Original paper

Extraction and Characterization of Gelatin from Bovine Lung

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Gelatin is extracted from animal tissues using heat usually with low yields, but pepsin may increase high quality gelatin yield per unit of tissue. Gelatin from bovine lungs was extracted using heat and pepsin and the resulting gelatins were characterized. Pepsin increased gelatin yield by about 9-fold that of heat extraction alone. All bovine lung gelatin contained protein as the major proximate component, with little ash and non-detectable fat. Bovine lung gelatin had pH, moisture and protein comparable to or less than that of commercial bovine gelatin and decreased ash. Transmittance of bovine lung gelatin was substantially reduced compared to that of commercial bovine gelatin but had increased water and fat-binding capacity, and comparable or increased gelling and melting temperature. Gel strengths of bovine lung gelatin were comparable to or lower than and foam stability and emulsifying activity were lower than commercial bovine gelatin. Increased imino acid (proline and hydroxyproline) content was associated with increased gelling and melting temperatures and was comparable to commercial bovine gelatin. Heat-extracted bovine lung gelatin contained predominantly collagen γ– chains, β– chains and α– chains (α1(I) and α2(I)), with some low molecular weight peptides, while the pepsin-extracted lung gelatins were characterized by comparatively decreased β– and α– chains and increased low molecular weight peptides. The gel strength of heat-extracted bovine lung gelatin was higher than that of pepsin-extracted gelatins, indicating that additional yield was associated with reduced gelatin quality. Bovine lung is a potential source of gelatin for application in diversified industrial fields and use of pepsin is a viable method for extracting additional gelatin after heat extraction of high quality (increased gel strength) gelatin from bovine lung.

Keywords: gelatin, bovine lung, extraction, pepsin, emulsifying, foaming

Introduction

Gelatin is a popular biopolymer used in food, pharmaceutical, cosmetic and photographic applications (Schrieber and Gareis, 2007) because of its unique functional properties (Zhou et al., 2006). Gelatin is derived from collagen, the major protein constituent of connective tissue, through partial hydrolysis using hot water, dilute acid or alkali treatments (Zhang et al., 2009). The global gelatin market volume was approximately 373,000 tons in 2013, with this market volume anticipated to grow by 3.8% every year to an estimated worth of $3,000,000,000 by 2020 (Grand View Research Inc., 2014). Gelatin derived from pig skin has accounted for the highest proportion available for use, followed by that from bovine hides and bovine bones (Karim and Bhat, 2009). Bovine Spongiform Encephalopathy (BSE) has been a concern with regard to the safety of gelatin from bovine sources despite the expectation that the probability of prion infectivity is low given the harsh chemical treatment of the raw material during gelatin production (Baziwane and He, 2003) and the Scientific Steering Committee of the European Union confirmed this (Schrieber and Gareis, 2007). As the cattle industry moves toward zero incidence of BSE and further research clarifies the BSE risk from gelatin, gelatin from cattle carcasses will become increasingly attractive and recent research has explored the use of low value organs as sources of functional proteins (Selmane et al., 2008).

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Acid treatment has been used to remove collagen from animal tissues with negligible peptide bond hydrolysis, leading to a high gel strength product (Slade and Levine, 1987). Because many collagen cross-links are resistant to thermal and acid treatment (Galea et al., 2000), a low gelatin yield is generally obtained. To increase the extractability, some proteases are used to solubilize the collagen (Chomarat et al., 1994). Pepsin has been reported to cleave collagen in its telopeptides so that the yield of gelatin can be increased (Jongjaroenrak et al., 2005). The extraction of bovine co–products using a pepsin–aided process should therefore increase gelatin yield without excessive proteolysis. The objectives of this study were to extract gelatin from bovine lung with different extraction conditions and to compare the physiochemical, thermal and rheological properties of the extracted gelatin and its solution to that of commercial bovine gelatin to estimate its potential uses.

Materials and Methods

Experimental samples and experimental design Ten fresh bovine lungs (BL), each from a different animal, were obtained from local abattoirs and transported to the laboratory. After arrival the whole lung was frozen at −20°C and stored frozen until processed. Each lung was thawed overnight at about 4°C, chopped into cubes, mixed to homogeneity, divided into 400 g portions and stored at −20°C until experimentation. From each lung, four approximately 400 g portions were randomly selected, two portions were randomly assigned to gelatin extraction using sequential heat treatment and a single pepsin digestion of 48 h for duplication of treatment within lung (Figure 1). Duplicate samples were used in all analyses including commercial bovine gelatin (CBG) (Sigma Aldrich, Cat No. G9382), which was considered as control.

Gelatin extraction

Isolation of connective tissue (CT) Each of the four frozen BL portion within each lung was thawed overnight at 4°C. The BL cubes were added to deionized (DI) water at a ratio of 1:4 (w/v) and blended (Waring 2-Speed Heavy-Duty Lab Blender, 120 VAC, Cole-Parmer, Canada) until homogenous. The homogenate was centrifuged at 5,000 g for 10 min at 4°C. The sediment was diluted with 0.5 M NaOH solution at a ratio of 1:10 (w/v) and incubated at 25°C for 30 min with stirring, then centrifuged at 5,000 g for 10 min and sediment was collected. This step was repeated once more. The alkaline sediment was then suspended in DI water for 10 min and centrifuged at 5, 000 g for 10 min and the sediment was collected. This step was repeated three times and then the sediment was suspended in DI water and neutralized with 6M HCl solution. The sediment was considered as lung CT.

