Effect of various acids on physicochemical and functional characteristics of gelatin from swim bladder of rohu (*Labeo rohita*)

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Abstract Influence of various acids (acetic acid, phosphoric acid, and propionic acid) at different concentrations (0.05, 0.1, and 0.2 M) on the extraction yield, physicochemical and functional properties of gelatin from *Labeo rohita* swim bladder were investigated. Highest gelatin yield (44.22%, dry weight basis) was obtained in a sample prepared by without acid pretreatment (GWA) of swim bladder as compared to acid pretreatment counterparts. Amongst the acid pretreatments, propionic acid (GPrA) showed the highest gelatin yield, followed by acetic acid (GAA) and phosphoric acid (GPA) at all concentrations used, respectively. Moreover, with increased concentrations of all acids, the decrease in gelatin yield was observed for all the acids. GWA showed higher protein and hydroxyproline content than that of acid counterparts ($P < 0.05$). Amino acid analysis of gelatins showed glycine as the major amino acid in all gelatins followed by proline, glutamic acid and alanine, respectively. GWA showed α (α_1 and α_2) and β -chains as the predominant components with low molecular weight peptides. However, GPrA, GAA, and GPA had α_1 and α_2 dominant constituents. FTIR spectra of gelatins revealed that the loss of the triple-helix was found in GPA, GAA, and GPrA, compared to GWA. Among gelatin samples, GWA showed the highest solubility at all pH tested followed by GPrA, GPA, and GAA respectively. Furthermore, GWA exhibited higher emulsifying, foaming and

gelling properties as compared to GPrA, GPA, and GAA, respectively. Therefore, the acid pretreatment of swim bladder had a negative impact on the extraction yield, physicochemical and functional properties of gelatin from rohu swim bladder.

Keywords Rohu · Swim bladder · Acid pretreatment · Gelatin · Physicochemical characterization · Functional properties

Introduction

Gelatin is the product of partial hydrolysis of collagen, which is widely used by various industries such as food, pharmaceutical, materials, and photography because of its techno-functional properties. Conversion of collagen into soluble gelatin is generally achieved by heating collagen, and the extraction process such as acid or alkali pretreatment, temperature, time and concentration can influence the length, functional, and biological properties of gelatin (Patil et al. 2000). Recovery of gelatin extraction depends on the method, in which collagens are pretreated. There are two types of gelatin with different characteristics, including type-A, acid-treated gelatin (isoelectric point at pH 6–9) and type-B, an alkaline treated (isoelectric point at pH 5) (Benjakul et al. 2009). Generally, the majority of commercial gelatin is derived from skin and bone of bovine and porcine. Due to sociocultural and religious constraints, outbreaks of bovine spongiform encephalopathy and foot-and-mouth disease, an alternative source for gelatin production, especially from aquatic sources have gained attention.

Fish processing operations; result in enormous amounts of by-products (fish viscera, head, swim bladder, skins, and

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scales, etc.) as wastes which constitute 36% of the total weight of the fish (Mohtar et al. 2010). However, these wastes so generated needs appropriate treatment or/utlization in order to minimize the environmental pollution. Additionally, the production of high-value products from these by-products could pave the way to gaining the higher benefit or revenue. The by-products generated by the fish-processing operations are a potential source for the production of gelatin. Recently, several studies have reported the extractions and properties of collagen and gelatin from fish skin, scale and bones (Chandra et al. 2013; Rawdkuen et al. 2013). Nevertheless, fish swim bladder has also been recognized as a promising alternative material for collagen and gelatin extraction in addition to fish skins and bones. Moreover, the pigments, especially from fish skin stance a color problem in the resulting gelatin. Therefore, swim bladder would be an alternative and promising raw material for gelatin extraction.

The swim bladder is an internal gas-filled organ that contributes to the ability of many bony fish to control their buoyancy (Chandra et al. 2013). It has been reported that swim bladders from a few marine fish species were used for the preparation of fining agents, referred to as isinglass (Chandra et al. 2013). However, swim bladders of fresh water carps are small in size, and it is not possible to utilize the same for isinglass production. Therefore, swim bladder from carps could be a good source for gelatin preparation. Nonetheless, the studies on gelatin extraction from fish swim bladder, especially for freshwater fish is very faint. Ironically, there is no report found in the extraction of gelatin from the swim bladder of rohu fish.

Rohu (*Labeo rohita*) is a fish species of the carp family found in rivers in South Asia. The rohu is economically important for India. It is the most important among the three Indian major carp species used in carp polyculture systems. In India, it has been cultured into almost all riverine systems, including the freshwaters of Andaman, where its population has successfully established. The fish swim bladder from rohu generally discarded as a processing waste. To fully exploit swim bladder from rohu, it can be used for the production of gelatin with high market value. However, the extraction conditions for gelatin production should be optimized. The effects of the acid pretreatment on gelatin yield and properties have been reported for the skins of many fish species, including Unicorn Leatherjacket (Ahmad and Benjakul 2011), Tilapia (Niu et al. 2013) and farmed Amur sturgeon (Nikoo et al. 2014). Hitherto, there is no information regarding the impact of acid pretreatment of rohu swim bladder on gelatin yield and properties. Therefore, the present study aimed to investigate the impact of different acids at various concentrations (0.05, 0.1 and 0.2 M) on extraction,

physico-chemical and functional properties of gelatin from rohu swim bladder.

