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Physicochemical Evaluation and Spectroscopic Characterisation of Gelatine from Shank and Toes of *Gallus gallus domesticus*

(Penilaian Fizikokimia dan Pencirian Spekstroskopik Gelatin daripada Kaki dan Jari Kaki *Gallus gallus domesticus*)

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ABSTRACT

Growing needs, inadequate supply along with health and religious issues are reasons for the increase in the demand for an alternative source of gelatine in the food and pharmaceutical industries. This study was conducted to optimize the process for producing halal-compliant gelatine from chicken waste; chicken shank and toes (CST). Double extraction process employed produced gelatine which was vitreous, lightweight and gossamer in appearance. Gelatine yield was 9.52%(w/w) with a pH of 3.85, moisture content of 7.17%, total protein content of 93.77%, total fat content of 0.93% and total ash of 1.57%. The Bloom strength of the CST gelatine (148.33 ± 5.51) was found to be slightly lower than the commercially available bovine gelatine (BS) (169.33 ± 58.53) (P > 0.05). Amino acid analysis showed that the CST gelatine (91.38 ± 1.01%) was comparable to BS gelatine (90.65 ± 1.56%)(P > 0.05). Double extraction has been shown to successfully increase the surface area to volume ratio of CST waste resulting in increased yield in gelatine and protein with lower total fat content obtained. The test results obtained showed that the CST gelatine produced through this method complies with pharmaceutical standards.

Keywords: Chicken shank; extraction; food and pharmaceutical industries; gelatine; protein

ABSTRAK

Keperluan yang meningkat dan bekalan yang terhad di samping isu kesihatan dan keagamaan merupakan penyebab permintaan yang tinggi untuk sumber alternatif gelatin dalam industri makanan dan farmaseutik pada masa ini. Kajian ini telah dijalankan untuk mengoptimumkan proses penghasilan gelatin halal daripada bahan sisa penyembelihan ayam iaitu bahagian kaki dan jari kaki ayam (CST). Proses dwi-pengekstrakan telah digunakan untuk menghasilkan gelatin yang bersifat lutsinar, ringan dan berserat umpama kaca. Jumlah gelatin CST yang diperoleh adalah 9.52% (berat/berat) dengan pH3.85, kandungan lembapan 7.17%, jumlah kandungan protein 93.77%, jumlah kandungan lemak 0.93% dan jumlah abu 1.57%. Kekuatan Bloom gelatin CST (148.33 ± 5.51) didapati lebih rendah daripada gelatin kulit lembu (BS) (169.33 ± 58.53) yang boleh didapati secara komersial di pasaran (P > 0.05). Analisis asid amino menunjukkan bahawa gelatin CST (91.38 ± 1.01%) adalah setanding dengan gelatin BS (90.65 ± 1.56%)(P > 0.05). Proses dwi-pengekstrakan didapati berjaya meningkatkan nisbah luas permukaan kepada isi padu CST menyebabkan jumlah hasil gelatin dan kandungan protein lebih tinggi serta kandungan lemak gelatin yang berkurang diperoleh. Ujian yang dijalankan telah menunjukkan bahawa gelatin CST yang dihasilkan melalui kaedah ini mematuhi piawaian farmaseutik.

Kata kunci: Gelatin; industri makanan dan farmaseutik; kaki ayam; pengekstrakan; protein

INTRODUCTION

In recent years, the strong global demand for an alternative source of gelatine has been driven by health, supply and religious issues. The primary raw materials required for gelatine production are animal bones, tissues and skin. Current sources of gelatine, namely bovine and porcine, are associated with health hazards and religious sentiments (Abdullah & Ireland 2012). Incidents of allergic reactions to bovine gelatine have occurred in individuals given vaccines for measles, mumps and rubella (MMR), varicella, yellow fever, rabies and influenza (Bourne, 1978 #382)(Johnson & Peebles 2004). The outbreak of Bovine Spongiform Encephalopathy (BSE) or Mad Cow Disease has also caused concern for the safety of bovine tissue-derived collagens and gelatines (Budka et al. 2006). Gelatine, a biopolymer, is widely used as hydrocolloids in food products and as an excipient in pharmaceutical, nutraceutical and cosmeceutical formulations. In food manufacturing especially in confectioneries, gelatine is utilized to provide chewiness, texture and foam stabilization. It is also used in the production of low-fat spreads as well as to provide creaminess and mouth feel. Comparatively, in dairy products it offers texture and stabilization, whereas in bakery products it is used as emulsifying and gelling agents. In meat products it provides water binding characteristics due to its high protein content

and low calories. Gelatine is recommended to enhance protein levels, particularly in an athletic diet such as body-building food. Whereas in pharmaceutical industry, it is an indispensable component in the manufacture of both hard and soft capsules, wound care products and plasma expanders for the treatment of hypovolaemic shock or burns. Moreover, its other function is as coating material or suspending agent, stabilizers in injections, tablet binder, matrix for tissue engineering and implants (Elzoghby 2013; Young et al. 2005). The type of gelatine used for these different pharmaceutical applications requires different physical properties and hence additional specialized processing techniques.