Gelatin extraction with heat The lung CT was heated at 70°C for 1h and centrifuged at 5, 000 g for 10 min. The supernatant (soluble gelatin) was collected, combined within animal, dialyzed (Spectra/Por 4 dialysis tubing, 12 – 14 K MWCO, Spectrum Laboratories , Canada) with DI water and then deionized with mixed ion exchange resin Rexyn I-300 (H-OH) beads (Across Chemicals, New Jersey, USA) until conductivity value was below
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50 μSiemens. The soluble gelatin fraction was subsequently separated from the resin by filtering through layered cheese cloth. The filtrate was frozen at −50°C, lyophilized, weighed and considered as bovine lung gelatin (BLG) and named BLG–70. After the 70°C extraction, the remaining CT sediment was suspended in DI water and heated at 80°C for 1h. The resultant solution was then centrifuged and the supernatant was collected by following the same steps as BLG–70 and considered as BLG–80.

**Gelatin extraction with pepsin** The CT sediment that remained after 80°C extraction from each BL portion was suspended in 0.5 M acetic acid at a ratio of 1:7 (w/v). Pepsin (from porcine gastric mucosa, Sigma Aldrich, St. Louis, MI) was added at a sediment to pepsin ratio of 100:1 (w/w) to each of two portions from each lung with stirring at 4°C for 24h. The pH of the resultant digest was adjusted to neutral using 2 M NaOH and the digest was heated at 80°C for 2h after which the supernatant was collected with centrifugation, considered as BLG extracted first 24h of pepsin digestion (BLG–F24h). The sediments of these samples were again digested with pepsin for a second 24h period, and the supernatant collected and designated as BLG–L24h (Figure 1). The remaining two sediments from each lung after 80°C heat treatment were extracted using pepsin digestion as described in Figure 1 and were considered gelatin extracted at 48h with pepsin digestion (BLG–48h).

All gelatins were processed as described for the heat-extracted gelatin.

**Yield of gelatin** The yield of gelatin was calculated as follows:

\[
\text{Yield (g/100g)} = \frac{[\text{Weight of freeze-dried gelatin (g)}]}{\text{Weight of raw bovine lung (g)}} \times 100 \quad \cdots \cdot \cdot \cdot \text{Eq. 1}
\]

**Proximate analysis** AOAC International (2000) methods were used to measure moisture (950.46), ash (920.153), and fat (960.39) contents of freeze-dried BL and BLG. Protein content was estimated using a TruSpec carbon/nitrogen determinator (Leco Corp., St. Joseph, MI) calibrated with EDTA and caffeine and calculated using a nitrogen conversion factors of 6.25 for lyophilized BL and 5.55 for lyophilized BLG (Sarbon et al., 2013).

**pH of gelatin solution** The pH value of BLG solution was measured with slight modification of the British Standard Institution, BSI 757 (1975) method in 2% (w/v) gelatin solution prepared in DI water. The pH was measured with a glass electrode (Orion 2 Star™, Fisher Scientific, Mississauga, ON) after standardizing the pH meter (Star Plus™, Fisher Scientific, Mississauga, ON) with pH 4.0 and 7.0 buffers.

**Transmittance** For transmittance determination, the spectrophotometer (Evolution™ 60S UV-Visible Spectrophotometer, Thermo Scientific, Canada) was calibrated at 620 nm to 100% transmittance with DI water as the blank. One mL of 2% gelatin solution was transferred to the cuvette and the percent transmittance was recorded at 620 nm following the method of Ninan et al. (2010).

**Colour** The colour of 6.67% (w/v) BLG gels was evaluated with a colorimeter (Konica Minolta CR-300, NJ, USA). The colorimeter, with an 8 mm aperture and illuminant D65, was calibrated using a standard white calibration tile provided by the manufacturer. The colour was described using the Commission Internationale de L’Eclairage (CIE) coordinates L* (lightness), a* (redness/greenness) and b* (yellowness/blueness).

**Water holding and fat binding capacities** Water holding capacity (WHC) of gelatin samples was estimated according to the method of Uriarte-Montoya et al. (2011) and fat binding capacity (FBC) was measured by slight modification of the method of Jellouli et al. (2011). Briefly, 0.25 (± 0.003) g of freeze-dried BLG was dissolved with 25 mL of DI water or sunflower oil in a 50 mL centrifuge tube and allowed to stand for 1h with brief stirring every 15 min. The residue was collected by centrifugation at 4, 500 g for 20 min at 4°C. The supernatant was discarded and the tubes were allowed to drain at a 45° angle for 30 min. The WHC and FBC of freeze-dried BLG were calculated as follows:

\[
\text{WHC or FBC(%)} = \frac{\text{Weight of the contents of the tube after draining (g)}}{\text{Weight of the freeze-dried gelatin (g)}} \times 100 \quad \cdots \cdot \cdot \cdot \text{Eq. 2}
\]

**Isoelectric point** The isoelectric point (pI) was estimated by measuring the percent transmittance of a 2% (w/v) gelatin solution at different pH values (pH 2 to 10) at 660 nm according to the method of Zhang et al. (2011). The pH value at which the gelatin solution had the lowest percent transmittance was considered the pI value.

**Foaming properties** Foam expansion (FE) and foam stability (FS) of BLG solutions were determined according to the method of Jridi et al. (2013). Twenty five mL of gelatin solutions of 0.5, 1.0 and 2.0% (w/v) were homogenized at 20, 000 rpm for 1 min at room temperature using an Ultra-Turrax Ika T-18 Basic Ultra Turrax Homogenizer (Cole-Parmer, Canada) to incorporate air. Immediately after homogenization, the solution was transferred to 50 mL graduated plastic tubes and the total volumes were measured at 0, 30 and 60 min after homogenization. The FE and FS were calculated by the following formula:

\[
\text{FE} (\%) = \frac{(V_f - V_b)}{V_b} \times 100 \quad \cdots \cdot \cdot \cdot \text{Eq. 3}
\]

\[
\text{FS} (\%) = \frac{(V_f - V_b)}{V_b} \times 100 \quad \cdots \cdot \cdot \cdot \text{Eq. 4}
\]

Where, \(V_f\) is the total volume (mL) after homogenization; \(V_b\) is the volume (mL) before homogenization; \(V_t\) is the total volume (mL) after standing at room temperature for 30 or 60 min. All determinations were means of two measurements.

