plepis, et al. (1996) reported that the ratio of approximately 1.0 reveals the triple-helical structure of collagens.

Thermal Transition

The maximum transition temperature (T_{max}) and enthalpy (ΔH) of ASC and PSC from the skin of rohu are shown in Figure 4. The T_{max} values of ASC and PSC were 36.40 and 35.48 °C, respectively. They were quite similar to that of collagen from clown featherback skin (35.23-36.28 °C) (Kittiphattanabawon, et al., 2015). However, they had slightly lower and quite higher T_{max} value than that of collagen from porcine skin (37 °C) and cod (15 °C), respectively (Duan, et al., 2009). The difference amongst T_{max} values correlated with their imino acid content and temperature of their normal habitat. The ΔH of ASC (1.01 J/g) was higher than that of PSC (0.31 J/g). The cleavage of telopeptide region by pepsin or removal of some of

291 those peptides might facilitate the denaturation of PSC induced by heat
292 (Kittiphattanabawon, et al., 2010).
293
294 *Zeta Potential*
295 Zeta potential of both collagen solutions at pH ranging from 2-12 is shown in
296 Figure 5. The zeta potential of both collagen decreased as pH increased. The zero
297 surface net charge of ASC and PSC was found at pH 5.9 and 5.3, respectively. A
298 protein in an aqueous system has a zero net charge at its isoelectric point (pI), when
299 the positive charges are balanced out by the negative charges (Bonner, 2007).
300 Therefore, those pHs were assumed to be pI of both collagens. The difference in pI
301 between both collagens was possibly by pepsin hydrolysis. Kaewdang, et al. (2014)
302 reported that the removal of telopeptide regions by pepsin might affect the protonation
303 or deprotonation of charged amino and carboxyl groups, respectively.
304
305 **CONCLUSIONS**
306 Collagen could be successfully isolated from the skin of rohu, especially acid
307 solubilisation process, with the total yield and recovery of 71 and 82.5%, respectively.
308 Use of pepsin slightly enhanced yield (about 6.8%) and did not affect to the triple
309 helical structure of collagen. T_{max} of both collagens was comparable to that of procine
310 collagen. Based on total yield and recovery and its T_{max}, rohu skin could be an
311 alternative source of collagen extraction.
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411 **FIGURE LEGENDS**

412

413 **FIGURE 1** Protein pattern of ASC and PSC from the skin of rohu under non-
414 reducing and reducing conditions. M and I denote high molecular weight protein
415 marker, and type I collagen from calf skin, respectively.

416

417 **FIGURE 2** Chromatogram of ASC from the skin of rohu on the TOYOPEARL® CM-
418 650M ion-exchange column. The fractions indicated by numbers were examined by
419 SDS-PAGE using 7.5% separating gel and 4% stacking gel. M denotes high
420 molecular weight protein marker.

421

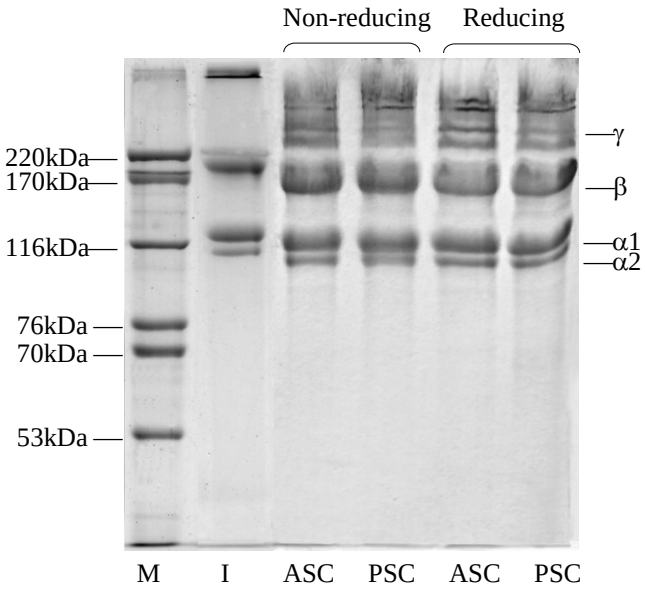
422 **FIGURE 3** FTIR spectra of ASC and PSC from the skin of rohu.

423

424 **FIGURE 4** DSC thermogram of ASC and PSC from the skin of rohu.

425

426 **FIGURE 5** Zeta (ζ) potential of ASC and PSC from the skin of rohu at different pHs.
427 Bars represent the standard deviation (n=3).