Materials and methods

Chemicals

Bovine serum albumin, wide range protein marker (Bio-Rad Laboratories, Hercules, CA, USA), Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, β -mercaptoethanol, N,N,N,N-tetramethyl ethylene diamine (TEMED), bromophenol blue, acrylamide, bisacrylamide (Bio-Rad Laboratories, Hercules, CA, USA), were procured. All other reagents and chemicals were of analytical grades.

Collection and preparation of swim bladder from rohu

Rohu (*Labeo rohita*) processing by-products containing swim bladder was collected from the local fish market, Mysuru, India. It was transported to the Department of Meat and Marine Sciences, Central Food Technological Research Institute, India within 30 min in chilled condition. Swim bladders (9–10 cm length) were manually separated from rohu processing by-products and washed with distilled water. The washed swim bladders were then cut into small pieces ($1 \times 1 \text{ cm}^2$), placed in polyethylene bags and stored at -20°C until used, but not longer than 1 month. Prior to extraction of gelatin the frozen swim bladders were thawed using running water until the temperature was $0\text{--}5^\circ\text{C}$.

Acid pretreatments and gelatin extraction

Prior to acid pretreatment, cuts from rohu swim bladders were soaked in 0.05 M NaOH at a swim bladder to solution ratio of 1:10 (w/v). The mixture was stirred at room temperature ($28 \pm 1^\circ\text{C}$) for 6 h using an overhead stirrer equipped with a propeller (Riviera, Mumbai, India). The alkaline solution was changed every 2 h to remove non-collagenous material. Alkaline-treated swim bladder residues were then washed with tap water until neutral pH of wash water was obtained. The washed swim bladder residues were mixed with various concentrations 0.05 or 0.1 or 0.2 M of propionic acid or acetic acid or phosphoric acid at a swim bladder to solution ratio of 1:10 (w/v). These mixtures were stirred for 6 h at room temperature ($28 \pm 1^\circ\text{C}$). The acidic solution was changed every 2 h to swell the collagenous material in the swim bladder matrix. Acid pretreated swim bladder residues were washed thoroughly with tap water until pH of wash water became neutral.

To extract gelatin, with acid pretreated or without acid pretreated swim bladder samples were mixed with distilled water at a solid to liquid ratio of 1:10 (w/v). These mixtures were heated at 60 °C for 6 h in a temperature controlled water bath with a continuous stirring at 200 rpm. The mixtures were then filtered using a muslin cloth (two-fold). The filtrates were freeze-dried using a freeze dryer (Cool-Safe 55, ScanLaf A/S, Lyngø, Denmark). Dried samples obtained were referred as propionic acid (GPrA), acetic acid (GAA), phosphoric acid (GPA) and without acid pretreatment (GWA) gelatins. All gelatin samples were weighed and extraction yield (% on dry weight basis) was calculated by the following equation and further subjected to analyses

$$\text{Yield\%} = \frac{\text{Weight of freeze dried (g)}}{\text{Weight of the initial dry swim bladder (g)}} \times 100$$

Analyses

Compositions and hydroxyproline content

The moisture, ash, protein and fat content of the raw material and the gelatin samples were determined following the method of AOAC (2000). The protein content was determined using Kjeldahl method. The nitrogen conversion factor 6.25 and 5.55 were used to calculate the protein content of swim bladder (raw material) and gelatin, respectively. Hydroxyproline contents of dried gelatin samples, residual acid solutions (used for pretreatment) and washed water were determined according to Bergman and Loxley (1963). Hydroxyproline content was calculated and expressed as mg/g sample.

Amino acid analysis

Amino acid compositions of all gelatin samples were determined according to Pal and Suresh (2017). The samples were hydrolyzed with 6 M HCl in an airtight vial at 110 °C for 24 h using a PICO-TAGTM workstation. Amino acid composition of extracted rohu swim bladder gelatin was determined using phenyl isothiocyanate (PITC) pre-column derivatization. The amino acid contents were presented as mg/g protein.

Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by following the method of Laemmli (1970) with slight modifications. Gelatin samples (0.5 mg/mL) were dissolved in 5% SDS and heated at 85 °C for 1 h using a temperature controlled water bath. To remove

undissolved debris, the dissolved gelatin solutions were centrifuged at 6000 g for 10 min using a microcentrifuge (Rotina 420R, Hettich zentrifuge, Germany) at room temperature. Gelatin samples were mixed with the sample buffer (0.05 M Tris-HCl, pH 6.8, 10% SDS and 20% glycerol) at a ratio of 1:1 (v/v). Samples (20 µg protein) were loaded on the polyacrylamide gels having 7.5% separating gel and a 4% stacking gel, then subjected to electrophoresis (ECOSDS-MIDI DUAL, Biobee, Bangalore, India) at a constant current of 50 mA/gel. After electrophoresis, the gel was stained with 0.04 g (w/v) Coomassie Blue R-250 in 100 mL of methanol and added 15 mL of glacial acetic acid and 85 mL of distilled water. The destaining solution was prepared with 50% methanol and 7.5% glacial acetic acid.

