



Title	Characteristics of Collagen from Rohu (Labeo rohita) Skin
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1 **TITLE** : **Characteristics of collagen from rohu (*Labeo rohita*) skin**

2 **RUNNING HEAD** : **Collagen from rohu (*Labeo rohita*) skin**

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6 **ABSTRACT**

7 Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were isolated
8 from rohu skin with the yield of 64.2 and 6.8% (dry weight basis), respectively. Both
9 collagens had glycine as the major amino acid with imino acid content of 196-202
10 residues/1000 residues and were characterised as type I collagen with molecular
11 composition of $(\alpha 1)_2\alpha 2$ -heterotrimer. FTIR spectra of both collagens were similar
12 with no shift in wavenumber of all amide bands. The T_{\max} value of ASC and PSC was
13 36.40 and 35.48 °C, respectively. The zero surface net charge of ASC and PSC was
14 found at pH 5.9 and 5.3, respectively.

15

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

17 INTRODUCTION

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

39

40

41

42 MATERIALS AND METHODS

43 Chemicals

44 All chemicals were of analytical grade. Sodium dodecyl sulphate (SDS),
45 Coomassie Blue R-250, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were
46 procured from Bio-Rad Laboratories (Hercules, CA, USA). Type I collagen from calf
47 skin and pepsin from porcine stomach mucosa (EC 3.4.23.1) were obtained from
48 Sigma Chemical Co. (St. Louis, MO, USA). High-molecular-weight markers and
49 TOYOPEARL[®] CM-650M were purchased from GE Healthcare UK Limited
50 (Buckinghamshire, UK) and Tosoh Corporation (Tokyo, Japan), respectively.

51

52 Preparation of Rohu Skin

53 Skin of rohu (*Labeo rohita*) with the size of 1-1.5 kg was obtained from a local
54 fish processing plant at Talaadthai in Pathumthani province, Thailand. The fresh skin
55 packed in polyethylene bags (1 kg/bag) was placed in ice at a ratio of skin to ice of
56 1:2 (w/w) using a polystyrene box as a container. The skin was transported to the
57 Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok,
58 Prachinburi province within 4 h. Upon arrival, the skin was washed with cold tap
59 water (≤ 10 °C). The residual meat on the skin was removed by knife and washed with
60 cold tap water. The clean skin was cut into small pieces (approximately 1.0×1.0 cm²)
61 using a pair of scissors. The prepared skin was placed in polyethylene bags (50-100
62 g/bag) and stored at -20 °C until used but not longer than 3 months. The moisture
63 content of prepared skin was 64.78% as determined by AOAC method (AOAC,
64 2000). Prior to collagen extraction, the frozen skin was thawed with running water
65 until the core temperature of the skin reached 8–10 °C.

66

67 **Extraction of Collagen from Rohu Skin**

68 Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were
69 extracted from the prepared skin following the method of Kittiphattanabawon et al.
70 (2010). All procedures were carried out at 4 °C.

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

77 To extract collagen, the pretreated skin was soaked in 0.5 M acetic acid with a
78 solid to solvent ratio of 1:15 (w/v) for 48 h with a continuous stirring, followed by
79 filtration with two layers of cheesecloth. The collagen in filtrate was precipitated by
80 adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M
81 Tris(hydroxymethyl) aminomethane, pH 7.5. The resultant precipitate was collected
82 by centrifugation at $20000 \times g$ at 4 °C for 60 min using a refrigerated centrifuge
83 (model Avanti[®] J-E, Beckman Coulter, Inc., Palo Alto, CA, USA). The pellet was
84 dissolved in a minimum volume of 0.5 M acetic acid. The solution was then dialysed
85 against 25 volumes of 0.1 M acetic acid for 12 h, followed by the same volume of
86 distilled water for 48 h. Then, the resulting dialysate was freeze dried using a freeze-
87 dryer (CoolSafe 55, ScanLaf A/S, Lyngø, Denmark). The obtained collagen from acid
88 solubilisation process was referred to as “acid soluble collagen, ASC”. The
89 undissolved residue obtained after acid extraction was used for pepsin soluble
90 collagen extraction. The residue was soaked in 0.5 M acetic acid with a solid to
91 solvent ratio of 1:15 (w/v). Porcine pepsin (20 unit/g of residue) in which proteolytic

92 activity was determined by the method of Nalinanon, Benjakul, Visessanguan and
 93 Kishimura (2008) was added. The mixtures were continuously stirred at 4 °C for 48 h,
 94 followed by filtration using two layers of cheesecloth. The filtrate was collected and
 95 subjected to precipitation and dialysis in the same manner with those used for ASC as
 96 previously described. The obtained collagen from pepsin solubilisation process was
 97 referred to as “pepsin soluble collagen, PSC”.