Religious standards have an important influence on the consumption of food and medicines. Currently, the available commercial sources for gelatine production might not meet the *halal* (Muslim) and *kosher* (Jewish) standards for consumption (Nur et al. 2010; Van der Spiegel et al. 2012). Other religions also impose particular inhibitions pertaining to food and medicines, for example, followers of Hinduism and Buddhism do not consume bovine-based gelatine products. The need to explore new alternative sources of gelatine that comply with health and religious standards is ever increasing and very challenging (Shah & Yusof 2014). The number of research focusing on solving the environmental problems of livestock has increased. Studies which aimed to utilize waste material from livestock have been carried out. Examples of livestock waste materials include skin of various fish and unconsumed by-products (such as skin) from poultry industries. These waste materials are economically and technologically viable sources for the extraction of collagen or gelatine. Chicken shank and toes (CST), one of the main unconsumed by-products of chicken processing, contains high levels of protein. Consequently, it was realized that these could be utilized for the production of halal gelatine (Liu 2002; Sarbon et al. 2013; Shiying 2005). There has been a steady annual increment of nearly 3 - 8% production growth in the South-East Asia poultry industry. In Malaysia, chicken meat is one of the most consumed food with per capita consumption rate of 33.2-40 kilogram per year from 2007-2014. Hence with the average growth of 1.5-2% in per capita consumption, it is projected that the poultry meat production will rise to 5-13.4 % by 2017-2018 (Abdul & Rittgers 2014; Statistics 2012). This tremendous growth in poultry production is mainly due to the transformation of consumer preference which resulted in an increase in the demand of poultry livestock and by-products (Smith et al. 2010; Windhorst 2006). Chicken meat demand has increased due to the versatility of the meat, relatively low cost in comparison to other meat products, acceptance of chicken meat to most religions and the perception that poultry meat is healthier than other meat products (Jayaraman et al. 2013; Norimah et al. 2008). Processing of chicken would lead to enormous amounts of by-products such as skin, bones and shank, which could be utilized as essential alternative sources for gelatine production. Synergistically, this would help to effectively manage the poultry industry by-products and preserving the environment (Jayathilakan et al. 2010; Lasekan et al. 2013; Rafieian et al. 2013).

Two main gelatine extraction processes utilized commercially are either the acidic process (type A) or the alkaline process (type B). In acidic process, the raw material is treated for 24 h for five days in 1-4% hydrochloric acid with pH maintained at less than 1.5. Alternatively in the alkaline process, the raw material are treated with lime, potassium or sodium carbonate to attain a pH above 7 for several weeks (Totre et al. 2011). Researchers have studied various experimental conditions using other parts of chicken by-products and treating them with different types of acids and its concentrations, also applying various treatment time from 6 days to 3 weeks (Almeida & Lannes 2013; Du et al. 2013; Lim et al. 2002; Liu & Xu 2004; Liu et al. 2001; Park et al. 2013; Prayitno 2011). This study investigated the employment of the double extraction method which is a simpler and less time consuming method of extraction which produced higher yield of pure, vitreous grade gelatine extract from by-products of halal slaughtered chicken waste (CST). Characterization of the physicochemical attributes of CST gelatine was made and compared to the commercially available bovine skin (BS). Positive results from the optimized extraction technique can initiate industrial production of gelatine from alternative sources such as CST in the future.

Materials and Methods

Halal CST were purchased from a local Muslim wholesaler, Laman Aqua Tani (Reg. No.: 001863146-V). For comparative studies bovine gelatine type B (Batch No: 126K00531) and β-mercaptoethanol (β-ME) were procured from Sigma Life Science (USA). Spectra™ multicolour high range protein ladder were purchased from Thermo Scientific (Rockford, USA). Folin-Ciocalteu's phenol reagent, hydrochloric acid and sodium hydroxide 0.1 N were obtained from Merck Malaysia Ltd. Sodium dodecyl sulphate (SDS), Coomassie Blue R-250 and N,N,N',N'-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents/chemicals used were of analytical grade and used as received.

Extraction of Chicken Shank and Toes Gelatine (CST)

Gelatine from CST was obtained using an improved extraction and purification process which is based on previously described conventional procedures found in the literature (Gomez-Guillen et al. 2009; Lim et al. 2002; Nicolas-Simonnot et al. 1997; Sarbon et al. 2013). About 600-620 g of CST was weighed (using Mettler-Toledo PB 3001-S, Switzerland weighing machine) and washed with water to remove the debris. The CST was chopped into small pieces and later washed several times with distilled water to remove any blood from the bone marrow and

other unwanted residues. It was then further crushed using a laboratory blender (Warning Commercial Laboratory Blender Model 32BL79 New Hartford, Connecticut, U.S.A) for about 1-3 min, till it turned into a fine mass (semi-solid portion). Next, the mashed CST was washed again with distilled water to further remove any remaining blood and debris.

The finely crushed mass was then soaked in 2 L of hydrochloric acid (0.5 N). It was continuously stirred at room temperature (25 ± 2°C) using a magnetic stirrer (Corning Laboratory Stirrer/Hotplate Model PC-620, U.S.A) for 1 h. During this treatment the wet ossein which is the starting material for gelatine was formed. The ossein formed was filtered and washed several times with distilled water. This careful washing was carried out to remove the remaining acid in the ossein and other impurities. Next, 600 mL distilled water was added to the wet ossein and heated at different temperatures (55 ± 2°C or 45 ± 2°C) with continuous stirring, using a Corning Laboratory Stirrer/Hotplate (Model PC-620, U.S.A) for one hour. During the heating, pH was adjusted by adding 0.5 mol/L sodium hydroxide (3-5 drops). The mixture was then filtered and the filtrate was collected and further filtered subsequently using a piece of muslin cloth.