Non-reducing
Reducing
220kDa
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76kDa
70kDa
53kDa
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α1
α2
M
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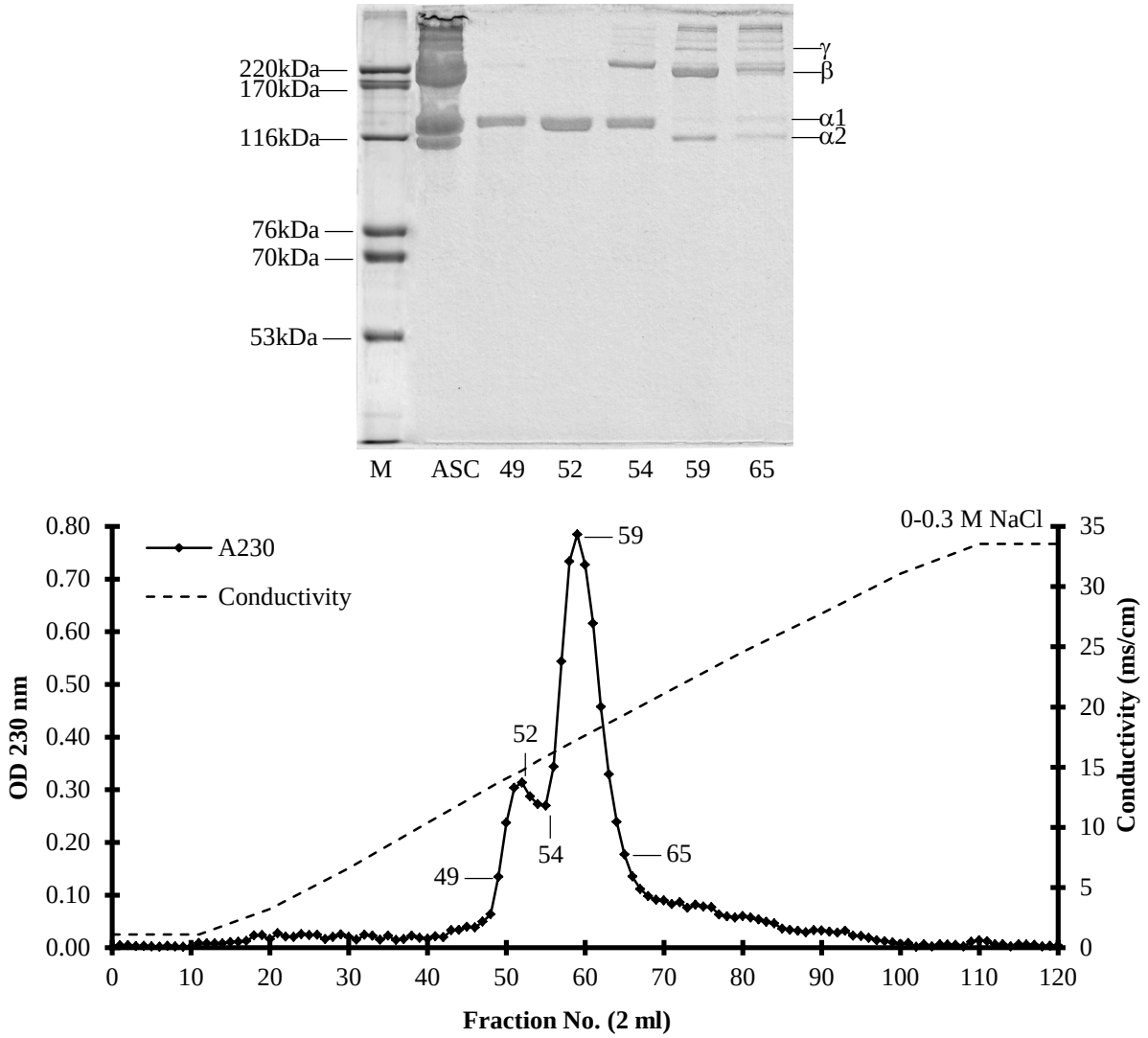
ASC
PSC
ASC
PSC

FIGURE 1


220kDa
170kDa
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76kDa
70kDa
53kDa
γ
β
α1
α2
M
ASC
49
52
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65
A230
Conductivity
0-0.3 M NaCl
OD 230 nm
Conductivity (ms/cm)
Fraction No. (2 ml)