98

99 **Yield and Recovery of Collagen**

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

$$102 \quad \% \text{ Yield} = \frac{\text{Weight of lyophilised collagen (g)}}{\text{Weight of dry skin (g)}} \times 100$$

103

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

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

107

108 **Characteristics of Collagen**

109 *Amino Acid Analysis*

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

115

116 *SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

117 SDS-PAGE was performed by the method of Laemmli (1970). The samples
118 were dissolved in 5% SDS solution. The mixtures were then heated in boiling water
119 for 1 min, followed by centrifugation at $8500 \times g$ for 5 min using a microcentrifuge
120 (MIKRO20, Hettich Zentrifugan, Germany) to remove undissolved debris. Solubilised
121 samples were mixed at 1:1 (v/v) ratio with sample buffer (0.5 M tris-HCl, pH 6.8
122 containing 4% SDS and 20% glycerol in the presence or absence of 10% (v/v) β ME).
123 Samples were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4%
124 stacking gel and subjected to electrophoresis at a constant current of 20 mA/gel. After
125 electrophoresis, gels were fixed with a mixture of 50% (v/v) methanol and 10% (v/v)
126 acetic acid for 30 min, followed by staining with 0.05% (w/v) Coomassie blue R-250
127 in 15% (v/v) methanol and 5% (v/v) acetic acid for 1 h. Finally, they were destained
128 with 30% (v/v) methanol and 10% (v/v) acetic acid for 1 h and destained again with
129 the same solution for 30 min. High-molecular-weight protein markers (GE Healthcare
130 UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of
131 proteins. Type I collagen from calf skin was used as standard collagen.

132

133 *TOYOPEARL[®] CM-650M Column Chromatography*

134 TOYOPEARL[®] CM-650M column chromatography was carried out according
135 to the method of Kittiphattanabawon et al. (2010). This technique, which is a cation
136 exchange chromatography, was used for identification of collagen type. Collagen
137 samples (30 mg) were dissolved in 3 mL of starting buffer (20 mM sodium acetate
138 buffer, pH 4.8) and boiled for 1 min. The mixtures were centrifuged at $8500 \times g$ at
139 room temperature (25-26 °C) for 10 min. The supernatants were applied onto a
140 TOYOPEARL[®] CM-650M column (1.8 x 20 cm) previously equilibrated with 10

141 volumes of the starting buffer at a flow rate of 3 mL/min. After loading, the unbound
142 proteins were washed by the same buffer until A_{230} was less than 0.05. Elution was
143 achieved with a linear gradient of 0-0.3 M NaCl in the same buffer at a flow rate of 2
144 mL/min with a total volume of 400 mL. The eluant was monitored at 230 nm and
145 fractions (4 mL each) were collected. The selected fractions were subjected to SDS-
146 PAGE using 7.5% separating gel and 4% stacking gel as previously described to
147 identify the subunit composition of collagen.

148

149 *Fourier Transform Infrared (FTIR) Spectroscopy*

150 FTIR spectra of collagens were obtained using a Bruker model EQUINOX 55
151 FTIR spectrometer (Bruker, Ettlingen, Germany). FTIR spectrometer (Bruker,
152 Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate
153 (DLATGS) detector. The Horizontal Attenuated Total Reflectance Accessory
154 (HATR) was mounted into the sample compartment. The internal reflection crystal
155 (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of
156 incidence to the IR beam. Spectra were acquired at a resolution of 4 cm⁻¹ and the
157 measurement range was 4000-650 cm⁻¹ (mid-IR region) at room temperature.
158 Automatic signals were collected in 32 scans at a resolution of 4 cm⁻¹ and were
159 ratioed against a background spectrum recorded from the clean and empty cell at 25
160 °C. Analysis of spectral data was carried out using an OPUS 3.0 data collection
161 software programme (Bruker, Ettlingen, Germany). Also, the ratio of amplitude of
162 amide III and 1454 cm⁻¹ band was calculated to evaluate the changes of triple helical
163 structure of collagen.