**Emulsifying properties** The emulsion activity index (EAI) and the emulsion stability index (ESI) of BLG were determined according to the methods of Aewsiri et al. (2013) and Jridi et al. (2013), respectively. Eight mL of gelatin solutions of 0.5, 1.0 and 2.0% were mixed with 2 mL of sunflower oil by Vortex and homogenized for 1 min at 24, 200 rpm at room temperature. Aliquots of each emulsion (50 μL) were taken from the bottom of the container at 0 and 30 min after homogenization and mixed with
5 mL sodium dodecyl sulphate (SDS) solution (0.1%, w/v). The absorbance was measured at 500 nm against a 0.1% SDS solution blank immediately (A₀) and 30 min (A₃₀) after emulsion formation. To calculate EAI and ESI, the following formula was used:

\[
EAI (m^2 / g) = \left(2 \times 2.303 \times \frac{A_{30} - A_{0}}{DF} \right) / (C \times \varphi \times 10^4) \quad \text{Eq. 5}
\]

\[
ESI (min) = \frac{(A_0 - A_\Delta T)}{\Delta A} \quad \text{Eq. 6}
\]

Where A₀₋₃₀ absorbance at 500 nm; DF, dilution factor (100); C, protein concentration (g/mL) before emulsification; \(\varphi\), oil volume fraction (ν/ν) of the emulsion and \(A_0\) and \(A_{30}\) represent the absorbance values at time zero and at 30 min, respectively with \(\Delta A = A_{30} - A_0\) and \(\Delta T = 30\) min.

**Gel strength** The gel strength of BLG was determined following the BSI 757 (1975) method with a slight modification. BLG (7.50 ± 0.01 g) was weighed into a standard Bloom jar of 150 mL (Schott, Mainz, Germany) with DI water (105 ± 0.2 g). The bottle was covered and stood at room temperature for 3h then held at 45°C for about 20 min with occasional shaking for complete dissolution. The bottles were allowed to cool for about 15 min at room temperature, and then chilled at 10 ± 0.1°C for 16 – 18h for gel maturation. Gel strength was measured with a 1.27 cm diameter flat-faced cylindrical Teflon plunger fitted to a TA-XT2 Texture Analyzer (Stable Micro Systems, Surrey, UK) with a 2 kN load cell. The plunger was set to move a distance 4 mm into the midpoint of the gel at a speed of 0.5 mm/sec. The maximum force (g) at the penetration distance of 4 mm at 0.5 mm/sec was recorded as the gel strength.

**Dynamic viscoelastic properties** Dynamic viscoelastic properties of BLG [6.67% (w/w)] were measured using a Physica MCR 301 rheometer (Anton Paar GmbH, Ashland, VA) with 25 mm parallel plate geometry and a gap of 1 mm between the plates. A Peltier element coupled to a thermo-electrical pump was incorporated for temperature control. Evaporation of the sample was prevented using a water–lock around the parallel plates of the rheometer.

The elastic properties and the viscous response in the structure of BLG were estimated using an amplitude sweep test (Giuseppe et al., 2009). The amplitude sweep test was performed with an increasing oscillatory strain (0.1 – 20%) while frequency (1 hertz) and temperature (15°C) were kept constant with the exception of the BLG–F24h samples, which was performed at 5°C.

A dynamic temperature sweep rheological test was used to determine gelling and melting temperatures. The analysis was performed under a constant strain of 5%, a constant frequency of 1 hertz, and the temperature was changed from 45 to 15°C and back to 45°C with a heating/cooling rate of 2°C/min, with the exception of the BLG–F24h samples, where the temperature was changed from 35 to 5°C and back to 35°C because of the low gelling temperature of this gelatin. The gelling temperature was taken to the temperature at which the storage modulus (\(G’\); Pa) began to dramatically increase in value. The melting point was determined in the same manner as the gelling point during the subsequent heating process, with melting considered to have begun when the storage modulus (\(G’\); Pa) decreased and the loss modulus (\(G’’\); Pa) increased. The temperature at which the \(G’\) and \(G’’\) converge during cooling was considered the solution-gel transition or the gel formation point (Gudmundsson, 2002).

**Molecular weight distribution** Protein molecular weight (MW) distributions of the gelatins were determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970). Gelatin solutions (5 mg/mL) diluted with sample buffer were prepared as both non-reduced and reduced (5% β-mercaptoethanol), denatured at 95°C for 5 min and clarified by centrifugation at 5, 000 g for 5 min. Ten µL of each sample and molecular weight markers (Bio-Rad Laboratories Inc., Hercules, CA) were loaded on a precast 7.5% Mini–PROTEAN® TGX™ ready gel (Bio–Rad Laboratories). The samples were run at a constant voltage of 200 V on a Power Pack Basic electrophoresis apparatus (Bio-Rad Laboratories). The gel was then stained using Coomassie Brilliant Blue R250 and de-stained in a DI water, methanol, and acetic acid solution in a ratio of 50:40:10 (v/v/v). Protein markers (Precision Plus Protein™ Dual Colour Standards, Bio-Rad Laboratories) ranged from 10 to 250 kDa. Gels were scanned using the Image Scanner (Amersham Biosciences U9908–HARO Flatbed Image Scanner, Uppsala, Sweden).

**Amino acid composition** Fifty µL (1 mg/mL) of BLG were hydrolysed with 6 M HCl containing 0.1% phenol for 1h at 160°C in a vacuum-sealed hydrolysis vial. Hydrolysates were labeled with AccQ–Tag Ultra Derivatization Kit (Waters, Milford, MA) according to the manufacturer protocol. Labeled amino acids were then separated by high performance liquid chromatography (HPLC, Agilent 1200 Series) using an AccQ–Tag reversed-phase C18 column (3.9 × 150 mm, Waters) and detected by absorbance at 254 nm. Norleucine (Sigma–Aldrich) was added as an internal standard for quantification and amino acid identification and quantification performed using Agilent Chemstation. Results were presented as percent mole.