FIGURE 2

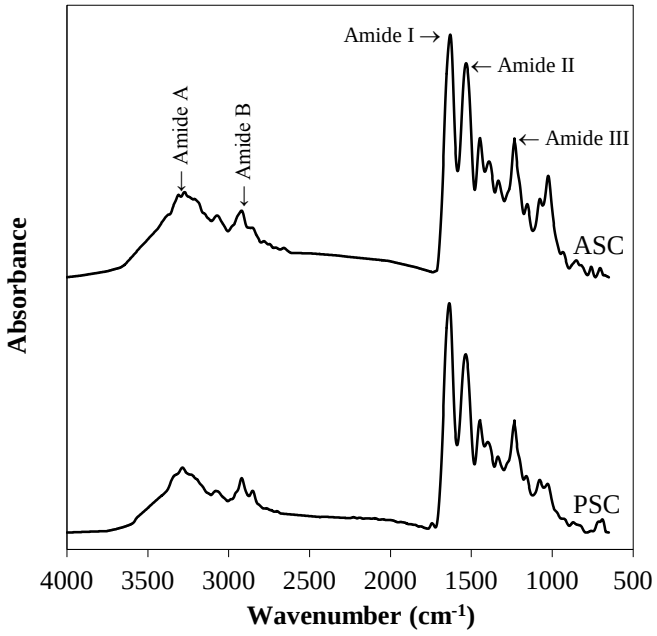
Amide I →
← Amide II
← Amide III
← Amide A
← Amide B
ASC
PSC
Absorbance
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)

FIGURE 3

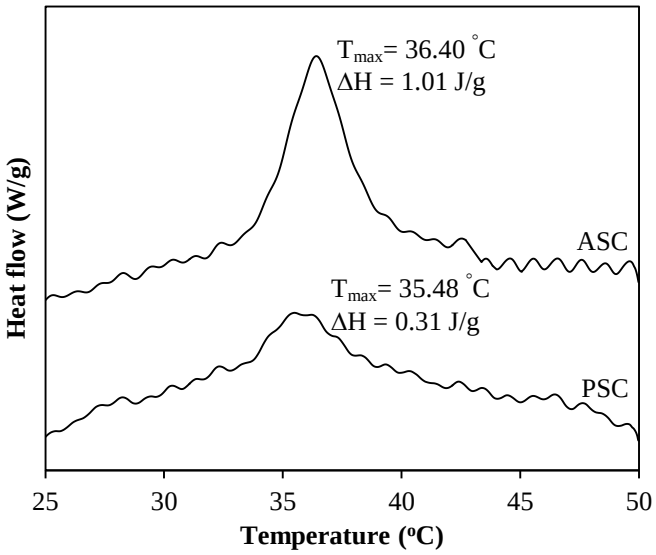
T$_{max}$= 36.40 °C
ΔH = 1.01 J/g
ASC
T$_{max}$= 35.48 °C
ΔH = 0.31 J/g
PSC
Heat flow (W/g)
25
30
35
40
45
50
Temperature (ºC)

FIGURE 4

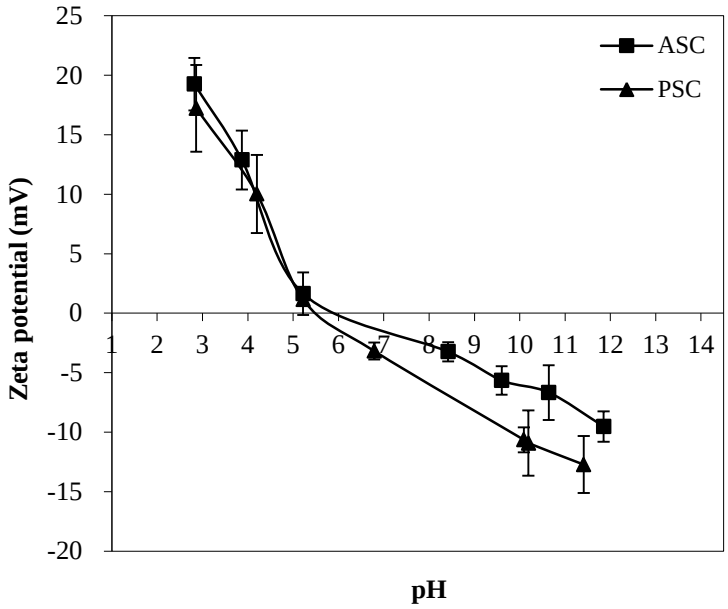
25
20
15
10
5
0
-5
-10
-15
-20
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Zeta potential (mV)
pH
ASC
PSC

FIGURE 5

TABLE 1
Amino acid composition of ASC and PSC from the skin of rohu (residues/1000 residues).

Amino acid	ASC	PSC
Alanine	119	121
Arginine	54	53
Aspartic acid/Asparagine	50	47
Cysteine	1	1
Glutamine/Glutamic acid	72	69
Glycine	317	330
Histidine	4	4
Isoleucine	13	11
Leucine	25	23
Lysine	28	26
Hydroxylysine	6	7
Methionine	12	12
Phenylalanine	15	13
Hydroxyproline	80	85
Proline	116	117
Serine	37	35
Threonine	24	23
Tyrosine	4	3
Valine	22	20
Total	**1000**	**1000**
Imino acid	196	202