Wide range molecular weight markers (7.1–209 kDa, Bio-Rad Laboratories, Hercules, CA, USA) were used to estimate the molecular weight of proteins.

Fourier transform infrared (FTIR) spectra analysis

FTIR spectra of freeze-dried gelatin samples were recorded using a PerkinElmer FTIR Spectrophotometer (Perkin Elmer Spectrum Version 10.03.09, Spectrum Two, Pike Miracle, Pike Technologies, Madison, USA) equipped with an attenuated total reflectance (ATR), deuterated triglycine sulphate (DTGS) detector, mid infra-red (MIR) source, single reflection horizontal ATR accessory (PIKE instruments) having a diamond ATR crystal fixed at incident angle of 45°. For the analysis of spectra, freeze-dried gelatin samples were placed on the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The FTIR spectra of extracted gelatins were collected in the range of 400–4000 cm⁻¹ and automatic signals gained were collected in 16 scans at a resolution of 4 cm⁻¹ against a background spectrum recorded from the clean empty cell at room temperature (28 ± 1 °C).

Functional properties

Solubility study The solubility of gelatin samples was investigated at different pH according to the method of Ahmad and Benjakul (2011) with slight modifications. The gelatin samples (0.5%, w/v) were dissolved in distilled water at 60 °C and the mixtures were stirred at room temperature (28 ± 1 °C) for 2 h. The samples were adjusted to different pH (3–10) with either 6 N HCl or 6 N NaOH. The volume of solutions was made up to 5 mL with distilled water, previously adjusted to the same pH of gelatin solution. The samples were centrifuged at 8500g for 10 min at room temperature. The supernatant was collected and subjected to determine the protein content by Lowry method (Lowry et al. 1951) using bovine serum albumin as

standard. The protein solubility (%) was calculated by comparing the pH at which it shows the highest solubility considered as 100%.

Emulsifying properties Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin samples were determined according to the method of Pearce and Kinsella (1978) with slight modification. Soybean oil (2 mL) and gelatin solution (1%, gelatin 6 mL) were homogenized using homogenizer at a speed of 10,000 rpm for 2 min. Thereafter, the formed emulsions were pipetted out at 0 and 10 min and diluted with 100-fold of 0.1% SDS. The mixture was mixed for 10 s using a vortex mixer. A_{500} was measured using a spectrophotometer. EAI and ESI were calculated using the following formula.

$$EAI \left(\frac{m^2}{g} \right) = \frac{(2 \times 2.303 \times A \times DF)}{1\phi C}$$

where $A = A_{500}$, DF = dilution factor (100), l = path length of cuvette (m), ϕ = oil volume fraction and C = protein concentration in aqueous phase (g/m^3)

$$ESI (min) = \frac{(A_0)}{(A_0 - A_{10})} \times \Delta t$$

where $A_0 = A_{500}$ at time of 0 min; $A_{10} = A_{500}$ at time of 10 min and $\Delta t = 10$ min.

Foaming properties Foam expansion (FE) and foam stability (FS) of gelatin sample solutions were determined as described by Shahidi et al. (1995) with slight modification. Gelatin solution (1%, w/v) was transferred into 100 mL measuring cylinders. The solution was homogenized at a speed of 10,000 rpm for 2 min at room temperature. The sample was allowed to stand for 0 and 60 min. FE and FS were then calculated using the following equations:

$$FE (\%) = \frac{V_T}{V_0} \times 100$$

$$FS (\%) = \frac{V_t}{V_0} \times 100$$

where V_T = total volume after whipping; V_0 = original volume before whipping and V_t = total volume after leaving at room temperature for different times (0–60 min).

Gel strength Gels of gelatin samples were prepared by the method of Ahmad & Benjakul (2011) with a slight modification. Gelatin samples (6.67%, w/v) were suspended in distilled water and heated at 60 °C for 30 min in a temperature controlled water bath with a continuous stirring to dissolve the gelatin completely. Solubilized gelatin solutions were cooled for 10 min at room

temperature (28 ± 1) and transferred to a cylindrical mold with 3 cm diameter and 2.5 cm height. The solutions were incubated at the refrigerated temperature (4 °C) for 16 h for gel maturation. The gel strength was determined at ambient temperature (22 °C) using a texture analyzer (LLOYD-LR-5K, Lloyd Instruments Ltd, UK) with a load cell of 5 kg and a standard probe. The Gel strength (maximum force) was recorded in grams (g) when the probe had penetrated 4 mm with a speed of 1 mm/s.