**Statistical analysis** Differences in gelatin properties were distinguished using statistical procedures within the Statistical Analysis System (SAS Institute, Cary, NC). One-way analysis of variance using the general linear models procedure (PROC GLM) determined significant differences (P < 0.05) in gelatin proximate components, pH, colour, water-holding and fat binding capacities, dynamic rheological measurements, amino acid proportions and gel strength due to extraction step in comparison to the CBG. Differences between means for this analysis were identified using the Student-Newman-Keuls test. Effects of gelatin extraction step, gelatin concentration and their interaction were determined using a 3 (% gelatin concentration) × 5 (gelatin extraction method) factorial analysis of variance within PROC GLM. For this analysis, differences between means were distinguished using
Table 1. Bovine lung gelatin (BLG) extraction method means (± standard error of the mean) for yield, proximate analyses and pH

<table>
<thead>
<tr>
<th>Components</th>
<th>Gelatin extraction methods(^1)</th>
<th>(n)</th>
<th>BLG-70</th>
<th>BLG-80</th>
<th>BLG-F24h</th>
<th>BLG-L24h</th>
<th>BLG-48h</th>
<th>CBG</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin yield (%)</td>
<td>(0.22 \pm 0.05) (^a) (0.07 \pm 0.05) (^b) (0.48 \pm 0.05) (^c) (2.38 \pm 0.05) (^d) (1.98 \pm 0.05) (^e) NA</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>(8.29 \pm 1.06) (^a) (3.33 \pm 2.42) (^b) (6.04 \pm 1.20) (^c) (3.61 \pm 0.76) (^d) (4.85 \pm 0.76) (^e) 9.19 \pm 1.67 (^f)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>0.0083</td>
<td></td>
</tr>
<tr>
<td>Crude Protein (%)</td>
<td>(92.05 \pm 1.18) (^a) (93.80 \pm 4.12) (^b) (82.20 \pm 1.84) (^c) (93.47 \pm 1.30) (^d) (90.90 \pm 1.30) (^e) 94.22 \pm 2.85 (^f)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>0.0009</td>
<td></td>
</tr>
<tr>
<td>Ash (%)</td>
<td>(0.40 \pm 0.07) (^a) (0.54 \pm 0.15) (^b) (0.96 \pm 0.07) (^c) (0.435 \pm 0.05) (^d) (0.327 \pm 0.05) (^e) 2.36 \pm 0.10 (^f)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Crude fat (%)</td>
<td>ND (^a) ND (^b) ND (^c) ND (^d) ND (^e) 0.41 (^f)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>(4.73 \pm 0.10) (^a) (4.84 \pm 0.16) (^b) (5.57 \pm 0.10) (^c) (5.27 \pm 0.07) (^d) (5.55 \pm 0.07) (^e) 5.23 \pm 0.16 (^f)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a,b,c,d}\) Means within a row with different letters are significantly different at P < 0.05 according to Student-Newman-Keuls multiple range test. 
\(^{a,b}\) Means within a row with different letters are significantly different at P < 0.05 according to paired t-tests.

\(^1\) BLG-70 = BLG extracted at 70°C for 1 h; BLG-80 = BLG extracted at 80°C for 1 h; BLG-F24h = BLG extracted with pepsin for 24 h; BLG-L24h = BLG extracted with pepsin for a second 24 h; BLG-48h = BLG extracted with pepsin for 48 h, CBG = commercial bovine gelatin.

Results and Discussion

Yield Extraction of gelatin using heat or acid can produce economically viable yields from fish (Slade and Levine, 1987; Karim and Bhat, 2009) and porcine co-products (Gudipati and Kannuchamy, 2014) and bovine skin and bone, but yields of gelatin from other mammalian tissues using these processes are limited by the relatively large concentration of heat-stable collagen cross-links (Galea et al., 2000; Gómez-Guillén et al., 2002). Extraction of gelatin using heat produces gelatin with the highest gel strength (Schrieber and Gareis, 2007). Because heat produces the best gelatin, it would be a standard first process for most mammalian tissue gelatin extraction with subsequent extractions applied to the same source. As a consequence, the quality of gelatin within a process series was considered in the present experiment and so heat extraction was preliminary to any other experimental treatment. In the current study, two successive heat extraction temperatures were used to achieve different gelatin grades, with the best grades expected at the lowest temperatures. Results of the current study indicated that most of the heat-soluble BLG was released with extraction at 70°C, with addition of small amount of gelatin obtained with increased temperature (Table 1). The yield of heat soluble gelatin may have potentially been increased by heating the connective tissue at 70 or 80°C for longer than 1h, but the objective of the experimental design was to obtain a measure of relative yield and that was achieved.

The use of enzyme technologies for protein recovery and modification is widespread (Kristinsson and Rasco, 2000). Of the enzymes available for use in the extraction of gelatin, pepsin is the most attractive (Chomarat et al., 1994) because it is an exopeptidase that cleaves amino acids located only in the collagen telopeptides as the triple helical structure of collagen prevents binding of the enzyme to the remainder of the protein polypeptide (Zeng et al., 2012). This allows for the length of the α-chains to be retained, which is conducive to a high strength gel (Elharfaoui et al., 2007). Results of the present study showed that pepsin successfully increased the gelatin yield from BL following heat treatment (Table 1). Comparison of a continuous 48h pepsin extraction with two sequential 24h pepsin digestion indicated that yields increased with the addition of new pepsin. Addition of fresh pepsin may solubilize additional collagen by replacing pepsin that has undergone autolysis (Funatsu and Tokuyasu, 1959), thereby cleaving additional peptide bonds and resulting in an increase in gelatin extraction within the same extraction time (Balti et al., 2011; Jridi et al., 2013).