The residue left was extracted again using the procedure mentioned earlier at a constant temperature of 65 ± 2°C for one hour. The pH of the clear supernatant obtained was checked and before freezing it at -80 ± 2°C (Electrolux Medical Refrigerator Model MRF 401/86, Luxembourg, Germany) for 4-5 days. The clear frozen supernatant was lyophilized using a Labconco Freeze Dry System (Model 77530-11, England) in a vacuum with an environment of 400×10^{-3} Mbars at -50 to -41°C for 100 h.

YIELD CALCULATION

The yield of gelatine was calculated based on wet weight of the crushed halal CST using the following formula:

$$Yield\ of\ gelatin\ (\%) = \frac{Weight\ of\ freezed\ dried\ (lyophilized)\ gelatin}{Wet\ weight\ of\ crushed\ CST} \times 100. \quad (1)$$

Identification Test Chemical tests were carried out to determine the presence of gelatine in the sample.

Identification gelatine content test Gelatine content was identified according to USP. Gelatine sample (1 g) was dissolved in 100 mL of hot water (35-40°C). To this, 10 mL of 3 N HCL was added. Next, 10 mL of 0.2 M potassium dichromate TS was added to the mixture. The presence of gelatine was identified qualitatively using standard colourimetry. Yellow precipitation indicates the presence of gelatine.

Identification of physical changes of gelatine Visual examination of the gelling behaviour of gelatine sample was carried out as outlined by the Gelatine Manufacturers Institute of America (GMIA 2012). Gelatine sample (1 g) was dissolved in 10 mL of hot water (35 - 40°C) and the solution was placed in a refrigerator at 2-10°C for 4 h. The gelled solution was then removed from the refrigerator and the container was placed in a water bath where the temperature was maintained at 60° ± 2°C. The unique interchangeable physical characteristics of gelatine under the influence of temperature (i.e. from gel state to liquid and back to gel state) were duly noted.

PROXIMATE ANALYSIS

Determination of Moisture Content The moisture content was determined using the Mettler Toledo HR 73 Halogen Moisture Analyzer (Mettler Toledo, Switzerland). Gelatine sample (3 g)(W_1) was evenly spread over the sample pan, weighed and recorded (W_2) heated to 105 ± 2°C using the Switch-Off Criterion 3 until dry. Uniform weights recorded (2) after successive weighing (average W_3) indicate complete removal of moisture.

$$Moisture\ Content\ (\%) = \frac{(W2 - W3)}{W1} \times 100. \quad (2)$$

Ash Content The test was carried out as outlined by the Gelatine Manufacturers Institute of America (GMIA 2013). Firstly, the crucibles were ignited in a muffle furnace at 550 ± 25°C for an hour. The crucible was removed from the furnace and was let to cool at room temperature (25° ± 2°C) in a desiccator. The empty crucible was weighed. 3 g of gelatine (W_1) was weighed in the crucible, the weight of sample and crucible was recorded (W_2). The gelatine samples were distributed evenly in the crucible and were charred on a heating mantle until no smoke evolved. Then it was transferred into a muffle furnace and heated to 550 ± 25°C (about 6 h) until the ash was grey in colour. The crucible and gelatine ash was weighed again (W_3). The content of ash was calculated as:

$$Ash\ (\%) = \frac{(W2 - W3)}{W1} \times 100. \quad (3)$$

Total Protein Content The total protein measurement was carried out using the Automated Kjeldahl Analyzer and Foss Tecator Digestion System (Model 2020/2300) based on the Kjeldahl method (AOAC 1995; Persson et al. 2008) for the analysis of total nitrogen content as a marker, using conversion factor of 5.4 to estimate protein content.

Total Fat Content The total fat content determination was carried out using the Soxtec 1047 System Hydrolyzing Unit and FOSS Soxtec Avanti 2055 Manual Extraction Unit. Celite 566 was added into glass thimbles (1 g in each thimble). Then 1.5 g ± 0.01 g of control sample was weighed into a glass thimble. In another glass thimble 1.5 g ± 0.01 g of the measured sample gelatine was weighed into it. The sample weight (W_1) was recorded. Empty aluminium extraction cups were weighed (W_2) and

extraction cup weight after fat extraction and cooling in desiccator (W_3). The total fat content was calculated as:

$$Fat\ (\%) = \frac{(W3 - W2)}{W1} \times 100. \quad (4)$$

pH DETERMINATION

The pH value for gelatine was determined using the Seven Easy pH Meter S20 (Mettler Toledo, Switzerland) (British Pharmacopoeia 2010). Gelatine solution 1% (w/v) of was prepared by dissolving 1 g of the gelatine in distilled water (100 mL) at 55° ± 2°C in water bath. The gelatine solution was then allowed to cool to room temperature (25° ± 2°C). Once the solution was in equilibrium with room temperature, pH of the gelatine solution was measured with the pH meter after standardizing the pH meter with buffers at pH 4.01, 7.0 and 9.21, respectively.