Statistical analyses

All experiments were performed in triplicate and a completely randomized design (CRD) was used. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie 1980). Analysis was performed using a SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Extraction yield, proximate composition and hydroxyproline content

The yield and proximate compositions of gelatin extracted from the swim bladder of rohu are presented in Table 1. The highest gelatin yield (44.22%, dry weight basis) was obtained in GWA (without acid pretreatment) as compared to acid treatments (GPrA, GAA, and GPA) ($P < 0.05$). During acid pretreatment, marked cleavage of peptide bonds might take place in collagen molecules, leading to the high content of loose collagen or peptides leached out during water washing, resulted in lower yield. This result in association with higher content of hydroxyproline in acids treated washed water (data not shown). Conversely, Ahmad and Benjakul (2011) reported that pretreatment of unicorn leatherjacket (*Aluterus monoceros*) skin with different acids leads to increase in gelatin yield. Sinthusamran et al. (2013) stated that structure of skin has more complex and strong fibrous connective tissue which is required to maintain a structure and to protect the body from the environments they reside, whilst swim bladder containing less complexed structure with lower collagen cross-links is located inside. In the present study, gelatin could be extracted with ease without acid pretreatment. Amongst acid pretreated, propionic acid showed the highest yield (11.91–16.54%) followed by acetic acid (7.62–12.06%) and phosphoric acid (4.37–10.97%), respectively ($P < 0.05$). This might be due to propionic acid which is weaker than acetic acid and phosphoric acid which resulted

Table 1 Yield, hydroxyproline content and proximate composition of gelatin from the swim bladder of rohu using different acid pretreatment at various concentrations

Sample	Yield (%) (dry weight)	Hydroxyproline content (mg/g gelatin)	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Swim bladder (wet basis)	–	–	76.81 ± 1.70	19.84 ± 0.48	0.62 ± 0.06	3.15 ± 0.42
GWA	44.42 ± 0.18 ^a	80.69 ± 1.23 ^a	3.91 ± 0.09 ^e	92.75 ± 1.12 ^a	0.78 ± 0.11 ^a	2.56 ± 0.08 ^e
GAA (0.05 M)	12.06 ± 0.16 ^{dA}	73.24 ± 1.28 ^{cd}	6.31 ± 0.78 ^{bcdA}	88.35 ± 0.78 ^{cdA}	0.67 ± 0.23 ^{abA}	4.61 ± 0.09 ^{eA}
GAA (0.1 M)	10.76 ± 0.61 ^{eB}	70.93 ± 1.87 ^{de}	6.72 ± 1.00 ^{abcA}	87.24 ± 0.49 ^{cdeAB}	0.21 ± 0.13 ^{cAB}	5.81 ± 0.81 ^{bB}
GAA (0.2 M)	7.61 ± 0.53 ^{eC}	69.70 ± 1.09 ^e	7.13 ± 0.26 ^{abA}	85.73 ± 1.30 ^{efB}	0.31 ± 0.21 ^{bcB}	6.83 ± 0.55 ^{aB}
GPA (0.05 M)	10.97 ± 0.91 ^{eA}	67.08 ± 0.78 ^f	5.98 ± 0.64 ^{cdB}	88.15 ± 1.34 ^{cdA}	0.52 ± 0.23 ^{abcA}	5.12 ± 0.34 ^{bcA}
GPA (0.1 M)	8.45 ± 0.36 ^{fB}	66.14 ± 2.10 ^f	6.23 ± 0.18 ^{bcdB}	86.91 ± 0.67 ^{deA}	0.48 ± 0.13 ^{abcA}	5.89 ± 0.10 ^{bB}
GPA (0.2 M)	4.37 ± 0.31 ^{hC}	64.42 ± 1.23 ^f	7.64 ± 0.23 ^{aA}	84.61 ± 1.28 ^{fB}	0.62 ± 0.31 ^{abA}	6.91 ± 0.23 ^{cC}
GPrA (0.05 M)	16.54 ± 0.11 ^{bA}	76.08 ± 1.45 ^b	5.37 ± 0.34 ^{dA}	90.45 ± 1.89 ^{bA}	0.39 ± 0.15 ^{bcA}	3.79 ± 0.17 ^{dA}
GPrA (0.1 M)	14.68 ± 0.53 ^{cB}	74.92 ± 0.54 ^{bc}	5.38 ± 0.70 ^{dA}	89.08 ± 0.59 ^{bcAB}	0.59 ± 0.19 ^{abcB}	4.95 ± 0.98 ^{cB}
GPrA (0.2 M)	11.92 ± 0.26 ^{dC}	71.49 ± 2.54 ^{de}	5.96 ± 0.11 ^{cdA}	87.74 ± 0.98 ^{cdeB}	0.44 ± 0.21 ^{abcB}	5.86 ± 0.03 ^{bB}

Values are given as mean ± SD (n = 3). The different lowercase letters in the same column indicate significant differences ($P < 0.05$). Different capital letters in the same column within same acid pretreatment denote significant difference ($P < 0.05$)

GWA Gelatin extracted without acid pretreatment; GPA Gelatin extracted with phosphoric acid pretreatment; GAA Gelatin extracted with acetic acid pretreatment; GPrA Gelatin extracted with propionic acid

in lower loss or leaching of collagen/peptide molecules during water washing. Furthermore, all acids showed sharp decrease in the gelatin yields as the increase in acid concentrations from 0.05 to 0.2 M. This was more likely due to higher concentration of acid, the solubilization of collagen might take place to a higher extent, which leads to the loss or leaching of loose collagens during swelling and washing process were enhanced. All gelatin samples exhibited protein content, moisture content, fat content and ash content (dry weight basis) were in the range of 84.61–92.75, 3.91–7.13, 0.21–0.78 and, 2.56–6.83%, respectively. The high protein and low moisture, fat and ash contents of gelatin samples, indicates the efficient removal of fat and minerals from the rohu swim bladder material. It was noted that GWA had slightly higher protein content (92.75%) as compared to acid pretreatment counterparts ($P < 0.05$). Conversely, gelatin samples extracted with acid pretreatment showed higher in ash and moisture contents as compared to GWA. Higher ash content more likely due to the presence of inorganic salt, which might be generated during the pretreatment with acid (Ahmad and Benjakul 2011). On the other hand, gelatin sample extracted without acid pretreatment had the lower ash content than the recommended maximum value (2.6/100 g) (Jones 1977). The moisture, protein, fat and ash content of rohu swim bladder were 76.81, 19.84, 0.62, and 3.15%, respectively.