164

165

166 *Differential Scanning Calorimetry (DSC)*

167 The collagens were rehydrated by adding the deionised water to dried samples
168 at a solid to water ratio of 1:40 (w/v). The mixtures were allowed to stand for 2 days
169 at 4 °C prior to analysis. Differential scanning calorimetry (DSC) was performed
170 using a differential scanning calorimeter model DSC 7 (Perkin Elmer, Norwalk, CT,
171 USA). Calibration was run using indium thermogram. The sample (5-10 mg) was
172 accurately weighed into aluminum pans and sealed. The sample was scanned at 1
173 °C/min over the range of 25-50 °C using iced water as the cooling medium. An empty
174 pan was used as the reference. The maximum transition temperature (T_{max}) was
175 estimated from the DSC thermogram. Total denaturation enthalpy (ΔH) was estimated
176 by measuring the area of the thermogram.

177

178 *Zeta Potential Analysis*

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

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

189

190

191 **Statistical Analysis**

192 The experiments were carried out in triplicate using three different lots of
193 samples. The difference between means was tested by T-test (Steel and Torrie, 1980).

194 The data were presented as means \pm standard deviation.

195

196 **RESULTS AND DISCUSSION**

197 **Yield and Recovery of Collagen**

198 ASC and PSC extracted from rohu skin showed the yield of 64.2 and 6.8%
199 (dry weight basis), respectively. The yield of ASC was 9.44 fold higher than that of
200 PSC, whilst that of ASC from the skin of clown featherback and black carp (27.6 and
201 15.5%, respectively) showed much lower than that of their PSC (44.6 and 26.5%,
202 respectively) (Jia, et al., 2012, Kittiphattanabawon, et al., 2015). The result indicated
203 that collagen from rohu skin might have lower intermolecular cross-links compared
204 with that from the skin of clown featherback and black carp, leading to the ease of
205 collagen solubilisation during acid extraction. Total recovery of collagen from rohu
206 skin was 82.5%. Similar result in total recovery of collagen was observed when
207 compared with that from clown featherback skin collagen (82.1%)
208 (Kittiphattanabawon, et al., 2015). It was implied that only approximately 18% could
209 not be extracted. However, the amount of unextractable collagen could be decreased
210 by increasing amount of pepsin used and taking longer time extraction during pepsin-
211 aided process (Nalinanon, et al., 2007).

212

213

214

215

216 **Characteristics of Collagen**

217 *Amino acid composition*

218 Amino acid composition of ASC and PSC from the skin of rohu is shown in
219 Table 1. Both collagens had glycine as their major amino acid (317-330 residues/1000
220 residues) and had high amount of alanine (119-121 residues/1000 residues), proline
221 (116-117 residues/1000 residues) and hydroxyproline (80-85 residues/1000 residues).
222 Very low amount of cysteine (1 residue/1000 residues), tyrosine (3-4 residues/1000
223 residues), histidine (4 residues/1000 residues) and hydroxylysine (6-7 residues/1000
224 residues) was found. Their amino acid composition was generally in agreement with
225 those of fish collagen from the skin of other freshwater fish species (Jia, et al., 2012,
226 Kittiphattanabawon, et al., 2015, Wang, et al., 2014, Wang, et al., 2014). Generally,
227 collagen contained glycine, imino acid (proline+hydroxyproline) and alanine about
228 33, 20 and 11% of the total amino acid residues, respectively (Balian and Bowes,
229 1977, Foegeding, et al., 1996, Pearson and Young, 1989). Imino acid content of both
230 collagens was about 196-202 residues/1000 residues, which is similar, lower and
231 higher to that of collagen from clown featherback skin (201-202 residues/1000
232 residues), cod skin (154 residues/1000 residues) and calf skin (215 residues/1000
233 residues), respectively (Duan, et al., 2009, Kittiphattanabawon, et al., 2015). The
234 difference in imino acid content amongst animals was associated with the difference
235 in the living environments of their sources, particularly habitat temperature
236 (Regenstein and Zhou, 2007). Hydroxyproline plays an important role in stabilization
237 of the helix structure by preventing rotation of the N–C bond, which correlated with
238 thermal stability of collagen (Foegeding, et al., 1996).