DETERMINATION OF GEL STRENGTH

The gel strength was carried out using QTS Texture Analyser (CNS Farnell, England) with a cylinder probe of 11.8 mm, as outlined by the (British Pharmacopoeia 2010). Gelatine samples were weighed into the Bloom bottles (7.5 g in each bottle) and 105 mL of distilled water were added to a final concentration of 6.67% (w/v). The mixture was swirled and let to stand for 30 min at room temperature (25 ± 2°C) to allow the gelatine to absorb water and swell. The Bloom bottles were then transferred to a water-bath maintained at 42 ± 2°C and were held for 30 min during which they were gently stirred with a glass rod intermittently. The sample bottles were removed from the bath and were allowed to cool at room temperature for 15 min. The bottles were sealed with rubber stoppers and were transferred to a thermostatically controlled bath at 10.0 ± 0.1°C and left for 17 ± 1 h before the gel strength was determined. The Bloom gel strength (g) was determined with the texture analyser set to make 10 mm depression at a rate of 0.5 mm/s at room temperature (25 ± 2°C). The Bloom bottles were placed in the centre on the platform of the apparatus so that the plunger contacts the sample as nearly at its midpoint as possible and the measurement were obtained (average of double measurements each).

VISCOSITY TEST

The viscosity test was carried out by using Brookfield DV III Ultra Rheometer (Model RV, USA), as outlined by the Brookfield Engineering Labs and Gelatin Manufacturers Institute of America (GMIA 2012). Gelatine solution 6.67% (w/v) was prepared by dissolving 7.5 g of the gelatine sample in distilled water (105 mL) at 55°C in water bath. The viscosity (in cP units) was determined with the above equipment, equipped with a CPE 41Z spindle and a sample cup CPE-44PSYZ. The test was carried out at 60 ± 2°C temperature within the spindle rotating range of 240 - 250 rpm. The data were obtained using Rheocalc V3.2 software.

SURFACE MORPHOLOGY

Gelatine samples were loaded into the chamber of the scanning electron microscope (Leica S440 SE Microscope, United Kingdom). Gelatine sample (0.1 mg) was loaded into the vacuum chamber by pumping gas molecules out of the chamber. Once the vacuum was ready, the beam was turned on and detector was selected. Accelerating voltage was set at 10.0 kV and probe current at 20 pA. The brightness and contrast of image was adjusted. The image was focused at working distance between 8 to 11 mm until sharp image was obtained at different magnification (Zhang et al. 2011).

THERMAL ANALYSIS

The thermal analysis was carried out using the differential scanning calorimetric, DSC (DSC 6000 Perkin Elmer, U.S) based on method by Noordin & Chung (Noordin & Chung 2004). It was connected to a chiller and a thermal gas station to control the flow of the purged gas. The DSC was set with nitrogen as the purging gas at a flow rate of 20 mL/min. Hermetically-sealed aluminium 20 μL pans were used. Indium and zinc were used to calibrate the DSC. Each samples weighed between 1.0-1.7 mg were scanned from -20°C to 370°C at a heat flow rate of 10°C/min. The heat of fusion (ΔH), glass transition and melting point were determined from the resulting thermo gram.

ANALYSIS OF AMIDES BY FOURIER TRANSFORM INFRARED (FTIR) SPECTRA

FTIR spectra of freeze-dried CST gelatine sample were recorded using a horizontal GladiATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) equipped with a Perkin Elmer Spectrum FT-IR/FT-Fir spectrometer (PerkinElmer, Inc., Waltham, MA, USA) at room temperature (25 ± 2°C). The samples were analysed by placing it on the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra were rationed in the range of 400 - 4000 cm^{-1} and automatic signals gained were collected at a resolution of 4.00 cm^{-1} against a background spectrum recorded from the clean empty cell at room temperature (25 ± 2°C). The number and location of bands were analyzed and registered in transmission/transmittance mode with resolution of 2 cm^{-1}.

DETERMINATION OF MOLECULAR WEIGHTS OF DIFFERENT AMINO ACIDS BY SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The extracted gelatine protein patterns were determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and Tris/HCl/glycine were used as a buffer system (Mahmoodani et al. 2012). The samples (1 g) were dissolved in 10 mL of 10% (w/v) SDS solution and then heated at 90 ± 2°C for 5 min. Supernatants were mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) βME) at the

ratio of 1:1 (v/v). The mixture was boiled for 5 min. Protein samples (15 µg) were loaded onto the polyacrylamide gel made with a 7.5% (v/v) running gel and a 4% (v/v) stacking gel and then subjected to electrophoresis at a constant voltage of 40 mamp for 1.5 h (power supply- Hoefer PS 300-B) using a Multiple Gel Castor – Hoefer SE250 (Hoefer.Inc, San Franciso, CA, USA). The loaded volume was 14 µL in all lines. After electrophoresis, the gel was removed and stained with 0.025% (w/v) Coomassie blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid and destained with 40% (v/v) methanol and 10% (v/v) acetic acid. Bovine skin (BS) gelatine was used as a reference standard. The protein marker Spectra ™ Multicolor High Range Protein Ladder (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) ranged from 40 to 300 kDa was used.