Proximate composition Few proximate composition differences existed between the gelatins extracted at the various steps of the extraction process, and the proximate analyses values for the gelatins were mostly comparable to those realized for the CBG and to those expected for gelatin (Table 1). Bovine gelatin contains 81.75 – 95.86% protein, 7.44 – 9.68% moisture, 0.21 – 0.24% fat and 0.29 – 1.16% ash (Balti et al., 2011; See et al., 2010; Sarbon et al., 2013). Notably, the protein content of BLG-F24h was lower than that of all the other extracted BLGs but was similar to that of Sarbon et al. (2013). Overall, moisture contents of the gelatins in the present study regardless of extraction method were well below the prescribed limit of 15% (GME, 2005) for edible gelatin. The ash contents of the BLG were in the range of 0.33 to 0.97% and these values were less than the recommended limit for food applications of 2% (GME, 2005). These results supported the conclusion that BLG was compositionally acceptable as a gelatin for food use.

Technological properties Generally, raw materials used to extract gelatin are pre-treated with dilute acid or saturated lime solution (concentrated calcium carbonate) and respectively produce...
two types of gelatin: Type A with pI range from 7 – 9; and Type B gelatin a pI of 4.8 – 5.1 (Cole, 2000). The pH of extracted gelatins in the present study varied from 4.73 to 5.57, indicating their category was Type B. Gelatin pH is an important technological characteristics as it has been reported that a pH of 5.0 is the most desirable for gelatin because at this pH gel viscosity is at a minimum but gel strength is at a maximum (Cole, 2000). In the present study, the pH values of the heat–extracted gelatins were lower than those of the pepsin-extracted gelatins most likely because the pepsin-extracted gelatin was treated twice with alkali (Table 2). All collagen was purified using alkali washes, but the gelatin extracted with pepsin was neutralized using alkali following pepsin extraction, resulting in two rather than one exposures to alkali, which may have elevated its pH values. It has been reported that alkali pre-treatment results in Type B gelatin with pH values in the range of 4 to 5 (Baziwane and He, 2003), and the results of the present study concur.

The pI is defined as the pH at which the net charge of the amphoteric molecule in solution is zero and its solubility is at the lowest point (Schrieber and Gareis, 2007). The pI of BL gelatins in the present study ranged between 4.0 and 5.0 (Figure 2), which is as expected for Type B gelatin. The heat-extracted BLG had pI values lower than that of the 48h pepsin-extracted BLG and the CBG. The pepsin-extracted BLG-L24h showed the lowest pI values at pH 4, and this may have arisen due to increased deamidation of asparagine and glutamine with the addition of new acidic pepsin solution after an initial 24h of pepsin digestion (Eastoe and Leach, 1977).

The clarity of gelatin, as indicated in the present study by the transmittance of light through the gelatin solution and by the colour of the gelled product, is important in food applications when gelatin is used as a thickening agent so that the final product quality is not adversely affected by unintended colour or opacity (Jamilah et al., 2011). Low transmittance values for gelatin are commonly caused by contaminating inorganic salts, proteins and mucopolysaccharides (Avena-Bustillos et al., 2006) and removed during gelatin extraction and purification (Muyounga et al., 2004). In this study, filtration was the only process performed to remove impurities from the gelatin, with the exception of the commercial gelatin as clarification had already been performed (Muyounga et al., 2004). In the present study, BLG-80 produced the clearest gelatin, although the clarity was not comparable to that of CBG (Table 2). BLG-80 gelatin was the most clear of the BLG because any contaminants were most likely removed from the gelatin sediment during the 70°C extraction that preceded it. This suggested that heat treatment of connective tissue at a temperature lower than 70°C might assist with the removal of contaminants without extraction of gelatin, which would improve the quality of the gelatin subsequently extracted at 80°C.

Gelatins extracted from BL had higher L* and lower a* values than the CBG (Table 2), concomitant with their increased opacity.

<table>
<thead>
<tr>
<th>Components</th>
<th>BLG-70</th>
<th>BLG-80</th>
<th>BLG-F24h</th>
<th>BLG-L24h</th>
<th>CBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water holding capacity (WHC)</td>
<td>147 ± 0.77</td>
<td>147 ± 0.77</td>
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<td>147 ± 0.77</td>
<td>147 ± 0.77</td>
</tr>
<tr>
<td>Fat binding capacity (FBC)</td>
<td>1518 ± 231</td>
<td>1518 ± 231</td>
<td>1518 ± 231</td>
<td>1518 ± 231</td>
<td>1518 ± 231</td>
</tr>
<tr>
<td>Gelling temperature (°C)</td>
<td>24 ± 0.47</td>
<td>24 ± 0.47</td>
<td>24 ± 0.47</td>
<td>24 ± 0.47</td>
<td>24 ± 0.47</td>
</tr>
<tr>
<td>Bloom strength (g)</td>
<td>174 ± 6.74</td>
<td>174 ± 6.74</td>
<td>174 ± 6.74</td>
<td>174 ± 6.74</td>
<td>174 ± 6.74</td>
</tr>
<tr>
<td>Mean values within a row with different letters are significantly different at P &lt; 0.05 according to Student-Newman-Keuls multiple range test.</td>
<td>a,b,c</td>
<td>a,b,c</td>
<td>a,b,c</td>
<td>a,b,c</td>
<td>a,b,c</td>
</tr>
<tr>
<td>BLG-70 = BLG extracted at 70°C for 1 h; BLG-80 = BLG extracted at 80°C for 1 h; BLG-F24h = BLG extracted with pepsin for 24 h; BLG-L24h = BLG extracted with pepsin for a second 24 h; CBG=commercial bovine gelatin.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The a* values were observed to be highest ($P < 0.0001$) in BLG–F24h and BLG–L24h pepsin-digestions compared with other BLG. BLG-70 had a mean b* value lower than that of the CBG and pepsin-extracted BLG, while BLG-80 and those from pepsin digestion regardless of digestion time had comparable b* values ($P = 0.0003$). The results suggested that the L* values decreased and a* and b* values increased of BLG as extraction progressed. Colour of a gelatin is an important aesthetic property and a light colour is preferable because it facilitates incorporation of the gelatin into any food item. The increased b* values of the pepsin-extracted BLG may have been due to at least three heat extractions being conducted on it, which would increase the opportunity for Maillard reactions between protein and carbohydrate moieties (Schrieber and Gareis, 2007). This postulation is supported by the increase in the mean b* values with each progressive extraction step. Also Poppe (1997) reported that gelatin solutions had their lowest transmittance at the pI because proteins aggregated and excluded water at the pI. No relationship was apparent between transmittance and pI in the current study, however, suggesting that this was not a factor contributing to the results observed.