239

240

241 *Protein Patterns and Subunit Compositions*

242 Protein patterns of ASC and PSC from the skin of rohu under reducing and
243 non-reducing conditions are shown in Figure 1. Both collagens consisted of α 1-, α 2-
244 and β -chains as major component and some of crosslinking components (γ -chain and
245 MW higher than γ -chain). ASC showed quite similar in protein pattern to PSC. The
246 result was in accordance with collagen from the skin of other fish species (Chen, et
247 al., 2015, Nalinanon, et al., 2010, Wang, et al., 2014). No difference in protein pattern
248 of both collagens determined under reducing and non-reducing conditions. The result
249 suggested that no disulphide bond was found in the collagens as accordance in their
250 amino acid composition (Table 1). The intensity of α 1-chain was found approximately
251 2-fold higher than that of α 2-chain. As shown in the chromatogram (Figure 2), the
252 fractions of ASC were eluted as 2 major peaks (fraction numbers 49-54 and 54-65).
253 The α 1-chain was found in the first peak, whilst α 2-, β - and γ -chains and crosslinking
254 components were found in the second peak. According to the protein pattern and
255 chromatogram, the results indicated that collagen from rohu skin was type I collagen
256 with molecular composition of $(\alpha$ 1)₂ α 2-heterotrimer. The similar results were found
257 in collagen extracted from the skin of clown featherback, squid, Amur sturgeon and
258 grass carp (Chen, et al., 2015, Kittiphattanabawon, et al., 2015, Veeruraj, et al., 2015,
259 Wang, et al., 2014).

260

261 *Fourier Transform Infrared (FTIR) Spectra*

262 FTIR spectra of ASC and PSC are shown in Figure 3. The major peaks of the
263 spectra were in amide band region, including amide I (1632-1635 cm⁻¹), amide II
264 (1533-1535 cm⁻¹), amide III (1233-1235 cm⁻¹), amide A (3274-3276 cm⁻¹) and amide
265 B (2919-2920 cm⁻¹). The amide I and amide II bands are associated with C=O

266 stretching vibration or hydrogen bond coupled with COO⁻ and N–H bending vibration
267 coupled with C–N stretching vibration, respectively (Krimm and Bandekar, 1986,
268 Payne and Veis, 1988). The amide III is associated with N–H deformation and C–N
269 stretching vibration (Muyonga, et al., 2004). The amide A band is associated with the
270 N–H stretching vibration and the existence of hydrogen bonds, whilst the amide B is
271 related to asymmetrical stretch of CH₂ stretching vibration (Abe and Krimm, 1972,
272 Doyle, et al., 1975). No shift in wavenumber of all amide bands was observed for both
273 collagens ($P>0.05$). A shift of amide I and II peaks to lower wavenumber is associated
274 with a decrease in the molecular order (Payne and Veis, 1988). The result indicated
275 that the pepsin did not affect to the structure of collagen. It was reconfirmed by the
276 ratio of amplitude of amide III and 1454 cm⁻¹ band, which revealed the triple-helical
277 structure of collagen, between ASC (0.99) and PSC (0.99) was not different. Plepis, et
278 al. (1996) reported that the ratio of approximately 1.0 reveals the triple-helical
279 structure of collagens.

280

281 *Thermal Transition*

282 The maximum transition temperature (T_{\max}) and enthalpy (ΔH) of ASC and
283 PSC from the skin of rohu are shown in Figure 4. The T_{\max} values of ASC and PSC
284 were 36.40 and 35.48 °C, respectively. They were quite similar to that of collagen
285 from clown featherback skin (35.23-36.28 °C) (Kittiphattanabawon, et al., 2015).
286 However, they had slightly lower and quite higher T_{\max} value than that of collagen
287 from porcine skin (37 °C) and cod (15 °C), respectively (Duan, et al., 2009). The
288 difference amongst T_{\max} values correlated with their imino acid content and
289 temperature of their normal habitat. The ΔH of ASC (1.01 J/g) was higher than that of
290 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 porcine
310 collagen. Based on total yield and recovery and its T_{\max} , rohu skin could be an
311 alternative source of collagen extraction.

312

313

314

315

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321

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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).