Hydroxyproline content of gelatins extracted from the swim bladder of rohu is shown in Table 1. Similarly, GWA had higher hydroxyproline content (80.69 mg/g) than that of acid pretreatment counterpart (64.42–74.08 mg/g)

($P < 0.05$). This was in accordance with higher gelatin yield and higher protein content in the former. The result reconfirmed that most of the loose collagen molecules or peptides leached out during water washing, resulted in low hydroxyproline content were found in gelatin samples pretreated with acids. Gelatin extracted with propionic acid showed highest hydroxyproline content as compared to gelatin samples extracted with acetic acid and phosphoric acid pretreatment (4.37–10.97%), respectively ($P < 0.05$). Moreover, all acids exhibited an apparent decrease in the hydroxyproline content as the increased in acid concentrations.

Amino acid composition

The amino acid compositions of gelatins extracted from the swim bladder of rohu without and with acid pretreatments are presented in Table 2. Generally, properties of gelatin are largely influenced by the amino acid composition and their MW distribution (Gómez-Guillén et al. 2009). Total amino acid contents of GWA, GPA, GAA, and GPrA were 91.30, 92.58, 93.05 and 92.04 mg/g, respectively. Low amounts of amino acids in GWA (91.30 mg/g) were coincidental the higher hydroxyproline content as compared to GPA, GAA, and GPrA (Table 1). It has been noticed that glycine (24.54–25.66 mg/g) is the major amino acid in all gelatins followed by proline (11.27–12.08 mg/g), glutamic acid (10.43–10.70 mg/g) or alanine (10.35–10.96 mg/g), and aspartic acid (5.27–5.70 mg/g), respectively. This implied that gelatins obtained were derived from its mother collagen. Chandra

Table 2 Amino acid compositions of gelatin from the swim bladder of rohu using different acid pretreatment at 0.05 M concentration

Amino acid	GWA (mg/g protein)	GPrA (mg/g protein)	GAA (mg/g protein)	GPA (mg/g protein)
Asp	5.27	5.36	5.58	5.70
Glu	10.45	10.43	10.57	10.70
Ser	3.37	3.41	3.43	3.41
Gly	24.24	24.89	25.43	25.66
His	0.49	0.45	0.46	0.58
Arg	7.17	7.18	7.13	7.81
Thr	2.55	2.60	2.84	2.82
Ala	10.44	10.64	10.96	10.35
Pro	12.08	11.8	11.54	11.27
Tyr	0.44	0.41	0.49	0.47
Val	2.46	2.42	2.45	2.47
Met	1.92	1.76	1.91	1.81
Cys	0.01	0.02	0.03	0.02
Ile	1.69	1.83	1.26	1.59
Leu	2.77	2.87	2.73	2.77
Phe	2.37	2.24	2.28	2.20
Lys	3.58	3.73	3.49	3.42
Total	91.3	92.04	92.58	93.05

Gelatin extracted without acid pretreatment; *GPA* Gelatin extracted with phosphoric acid pretreatment; *GAA* Gelatin extracted with acetic acid pretreatment; *GPrA* Gelatin extracted with propionic acid

and Samsunder (2015) reported that gelatin from *Catla catla* swim bladder contained glycine, glutamic acid and proline were 18.9, 15.8 and 15.4 mg/g, respectively. GPA, GAA, and GPrA had the higher glycine content than GWA. The higher glycine in GPA, GAA, and GPrA might be caused by free glycine, which was released to a high extent during acid pretreatment. Histidine (0.45–0.58 mg/g) and tyrosine (0.41–0.49 mg/g) were found in all gelatins at very low levels. Moreover, cysteine (0.1–0.3 mg/g) was present in negligible amount in all gelatins. Therefore, the amino acid composition might have the impact on functional properties of gelatin extracted.