WHC and FBC, gelling and melting temperature, storage and loss moduli, and gel strength measurements (Table 2) indicated that highly functional gelatin was obtained in BLG-70. The WHC of gelatin is a desirable trait in foods such as sausages, custards and dough because these products require the gelatin to soak in water without dissolving, thereby acting as a thickening agent and increasing product viscosity (Rawdkuen et al., 2013). In ground meat formulations, high FBC of gelatin is desired because it helps to retain flavour, improve palatability, and extend the shelf life of meat products (Rawdkuen et al., 2013). The BLG-80 had a higher mean WHC than gelatin extracted at all other steps and the CBG but a lower mean FBC ($P \leq 0.0001$) than BLG-48h or BLG-L24h (Table 2). Also, BLG-F24h showed the lowest WHC compared to that from all other extraction methods and CBG. BLG-48h had the greatest FBC (Table 2). FBC is postulated to be associated with the proportion of available hydrophobic, electrostatic and hydrogen bonds (Lawal, 2004), and particularly with hydrophobic amino acid residues such as tyrosine, leucine, valine and isoleucine (Ninan et al., 2011). No associations between FBC and amino acid content were noted in the present study, nor were there any differences in calculated non-polar hydrophobic and polar uncharged amino acid contents (Table 3).

The G’ and G” curves of the BLG-70 gelatin as a function of temperature during cooling indicated that this gelatin could form gels at higher temperatures than CBG and gelatins from the other extractions, suggesting that it was the most stable (Figure 3). Also, with subsequent heating, the G’ and G” curves of the BLG-70 gelatin gels began to decrease at higher temperatures than the gelatins from the pepsin extractions, substantiating the increased thermo-stability of this gelatin. Melting and gelling temperatures were also highest in BLG-70 (Table 2), indicating that this gelatin would have the greatest range of applications (Gudmundsson,
Table 3. Amino acids profile (mean % mol ± standard error of the mean) of bovine lung gelatins (BLG) extracted with various methods

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>BLG-70</th>
<th>BLG-80</th>
<th>BLG-F24h</th>
<th>BLG-L24h</th>
<th>BLG-48h</th>
<th>CBG</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>9.80 ± 0.23</td>
<td>9.96 ± 0.53</td>
<td>9.90 ± 0.27</td>
<td>10.44 ± 0.17</td>
<td>10.37 ± 0.17</td>
<td>10.00 ± 0.36</td>
<td>0.1926</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.81 ± 0.21</td>
<td>5.49 ± 0.48</td>
<td>5.05 ± 0.23</td>
<td>5.07 ± 0.15</td>
<td>4.80 ± 0.15</td>
<td>6.00 ± 0.33</td>
<td>0.0037</td>
</tr>
<tr>
<td>Asparagine /Aspartic acid</td>
<td>5.53 ± 0.16 &amp; 6.13 ± 0.36</td>
<td>7.86 ± 0.18 &amp; 5.68 ± 0.12</td>
<td>5.96 ± 0.12</td>
<td>6.00 ± 0.25</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine/Glutamic acid</td>
<td>7.85 ± 0.18</td>
<td>7.91 ± 0.41</td>
<td>9.43 ± 0.21</td>
<td>7.96 ± 0.13</td>
<td>8.37 ± 0.13</td>
<td>8.00 ± 0.28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glycine</td>
<td>32.20 ± 0.48</td>
<td>32.62 ± 1.08</td>
<td>21.05 ± 0.54</td>
<td>31.37 ± 0.34</td>
<td>30.36 ± 0.34</td>
<td>32.00 ± 0.74</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.12 ± 0.15</td>
<td>1.02 ± 0.33</td>
<td>1.65 ± 0.17</td>
<td>1.01 ± 0.10</td>
<td>0.92 ± 0.10</td>
<td>1.00 ± 0.22</td>
<td>0.0247</td>
</tr>
<tr>
<td>Hydroxyproline (Hyp)</td>
<td>7.89 ± 0.26</td>
<td>8.14 ± 0.58</td>
<td>4.04 ± 0.29</td>
<td>7.22 ± 0.18</td>
<td>7.09 ± 0.18</td>
<td>8.00 ± 0.40</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.57 ± 0.10c</td>
<td>1.33 ± 0.22c</td>
<td>3.12 ± 0.11c</td>
<td>1.88 ± 0.07bc</td>
<td>2.00 ± 0.07bc</td>
<td>1.50 ± 0.18bc</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.99 ± 0.18c</td>
<td>2.62 ± 0.41b</td>
<td>5.98 ± 0.20c</td>
<td>3.25 ± 0.13c</td>
<td>3.54 ± 0.13c</td>
<td>3.00 ± 0.28c</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.68 ± 0.07bc</td>
<td>2.67 ± 0.15b</td>
<td>4.07 ± 0.07bc</td>
<td>2.69 ± 0.05bc</td>
<td>2.90 ± 0.05bc</td>
<td>3.00 ± 0.10bc</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.93 ± 0.10b</td>
<td>1.30 ± 0.21b</td>
<td>0.85 ± 0.10b</td>
<td>0.94 ± 0.07b</td>
<td>0.84 ± 0.07b</td>
<td>1.00 ± 0.14bc</td>
<td>0.3903</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.57 ± 0.08c</td>
<td>1.34 ± 0.18c</td>
<td>2.57 ± 0.09c</td>
<td>1.66 ± 0.06bc</td>
<td>1.74 ± 0.06bc</td>
<td>2.00 ± 0.12bc</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Proline (Pro)</td>
<td>11.55 ± 0.18c</td>
<td>11.42 ± 0.40c</td>
<td>9.38 ± 0.20bc</td>
<td>11.27 ± 0.12c</td>
<td>11.25 ± 0.13c</td>
<td>11.50 ± 0.29c</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serine</td>
<td>3.31 ± 0.09c</td>
<td>3.30 ± 0.20bc</td>
<td>4.20 ± 0.10c</td>
<td>3.31 ± 0.06bc</td>
<td>3.49 ± 0.06bc</td>
<td>3.00 ± 0.13bc</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.95 ± 0.10b</td>
<td>1.74 ± 0.22b</td>
<td>3.08 ± 0.11b</td>
<td>1.90 ± 0.07b</td>
<td>2.00 ± 0.07b</td>
<td>2.00 ± 0.15bc</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.61 ± 0.07c</td>
<td>0.60 ± 0.16c</td>
<td>1.56 ± 0.08c</td>
<td>0.76 ± 0.05c</td>
<td>0.76 ± 0.05c</td>
<td>1.00 ± 0.11bc</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Valine</td>
<td>2.66 ± 0.16b</td>
<td>2.39 ± 0.35c</td>
<td>6.24 ± 0.17c</td>
<td>3.57 ± 0.11c</td>
<td>3.62 ± 0.11c</td>
<td>3.00 ± 0.24bc</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Imino acids (Hyp + Pro)</td>
<td>19.43 ± 0.38</td>
<td>19.56 ± 0.85</td>
<td>13.42 ± 0.42</td>
<td>18.49 ± 0.27</td>
<td>18.33 ± 0.27</td>
<td>19.50 ± 0.59</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Non polar hydrophobic</td>
<td>27.07 ± 0.41</td>
<td>25.89 ± 0.93</td>
<td>27.79 ± 0.29</td>
<td>33.17 ± 0.46</td>
<td>27.64 ± 0.29</td>
<td>27.27 ± 0.54</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Polar unchanged</td>
<td>38.05 ± 0.33</td>
<td>38.27 ± 0.74</td>
<td>36.60 ± 0.23</td>
<td>29.88 ± 0.37</td>
<td>37.33 ± 0.23</td>
<td>37.73 ± 0.43</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