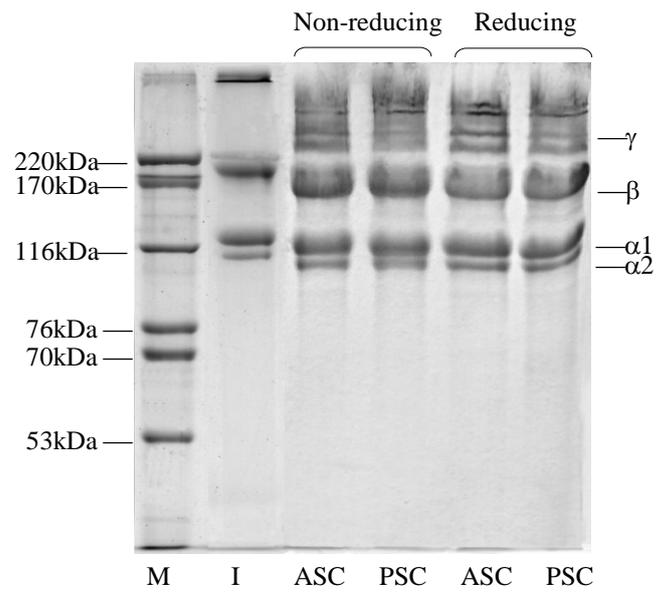


FIGURE 1

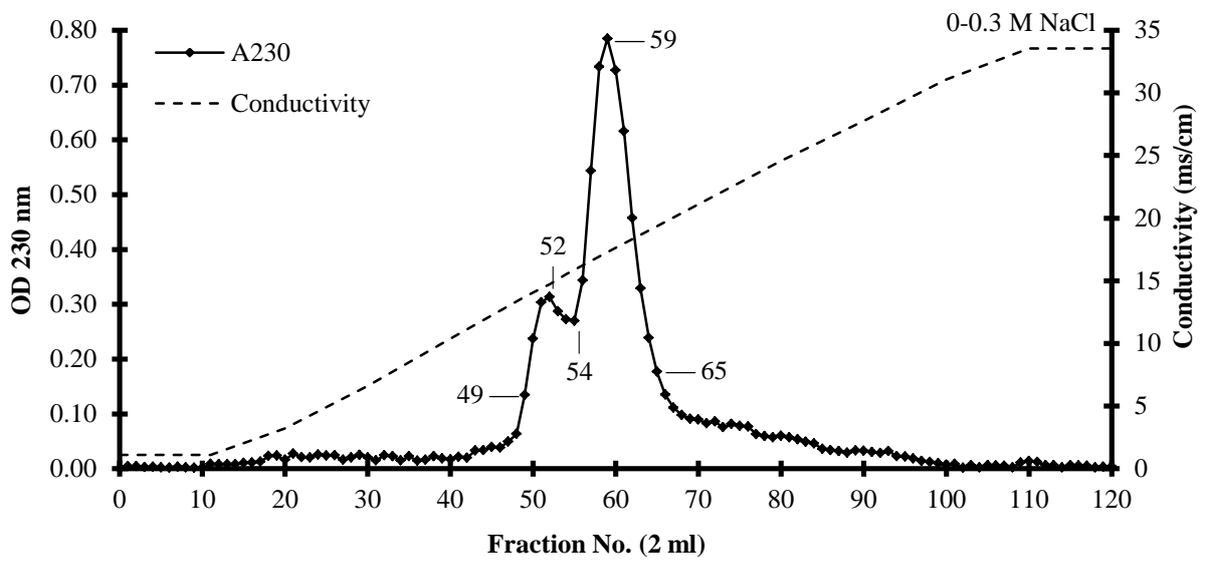
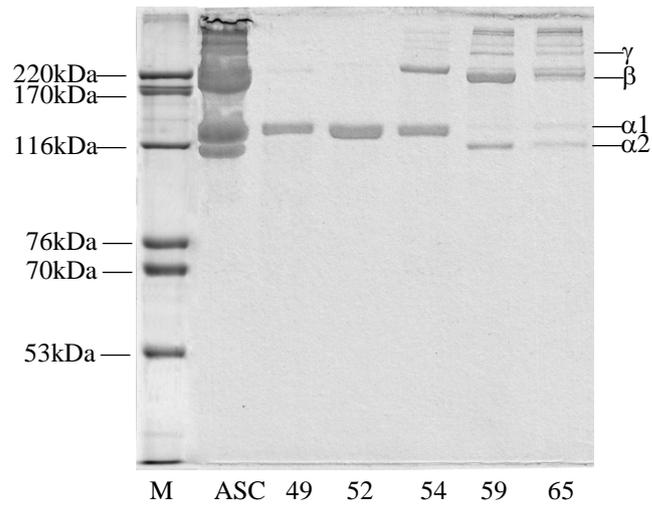