POLYPEPTIDE PROFILE OF THE AMINO ACIDS

The gelatine were weighed approximately within the range of 0.1 to 0.2 g and mixed with 5 mL of 6 N HCL. The gelatine solutions were hydrolysed in an oven at 110°C for 24 h. The chromatographic system consisted of High Performance Liquid Chromatography Waters (Model 2695, Massachusetts, USA) equipped with online degasser, auto injector and a multi-wavelength Waters fluorescence detector (Model 2475, Milford, Massachusetts, USA) was used. Waters Act Tag column (3.9 × 150 mm) was used with temperature for amino acid separation. The column was set at 31°C and the injection volume was 10 µL. The AccQ Tag^{tm} Eluent A concentrate and 60% acetonitrile were filtered using a 0.45 µm regenerated cellulose membrane filter prior to injection onto HPLC system. A flow rate was set at 1 mL/min. Data was acquired using Waters Empower Pro^{tm} software. The methodology was referred from method for hydrolyse amino acid analysis (Mahmoodani et al. 2012). Samples for cysteine analysis were oxidized by adding 0.1 g of each sample with 5 mL of performed acid before hydrolysis (0.1 g of each sample was hydrolysed with me of 6 N HAL in a closed test tube and then kept in oven for 24 h at 110°C). The analysis was carried in triplicate.

DETERMINATION OF PROTEIN BIOMARKERS BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION SPECTROMETRY (MALDI-TOF-TOF™)

Samples were analysed from modified procedure of "Pierce In- Solution Tryptic Digestion Kit" and using tandem time-of-flight mass spectrometer systems with reflectron mode (ABSCIEX 4800 PLUS MALDI-TOF-TOF™ Analyzer, US) that provides simultaneous identification and quantitation of low-abundance, protein biomarkers (DNA contents). The laser diode-pumped Nd: YAG at 355 nm, with pulse rate up to 200 Hz with <500psec duration/ pulse. The accelerating voltage was set at 20kv and nitrogen gas was introduced in the chamber of Collision-Induced Dissociation (CID) for fragmentation. The data were obtained by software GPS Explorer software version 3.6 (ABSCIEX, US). Gelatine samples (CST/BS gelatine) 2.5 mg were weighed and diluted in purified water. Trypsin (25 uL) in 100 mM ammonium bicarbonate was then added. Each gelatine sample vial was incubated at 37°C overnight for about 16 to 20 h. Then equal volumes of 1 mL 10mg/mL cinnamic acid matrix and 1 mL digested gelatine were added in another vial. The mixture was then vortexed using a vortex mixer (Vortex IKA, USA) at speed of 1000 rpm. The sample mixture (0.7 uL) was then pipetted out and spotted on the MALDI plate.

STATISTICAL ANALYSIS

All measurements were analyzed in triplicates and the probability value of $P < 0.05$ was considered as significant. Descriptive statistics, one way Anova and non-parametric test was applied using SPSS 20.0 (IBM SPSS, Chicago, IL, USA). The median values of CST and BS gelatine for pH and bloom strength were compared using the Mann Whitney test in the above mention program. Median min and max values was applied to present numerical variables.

RESULTS AND DISCUSSION

EXTRACTION OF CST AND YIELD

The yield and properties of gelatine depend on source, pre-treatment and process used in its preparation. The CST gelatine extraction was carried out using the acidic method with HCL (0.5 M), at different temperature. The method of extraction showed an enhance yield as the collagen rod formed from treatment solubilizes without changing its original triple-helix structure. Amidst extraction in water with the presence of thermal application resulted in the disruption of hydrogen and covalent bonds, thus destabilizing the triple helix through a helix-to-coil transition and converting it into gelatine (Mahmoodani et al. 2012). Table 1 indicates double extraction at initial temperature of 45 ± 2°C for first extraction followed by a second extraction at 65 ± 2°C was better compared to single extraction at 55 ± 2°C and or 45 ± 2°C. The average percentage yield by double extraction was about 9.52 ± 0.18% (w/w) which was significantly more compared to the yields from single extractions at two different temperature i.e 55 ± 2°C and 45 ± 2°C ($P < 0.000$). The moderately higher gelatine yield quality may also be due to the reduction of particle size of CST waste during the chopping and crushing process, thus increasing the surface area of CST waste to volume ratio during treatment stage in the optimized extraction method. In contrast to other sources being researched mainly marine gelatine, the extraction yield from the different fish skins ranged about 5.5-21% of the weight of raw material (Herpandi et al. 2011; Mahmoodani et al. 2014; Sarbon et al. 2013). Therefore, this moderate yield in CST gelatine (Table 2) could be due to incomplete hydrolysis or loss of collagen during washing process.

TABLE 1. Comparison of extracting chicken shank and toes (CST) gelatine by single or double extraction method

	Single CST extraction 55°C (*n*=3)	Single CST extraction 45°C (*n*=3)	Double CST extractionn45°C/65°C (*n*=3)	P value[b]
Yield (g)	24.06 ± 0.07[a]	25.89 ± 0.49[a]	62.84 ± 10.98[a]	0.000
% Yield	3.85 ± 0.09[a]	4.33 ± 0.02[a]	9.52 ± 0.18[a]	0.000

[a] Mean (SD)
[b] One Way Anova test

TABLE 2. Physical characteristics between chicken shank and toes (CST) gelatine (double extraction method) and bovine skin (BS) gelatine

	Chicken shank and toes (CST) gelatine (*n*=3)	Bovine skin (BS) gelatine (*n*=3)	z[b]	P value[b]
Yield (g)	62.84 ± 10.98[a]	NA	-	-
% Yield	9.52 ± 0.18[a]	NA	-	-
pH	3.85 ± 0.12[a]	4.94 ± 0.17[a]	-2.121	0.034
Bloom strength (g)	148.33 ± 5.51[a]	169.33 ± 58.53[a]	-1.000	0.317

[a] Mean (SD)
[b] Mann Whitney test

IDENTIFICATION OF GELATINE

In identifying the gelatine content, it was found that a yellow precipitate developed upon the addition of potassium dichromate to the acidified CST gelatine solution. This yellow precipitate is due to hydroxyproline which confirms the presence of gelatine. Concurrently, the gelling behaviour of gelled CST gelatine solution reverted to the original liquid state when heated and stirred in the water bath at temperature of 60°C. This unique behaviour signified the presence of gelatine in the CST gelatine sample (GMIA 2012).