Protein patterns

Protein patterns of extracted gelatins from the swim bladder of rohu by using different acid at 0.05 M pretreatments and without pretreatment are shown in the Fig. 1. GWA showed α -(α_1 and α_2) and β -chains (α -chain dimmers) as the dominant constituents ranging from 72 to 200 kDa. In addition α - and β -chains, GWA exhibited a number of peptide bands with MW ranging from 20 to 69 kDa, which might have arisen due to degradation of gelatin during extraction at 60 °C. The protein patterns of gelatin were similar to those found in the gelatin extracted from the swim bladder of *Catla catla* reported by Chandra and Shamsunder (2015). In general, gelatin with a higher content of α -chains exhibited better functional properties including gelling, emulsifying and foaming properties,

Fig. 1 SDS-PAGE of gelatin from the swim bladder of rohu using different pretreatment at 0.05 M concentration. *GWA* Gelatin extracted without acid pretreatment; *GPA* Gelatin extracted with phosphoric acid pretreatment; *GAA* Gelatin extracted with acetic acid pretreatment; *GPrA* Gelatin extracted with propionic acid; *M* Standard protein molecular weight marker

Gómez-Guillén et al. (2002). On the other hand, GPA, GAA, and GPrA had α_1 and α_2 with a MW of 93 and 80 kDa, respectively. Disappearance of β -chains (~ 200 kDa) and other some bands in GPrA, GAA, and GPA more likely due to marked degradation of collagen molecules induced by acid pretreatment process, resulted in leached out during water washing. This result was in

accordance with the lower gelatin yields in GPrA, GAA, and GPA (Table 1). Nevertheless, GPrA, GAA, and GPA showed two low MW peptides of ~ 40.2 and ~ 32.4 kDa. Appearance of low MW peptides (~ 40.2 and ~ 32.4 kDa) was plausibly due to the degradation of higher MW (β -chains) protein induced by acids. No marked differences in protein patterns were observed among GPrA, GAA, and GPA. However, GPA showed slightly decreased in band intensity of the α -chains and small MW proteins (~ 40.2 and ~ 32.4 kDa). This might be caused by more degradation induced by phosphoric acid. From the result, acids used for pretreatment of rohu swim bladder were more punitive and induced pronounced degradation of collagen molecules leading to removal of protein (gelatin) molecules during water washing.

FTIR spectra

FTIR spectra of gelatins extracted from the swim bladder of rohu without and with different acid pretreatments are depicted in Fig. 2. FTIR spectroscopy has been used to monitor the changes in the functional groups and secondary structure of gelatin samples (Muyonga et al. 2004). Several major bands were observed in the region of 4000 – 500 cm^{-1} . Similar FTIR spectra were found in all gelatin except some differences in amplitude. All gelatin samples exhibited amide A band at 3289 – 3304 cm^{-1} . Amide A represents N–H stretching coupled with hydrogen bonding. Generally, a free N–H stretching vibration occurs in the range of 3400 – 3440 cm^{-1} . When the N–H group of a peptide is involved in a hydrogen bond, the position shift to lower frequencies (Doyle et al. 1975). Amide A peaks appeared at 3288 , 3302 , 3301 , and 3301 cm^{-1} for GWA, GPA, GAA, and GPrA, respectively. In amide-A region, the lower wavenumber as well as lower amplitude was

found in GWA as compared to GPA, GAA, and GPrA, suggesting the N–H group of shorter peptide fragments in GWA was involved in hydrogen bonding. The result was in agreement with smaller peptides appeared in GWA (Fig. 1). The amide B was observed at 2928 , 2935 , 2930 , 2928 cm^{-1} for GWA, GPA, GAA, and GPrA, respectively. Amide B corresponds to asymmetric stretch vibration of $=\text{C}-\text{H}$ as well as $-\text{NH}_3^+$. Furthermore, all gelatin samples exhibited amide I band at the wavenumber of 1632 – 1634 cm^{-1} . The major amide I peak appeared at wavenumbers of 1634 , 1633 , 1632 and 1634 cm^{-1} for GWA, GPA, GAA, and GPrA, respectively. The amide I vibration mode is primarily stretching vibration of $\text{C}=\text{O}$, which belongs to the amide groups weakly coupled with in-plane NH bending and CN stretching (Carbonaro and Nucara 2010). The absorption peak at amide I was characteristic for the coil structure of gelatin (Yakimets et al. 2005). The spectral differences in the amide I of different gelatin samples were largely attributed to different conformation of polypeptide chains. Higher amplitude of Amide I peaks were observed in GPA, GAA, and GPrA compared to GWA, respectively. The results indicated that greater loss of triple helix due to the pronounced disruption of the inter chain interaction induced by acidic conditions. This resulted into generation of low MW components which leached out during washing in GPA, GAA, and GPrA. This coincident with the loss of α -chains and some other small MW proteins in GPA, GAA, and GPrA compared to GWA (Fig. 1). Lower amplitude at amide I was associated with the higher extent of molecular order due to interaction of $\text{C}=\text{O}$ with adjacent chains via hydrogen bonds. Nevertheless, GPA showed higher amplitude than GAA, and GPrA, respectively, indicating the greater disruption of intra-molecular bonding by phosphoric acid than acetic acid and propionic acid resulted in loss of the