FIGURE 2

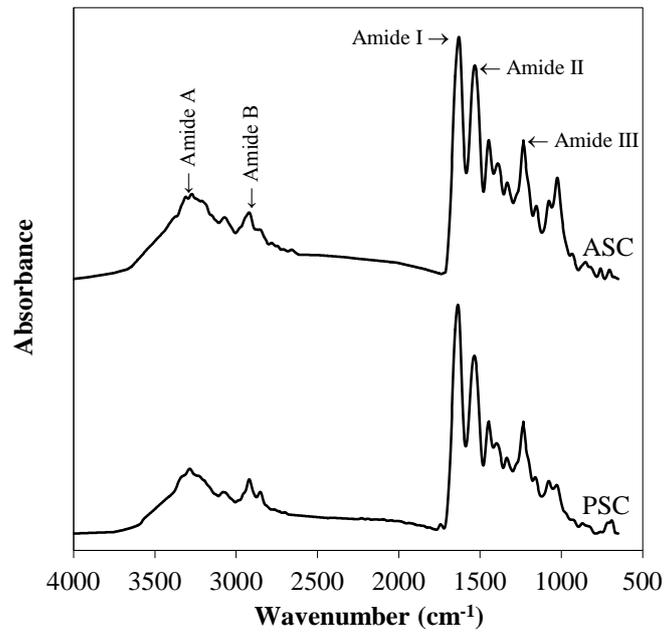


FIGURE 3

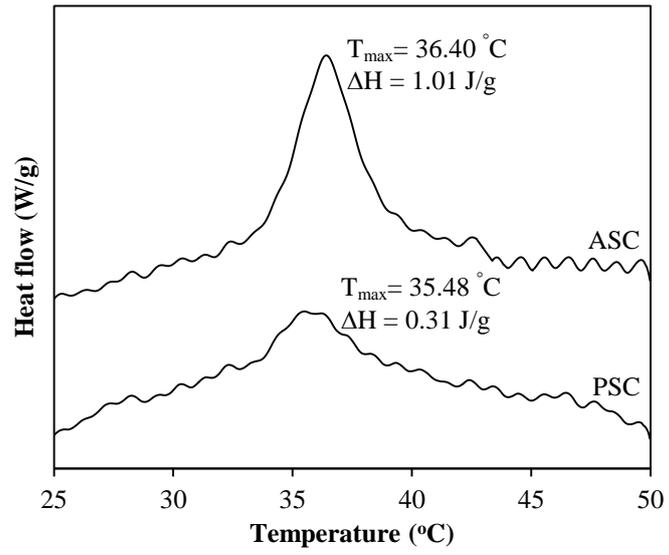


FIGURE 4

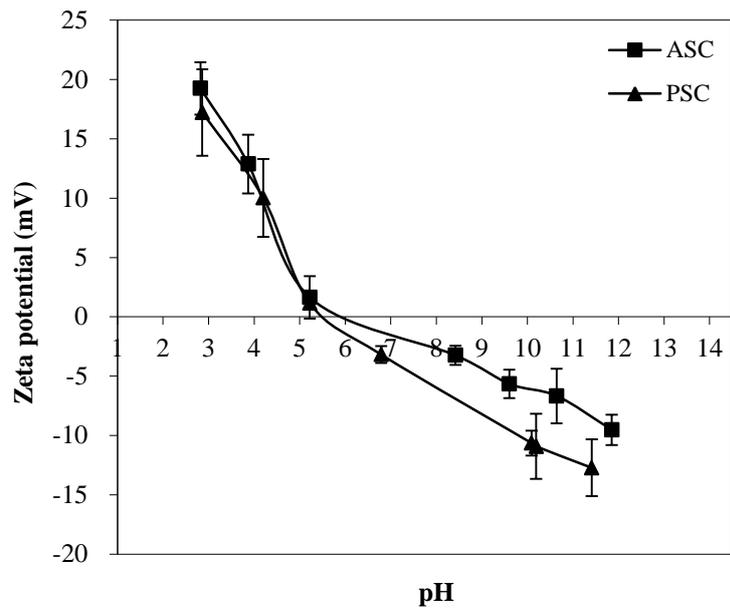


FIGURE 5

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