PROXIMATE ANALYSIS

The proximate analysis of chicken leg gelatine showed comprised 93.77 ± 2.99% protein and 0.93 ± 0.15% fat. The high protein content showed that the gelatine obtained is highly untainted. Both the protein (<15) and fat ≤ (< 2) contents were within an acceptable range and could be reduced further by carrying out degreasing process before extraction (Boran et al. 2010). Moisture and ash contents determined were 7.17 ± 1.94% and 1.57 ± 1.10%, respectively. The moisture content indicated the presence of moisture in the sample and this not only depends on the extent of drying, but also the introduction of humidity during handling and storage. Low ash content showed that pre-treatment process was efficient in demineralizing the bone.

pH

The pH of gelatine is characterized by their mode of manufacture such as alkaline or acidic. The pH of both CST and BS are acidic was due to the process involved. The difference of pH between the CST gelatine and BS gelatine were found to be significant (P<0.05 Mann Whitney test) (Table 2). Variation in pH could be contributed by the different types of chemicals at different concentration used during the pre-treatment (acid or alkaline). Whereby usually Type A gelatine (pH3.8–6.0; isoelectric point 6–8) is derived by acidic hydrolysis of pork skin and contributes rheological properties to the blend. Type B gelatine (pH 5.0–7.4; isoelectric point 4.7–5.3) is derived by basic hydrolysis of bones and animal skin. It also showed that the washing steps place an important role in removing the acid and or alkaline residues. While during the formulation process in various industrial applications the pH will be altered in order to achieve the desired pH range (Singh et al. 2002).

GEL STRENGTH

Bloom or gel strength is a measure of hardness, stiffness, strength, firmness and compressibility of the gel at a particular temperature and is influenced by the concentration and molecular weight. The higher the Bloom value the stronger is the gel strength. Bovine skin (BS) gelatine (169.33 ± 58.53) showed a higher value as compared to CST (148.33 ± 5.51) (Table 2) and the values were found to be similar (P>0.05 Mann Whitney test). High Bloom strengths could be due to high molecular weight as it was well established that hydrogen bonds between water molecules and free hydroxyl groups of amino acid in gelatine are essential for the gelatine's gel strength. Presence of hydroxyproline was shown to produce higher gel strength of the gelatine (Sarabia et al. 2000). In this study, it was shown that BS has higher hydroxyproline content as compared to CST gelatine (Figure 6).

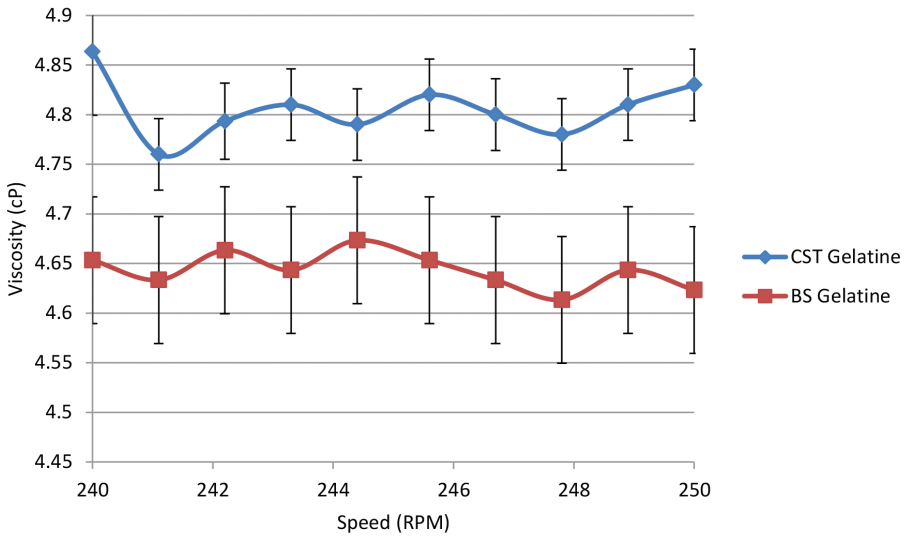
VISCOSITY TEST

Shear stress to shear rate data of gelatine solution were tested for various rheological models using the software provided along with a rheometer. Some researchers