a,b,c Means within a row with different letters are significantly different at P < 0.05 according to Student-Newman-Keuls tests.

Notes: Means within a row with different letters are significantly different at P < 0.05 according to paired t-tests.

1 BLG-70 = BLG extracted at 70°C for 1 h; BLG-80 = BLG extracted at 80°C for 1 h; BLG-F24h = BLG extracted with pepsin for 24 h; BLG-L24h = BLG extracted with pepsin for a second 24 h; BLG-48h = BLG extracted with pepsin for 48 h. CBG = commercial bovine gelatin.

2002). Gel strength is one of the most important quality properties used in the gelatin industry to differentiate gelatins (Boran and Regenstein, 2010) and can be classified as low (< 150 g), medium (151 – 220 g) and high (221 – 300 g) (Johnston-Banks, 1983). The three dimensional hydrogen bond formations between the water molecules and free hydroxyxyl groups of amino acid in gelatin confer the strength and rigidity of the gelatin gel (Karim and Bhat, 2009). Other factors that contribute to gel strength are the interactions between imino groups, the ratio of α-chains, the amount of β-chains and the content of free hydroxyl group of amino acids (Arnesen and Gildberg, 2002). In the present study, heat-extracted gelatins had mean G’ and G” and gel strengths greater than or comparable to that of the CBG and greater than those of gelatins extracted using pepsin (Table 2). This may have been related to the imino acids content of the gelatin as gelling points are related to the proportion of proline and hydroxyproline in the original collagen molecule (Ledward, 1986; Gilsenan and Ross-Murphy, 2000; Ninan et al., 2011; Rawdkuen et al., 2013). According to Arnesen and Gildberg (2002), a high hydroxyproline content of gelatin is the major reason for high gel strength but this was only true in the present study for BLG-F24h gelatin, which had the lowest imino acids content and the lowest gel strength (Table 3). Gudmundsson and Hafsteinsson (1997), however, suggested that the gel strength may depend on the pI of gelatin and may be controlled to a certain extent by adjusting the pH. This conclusion was not supported, however, by the differences in gelling and melting temperatures and G’ and G” values between BLG-70 and BLG-80, which had similar pH values (Table 2).

Gel strength and melting characteristics of gelatin can also be affected by their molecular weight profile, as gelatins consisting of low MW peptides melt at lower temperatures than gelatins with high MW peptides (Gilsenan and Ross-Murphy, 2000). Silva et al. (2014) reported that increased proportions of β-chains and γ-components were associated with increased gel strength, while Gómez-Guillén et al. (2002) observed weak gelatin gels were associated with increased content of small peptide fragments. Ledward (1986) and Kittiphatthanabawon et al. (2010) reported that short α-chain peptides in gelatin could not form the electrostatic junctions required for a strong protein network, and this was evidenced by the lowered bloom strength those authors observed. All collagen fractions that have a minimum MW of 30 kDa can be considered gelatin (Eastoe and Leach, 1977) because collagen fractions with MW lower than 30 kDa are unable to form contiguous structures of sufficient length for gel rigidity (Boran and Regenstein, 2009). The protein MW profiles of gelatin characterized by SDS-PAGE in the current study support this
conclusion (Figure 4), and agree with the results of Gilsenan and Ross-Murphy (2000); specifically, BLG having increased contents of α-chains showed the best gel strength. This result directly agreed with that of Schrieber and Gareis (2007), who reported that gel strength, was mainly dependent on the proportion of α-chains and that of Nagarajan et al. (2012), who reported that squid gelatin with increased content of α-chains possessed improved functional properties and gel strength. The molecular components of BLG–70
and BLG–80, which had the highest gel strengths and were comparable to CBG, consisted primarily of γ-, β- and α-chains, while those of the BLG–L24h, which had the lowest gel strength, was mostly decreased β- and α-chains with increased low MW peptides (Figure 4). The results of this study agreed with those of Gómez–Guillén et al. (2002), who found that the fraction of high MW molecules (γ- or β-chains) in gelatin was positively correlated with gel strength.