Fig. 2 Fourier transform infrared spectra of gelatin from the swim bladder of rohu using different acid pretreatment at 0.05 M concentration. GWA Gelatin extracted without acid pretreatment; GPrA Gelatin extracted with propionic acid; GAA Gelatin extracted with acetic acid pretreatment; GPA Gelatin extracted with phosphoric acid pretreatment

secondary structure at higher extent. For the amide II band, GWA, GPA, GAA, and GPrA showed the peaks at wavenumber of 1545, 1549, 1539, and 1547 cm^{-1} , respectively. The amide II vibration modes are attributed to an out-of-phase combination of the N–H in plane bend and the C–N stretching vibration with smaller contributions from the C–O in plane bend and the C–C and N–C stretching vibrations (Jackson et al. 1995). Furthermore, peaks for amide III were detected at wavenumber of 1240, 1239, 1236, and 1239 cm^{-1} for GWA, GPA, GAA, and GPrA, respectively. Amide III bands were associated with the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Jackson et al. 1995). Nevertheless, GPA, GAA, and GPrA exhibited slightly higher amplitude peaks at amide II and III region than did GWA. This indicated that the greater disorder of molecular structure due to transformation of α -helical to a random coil structure. Therefore, it can be concluded that during acid pretreatment of the swim bladder, the triple-helix structure of collagen converted into a higher extent of unordered and low MW peptides, led to removal of proteins during washing with water.

Solubility

Protein solubility of all gelatins extracted from the swim bladder of rohu without and with different acid pretreatments are depicted in Fig. 3. Solubility of all gelatin samples showed U-shaped curves in the pH range of 3–10 (Fig. 3). These trends in solubility are in agreement with Lassoued et al. (2014), who studied the influences of different pH on solubility of gelatin from thornback ray skin obtained by pepsin-aided process. The lowest solubility of all gelatin samples was found at pH 6–7 ($P < 0.05$), which may be nearer to its isoelectric point (pI). At pH close to pI, hydrophobic interaction between gelatin molecules increased and the total net charge of protein molecules became zero, consequently more protein–protein interactions and fewer protein–water interaction occurs. Gelatin is an amphoteric protein with isoelectric point between 5 and 9 depending on raw material and method of manufacture (Johnston-Banks 1990; Poppe 1997). GWA revealed minimum solubility (65.65%) at pH 6.0 while GPA, GAA, and GPrA showed minimum solubility at pH 5.0. This might be due during acid pretreatment of the swim bladder, some glutamine and asparagine can be converted to their acidic forms, i.e. glutamic acid and aspartic acid, respectively leads to increases the number of carboxyl groups in the gelatin molecule and thus lowers the isoelectric point (Jamilah and Harvinder 2002). The degree of conversion is related to the severity of the pretreatment process. On

Fig. 3 Solubility of gelatin from the swim bladder of rohu using different acid pretreatment at 0.05 M concentration. GWA Gelatin extracted without acid pretreatment; GPA Gelatin extracted without acid pretreatment; GPrA Gelatin extracted with propionic acid; GAA Gelatin extracted with acetic acid pretreatment; GPA Gelatin extracted with phosphoric acid pretreatment

either side of isoelectric point (pH 6–7), all gelatin samples showed increased solubility. The high solubility at pH far away from pI can be attributed to increased electrostatic repulsion of the protonated or deprotonated gelatin molecules, led to the increased solubility. Furthermore, all gelatin samples exhibited maximum solubility at pH 10 ranges from 92 to 98%. Among the gelatin samples, GWA showed the highest solubility at all pH tested followed by GPrA, GPA, and GAA, respectively ($P < 0.05$). The difference in solubility of different gelatins might result from the differences in MW and the content of polar and non-polar groups in amino acids (Zayas 1997). Therefore, gelatin solubility at different pH may serve as a useful indicator for the performance of the gelatin incorporated in the food systems, since solubility is a prerequisite for most functionality of food proteins.

Emulsifying property

Emulsion activity index (EAI) and emulsion stability index (ESI) of GWA, GPrA, GAA, and GPA from the swim bladder of rohu are presented in Table 3. GWA exhibited higher EAI and ESI than those of GPrA, GAA, and GPA ($P < 0.05$). This was more likely due to GWA had a more proportion of longer peptides (Fig. 1) could stabilize the protein film at the interface more effectively. Surh et al. (2006) found that the oil-in-water emulsion prepared with high MW fish gelatin (~ 120 kDa) was more stable than that prepared with low MW fish gelatin (~ 50 kDa). Furthermore, the high solubility of GWA in the dispersing phase increases the emulsifying efficiency, because the protein molecules should be able to migrate to the surface of the fat droplets rapidly (Sikorski 2001). Lower EAI and

Table 3 Emulsion, foaming and gelation properties of the gelatin from the swim bladder of rohu using different acid pretreatment at 0.05 M concentration

Sample	EAI (m ² /g)	ESI (min)	FE (%)	FS (%)	Gel strength (g)
GWA	31.46 ± 0.9 ^a	19.45 ± 0.8 ^a	145.15 ± 1.5 ^a	133.50 ± 2.1 ^a	69.23 ± 3.6 ^a
GPrA	28.45 ± 0.6 ^b	16.45 ± 0.6 ^b	137.32 ± 2.2 ^b	120.11 ± 1.7 ^b	55.37 ± 2.2 ^b
GAA	25.46 ± 1.6 ^c	15.34 ± 0.5 ^b	125.23 ± 1.9 ^c	116.45 ± 1.1 ^c	41.81 ± 2.8 ^c
GPA	16.23 ± 1.1 ^d	8.65 ± 1.5 ^c	121.45 ± 2.1 ^d	111.50 ± 1.4 ^d	35.67 ± 3.1 ^d