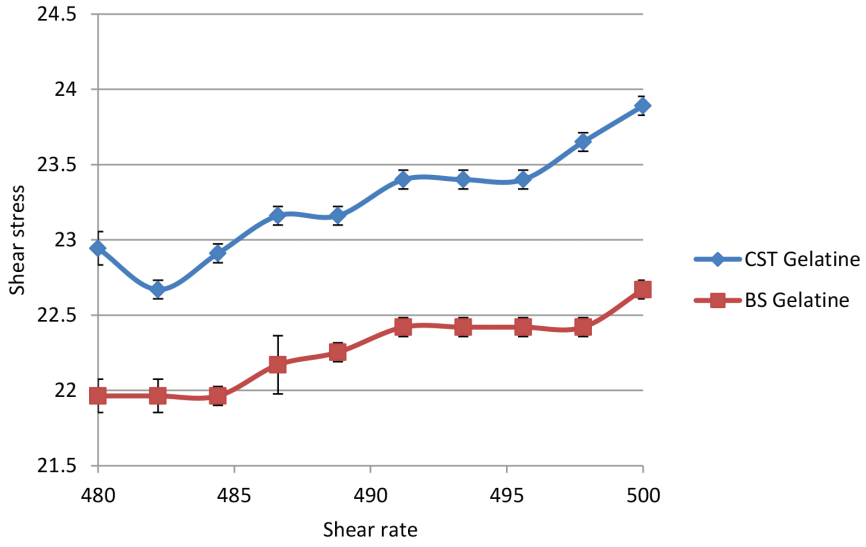
have reported a clear Newtonian behaviour for gelatine (Marcotte et al. 2001). The viscosity for the CST gelatine at 60°C was in the range of 4.76- 4.86 cP, while the BS gelatine was in the range of 4.61- 4.67 cP (Figure 1(a)). Viscosity of CST gelatine is found to be higher may be due to presence of impurities as the sample used is still in its crude nature and not filtered. The BS gelatine used as standard was a commercially available product hence has gone through refined filtration process. The high standard deviation shown was most probably due to the small number of samples used (n=3). Although the viscosity values of CST and BS gelatine samples were different, both of these gelatine products exhibit similar flow characteristic which are Newtonian (Figure 1(b)). Newtonian flow characteristic is shown when the shear stress measured increases proportionately to shear rate.

SURFACE MORPHOLOGY (SCANNING ELECTRON MICROSCOPY)

Scanning electron microscopy SEM showed highly ordered three dimensional scale-like structure of CST gelatin (Figure 2). Each scale consist of two distinct regions - an external (osseous) layer and an internal fibrillary plate. The flaky crystals of apatite in random orentation were observed in the outer layer (Zhang et al. 2011). The flake like structure is due to demineralisation and lyophilization of CST gelatin which makes it easy to dissolve in water faster when compared to powdered gelatin. Demineralised and lyophilized CST gelatin could thus be used in pharmaceutical applications due to its consistant solubility,dispersibility and viscous fluidity as shown in Figure 1(b).



(a)



(b)

FIGURE 1. Rheological properties of CST gelatine compared with BS gelatine (a) Newtonian properties of CST gelatine and BS gelatine (b)

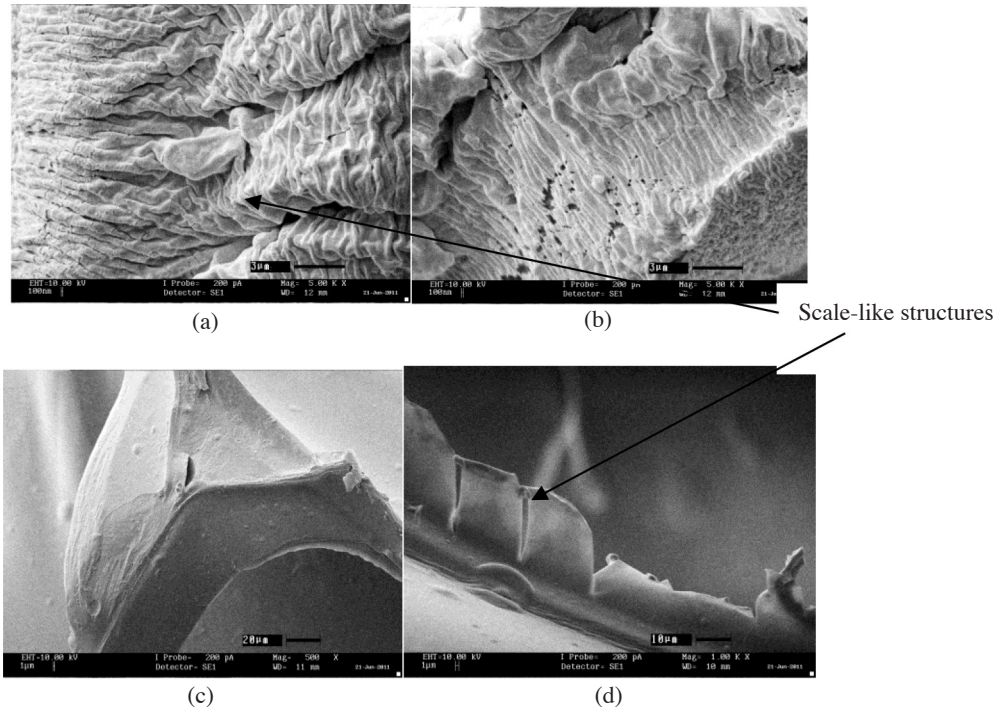


FIGURE 2. Chicken shank and toes (CST) gelatine by SEM Micrograph (a) & (b): the lyophilized CST gelatine and (c) & (d): perpendicular cut surface of CST gelatine (Note the scale-like structures)