Lengthening gelatin extraction processes to increase yield has been associated with decreased MW peptide size. According to Gómez–Guillén et al. (2002), extraction yield was inversely proportional to the quantity of β-components in fish gelatin samples. Kittiphattanabawon et al. (2010) also observed that gelatin extracted at prolonged high temperatures or after extensive processing exhibited decreased gel strength. The results of this study indicated that extraction at a high temperature for a short period produced the strongest gels while continued extraction with extended pepsin digestion negatively affected the gel strength of any additional BLG. In the present study, the initial thermal treatment may have disrupted the hydrogen bonds that stabilize the triple helical structure of the collagen newly–synthesized or weakly cross-linked molecules, resulting in conversion of this collagen to soluble gelatin. The first fraction of gelatin may therefore simply be collagen molecules in which the α–chains are released by thermal disruption of internal hydrogen bonds, while the later extracts are obtained as a consequence of hydrolysis or digestion of the collagen telopeptides (Ledward, 1986; Schrieber and Gareis, 2007). The increased content of low MW compounds in the pepsin-extracted gelatin was most likely the result of the increased hydrolysis of peptidyl bonds by the enzyme (Figure 4).

Foam stability in particular is an important property of gelatin when it will be used in foods such as marshmallows (Zufiga and Aguilera, 2009). The CBG standard had the highest foam stabilities at 0.5 and 2% gelatin concentrations and EAI values at the 1 and 2% gelatin concentrations (Table 4). Jellouli et al. (2011) reported that the higher foaming capacity of grey trigger fish gelatin compared to that of bovine gelatin was due to the increased content of hydrophobic amino acid contents (alanine, valine, isoleucine, leucine, proline, methionine, phenylalanine and tyrosine) in trigger fish gelatin. Results of the present study do not agree with Jellouli et al. (2011) as there was no clear pattern of differences in the hydrophobic amino acids between heat and pepsin extracted gelatins (Table 3). Foams with high concentrations of proteins have increased foaming densities and stabilities (Zayas, 1997) and emulsifying ability is governed by protein peptide size, with small peptides being more soluble and having a greater ability to form a film around an oil droplet than large peptides (Kittiphattanabawon et al., 2012). The ability of the CBG to do this would be enhanced by the increased proportion of low MW proteins observed during SDS–PAGE (Figure 4).

**Table 4.** Means with standard errors of the means for foaming and emulsifying properties of bovine lung gelatins (BLG) extracted using different methods.

<table>
<thead>
<tr>
<th>Components</th>
<th>Gelatin extraction methods</th>
<th>BLG-70</th>
<th>BLG-24h</th>
<th>BLG-80</th>
<th>CBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of gelatin (%)</td>
<td>2.0</td>
<td>38.52 ± 4.37</td>
<td>39.60 ± 9.78</td>
<td>44.88 ± 4.37</td>
<td>38.40 ± 9.99</td>
</tr>
<tr>
<td>Foam Expansion (%)</td>
<td>1.0</td>
<td>37.20 ± 4.19</td>
<td>50.40 ± 9.12</td>
<td>47.60 ± 4.80</td>
<td>43.70 ± 3.39</td>
</tr>
<tr>
<td>Foam Stability (%)</td>
<td>0.5</td>
<td>31.98 ± 4.46</td>
<td>35.80 ± 9.90</td>
<td>29.78 ± 4.03</td>
<td>31.94 ± 3.20</td>
</tr>
<tr>
<td>Emulsifying Index (%)</td>
<td>0.5</td>
<td>26.72 ± 4.03</td>
<td>40.00 ± 10.14</td>
<td>38.52 ± 4.53</td>
<td>35.94 ± 2.30</td>
</tr>
<tr>
<td>Stability Index (m/ g)</td>
<td>0.5</td>
<td>10.44 ± 3.50</td>
<td>24.50 ± 8.20</td>
<td>27.52 ± 3.49</td>
<td>27.80 ± 3.20</td>
</tr>
<tr>
<td>Emulsifying Activity Index (minutes)</td>
<td>0.5</td>
<td>74.20 ± 3.80</td>
<td>84.00 ± 31.67</td>
<td>71.00 ± 13.67</td>
<td>75.00 ± 13.67</td>
</tr>
</tbody>
</table>

Means within a row with different letters are significantly different at P < 0.05 according to Student-Newman-Keuls test.

1. BLG–70: BLG extracted at 70°C for 1 h; BLG-24h = BLG extracted with pepsin at 80°C for 24 h; BLG–80 = BLG extracted with pepsin at 48 h; CBG = commercial bovine gelatin.

2. Means within a row with different letters are significantly different at P < 0.06 according to Student-Newman-Keuls test.
Conclusions

Based on the results of this study, BL is a promising source for low yield heat extraction of high quality gelatin with gel strengths suitable for use as a stabilizer in dairy products, frozen foods, confections and baked goods. Pepsin digestion for 48h after initial heat extraction can improve gelatin yield, although subsequent gelatin quality is not equivalent to that realized using heat extraction. It is however of a quality suitable for soft gelatin capsules, tablets and technical applications such as bacterial growth media.

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