Values are mean ± SD (n = 3). Different superscripts within the same column denote the significant difference ($P < 0.05$)

GWA Gelatin extracted without acid pretreatment; GPA Gelatin extracted with phosphoric acid pretreatment; GAA Gelatin extracted with acetic acid pretreatment; GPrA Gelatin extracted with propionic acid

ESI found in GPrA, GAA, and GPA was more likely related to the low solubility of proteins, as a consequence, insolubilized proteins could not unfold rapidly at the interface and could not form a film around an oil droplet effectively. GPrA, and GAA had slightly higher EAI and ESI than that of GPA. This was more plausibly due GPrA, and GAA exhibited additional longer peptides than GPA. Additionally, higher solubility of GPrA, and GAA facilitated more protein migration and adsorption at interfaces, in which thicker and stronger films could be formed. However, no difference in EAI and ESI was found between GPrA, and GAA ($P > 0.05$).

Foaming property

Foam expansion (FE) and foam stability (FS) of GWA, GPrA, GAA, and GPA from the swim bladder of rohu are presented in Table 3. GWA exhibited higher FE and FS than GPrA, GAA, and GPA ($P < 0.05$). The higher FE and FS of GWA more likely underwent a desirable structural change, which led to the higher adsorption, conformational change and rearrangement at the air–water interface. Moreover, GWA had higher protein solubility and longer peptides than GPrA, GAA, and GPA. High protein solubility is a prerequisite to achieve better foaming capacity and foam stability. In general, the foaming ability of proteins is correlated with their film-forming ability at the air–water interface. When proteins are rapidly adsorb at the newly created air–liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, a better foaming ability can be obtained, compared with proteins that adsorb slowly and resist unfolding at the interface (Damodaran 1997). Amongst acid pretreatment samples, the GPrA displayed higher FE and FS followed by GAA, and GPA, respectively ($P < 0.05$). This was more likely due to propionic acid which induced desirable structural changes in the protein molecules which led to rapid adsorb at the newly created air–liquid interface and formed stable foam. The stability of foams depends on various parameters, such as the rate of attaining equilibrium surface tension, bulk and surface viscosities, steric

stabilization, and electrical repulsion between the two sides of the foam lamella (Foegeding et al. 2006).

Gel strength

The gel strengths of gelatin extracted from the swim bladder of rohu without (GWA) and with different acid pretreatments (GPrA, GAA, and GPA) are depicted in Table 3. Gel strength is one of the important properties of gelatin gels, and the specific application of gel is determined by the range of gel strength values (Cho et al. 2005). GWA had highest gel strength (69.23 g), compared to GPrA, GAA, and GPA (33.67–55.37 g). Higher gel strength in GWA might be due to the presence of higher MW peptides as compared to gelatins extracted with acid pretreatments. It has been postulated that the gelatin with higher MW peptides or protein presented the higher gel strength (Kaewruang et al. 2013). Gelatin with the lower hydrolysis more likely had the longer chain peptides which undergo interaction via inter-junction zone resulted in the stronger network was formed as indicated by the higher gel strength (Kaewruang et al. 2013). Apart from high MW peptides, GWA exhibited the imino acid content, especially hydroxyproline content higher than GPrA, GAA, and GPA (Table 3). Kaewruang et al. (2013) proposed the imino acid content of gelatin determine the gel strength by introducing pyrrolidine rings for bridging between chains, apart from H-bondings, which resulted in increased gel strength. However, lower gel strength in GPrA, GAA, and GPA more likely presence of low MW peptides (Fig. 1) and lower hydroxyproline content (Table 3). In addition, lower number of peptides in GPrA, GAA, and GPA due to leached out during water washing, resulted in lower gel strength. This correlated well with the absence of higher and some smaller chain peptides in GPrA, GAA, and GPA (Fig. 3). Amongst the GPrA, GAA and GPA, the GPrA exhibited higher gel strength (55.37 g), followed by GAA (41.81 g), and GPA (33.67 g). Higher gel strength in GPrA presented by higher hydroxyproline content and high intensity peptides compared to GAA and GPA, respectively. The results suggested that the acid pretreatment

during gelatin extraction affects the gelation properties. Furthermore, stronger acid which cleaves collagen molecules intensely during acid pretreatment of swim bladder led to impaired gel formation.

Conclusion

From the investigations of the present work, it can be concluded that the phosphoric acid, acetic acid, and propionic acid at all concentrations used for pretreatment of rohu swim bladder were affected the gelatin yields. The acid pretreatment leads to a significant decrease in the gelatin extraction due to pronounced degradation of collagen molecules. Additionally, gelatin extracted using aforementioned acid pretreatment exhibited lower emulsifying, foaming and gelling properties. Among the acids, gelatin extracted with propionic acid had slightly higher gelatin yield and better functional properties compared to other two acid (acetic acid and phosphoric acid) pretreatment. Gelatin extracted without acid pretreatment showed a higher gelatin yield as well as superior emulsifying, foaming, and gelling properties than those of gelatins extracted with acid pretreatments. Therefore, the acid pretreatment of swim bladder was not significant and had a negative impact on the extraction yield and functionalities of gelatin.

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