THERMAL ANALYSIS

The DSC study (Figure 3(a)) for CST gelatine and BS gelatine showed almost comparable thermal characteristics of onset and peak melting points. BS gelatine showed a large initial mass loss followed by a mass plateau indicating evaporation of volatile components used during polymerization, drying and or desorption process. There was then a subsequent, mass increase in the presence of an interacting atmosphere at 249°C. In CST gelatine, evaporation of volatile components occurred at a lower temperature of 238°C. Some endothermic transformations of both CST gelatine and BS gelatine can be observed at 240° - 252°C. These transformations are clearly accompanied by irreversible chemical deterioration. When observed using a microscopic video camera attached to the DSC system, it was found that at the temperature corresponding to the smaller peak (201°C), CST gelatine began to vibrate or undergoes an endothermic transformation in the pan, without any physical changes (Figure 3(b)). At the peak temperature of 211 - 213°C, it showed clearly that the CST gelatine was undergoing denaturation (Figure 3(b) (g-h)) followed by degradation of the samples (Figure 3b(i)). The endothermic transition observed just before denaturation of the gelatine is believed to be the unfolding of the protein structure of the gelatine as shown in Figure 3(a) by a sharp peak difference between the CST gelatine and BS gelatine (Al-Saidi et al. 2012). This showed that the lyophilized lightweight dry form of CST gelatine contains pure amino acid as there is no evaporation of volatile components and it is an amorphous substance with long range melting point. At the peak temperature, CST gelatine undergoes straight denaturation or degradation. In DSC, the heat flow (or heat capacity) of a sample was measured as a function of temperature. The technique has been useful for studying the unfolding of secondary structures of protein molecules and for characterizing the conformational stability of proteins in different conditions, such as solid and liquid states. While protein unfolding produces endothermic peaks as measured by DSC, protein aggregation is detected as an exothermic event. With DSC the melting or denaturation temperatures (T_m) of different protein samples can be compared, giving insight to the differences in their secondary structures.

ANALYSIS OF AMIDES

The FTIR spectra of both gelatines indicated some differences in the peptide secondary structure at 3600-1900, 1700-800 and 600-400 cm^{-1} as shown in Figure 4. The main transmittance vibration peaks for CST gelatine are amide A (3287.16), amide B (2929.05), amide I (1628.32), amide II (1536.61) and amide III (1236.37) as compared to BS gelatine; amide A (3283.10), amide B (2943.95), amide I (1629.40), amide II (1528.37) and amide III (1237.30). Whereas for chicken feet gelatine, it was observed that vibration peaks for amide A (3399.56), amide B (2923.72), amide I (1652.01), amide II (1539.87) and amide III (1241.29) (Almeida et al. 2012) (Table 3(a)). Therefore, it was observed that amide A may change peak due to stretching with the CH_2 groups. The amide B peaks indicated the interaction of $-NH_3$ groups between

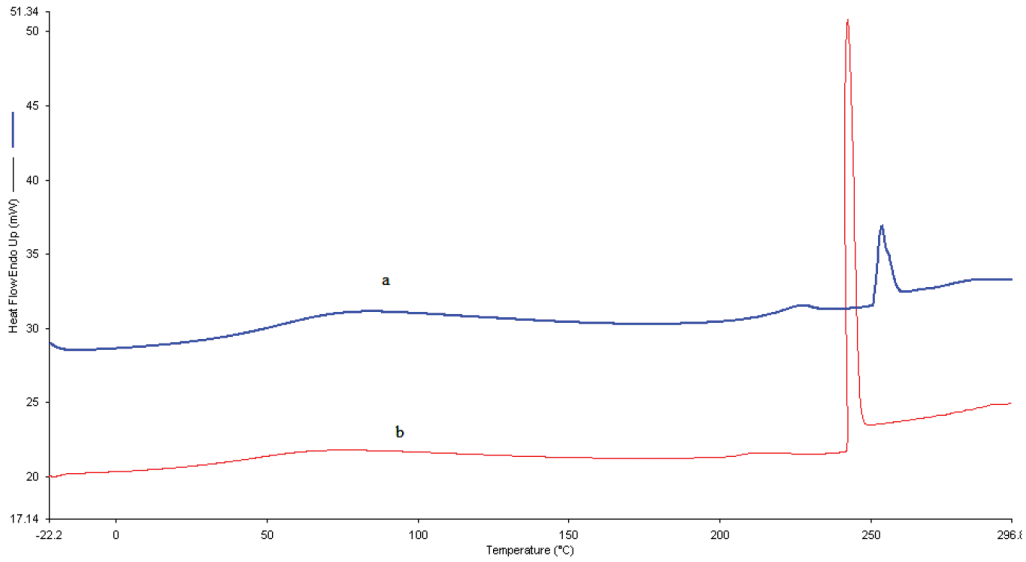
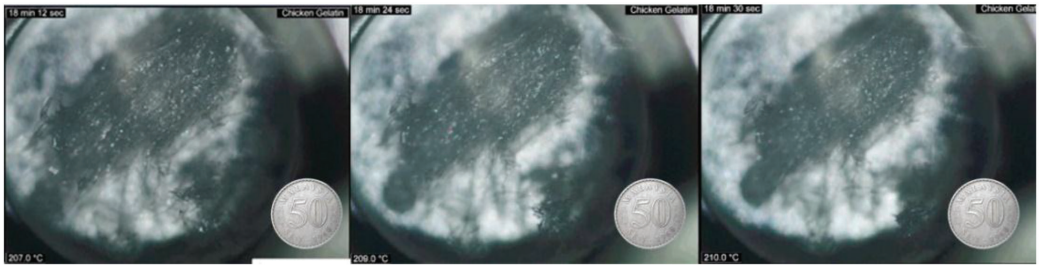
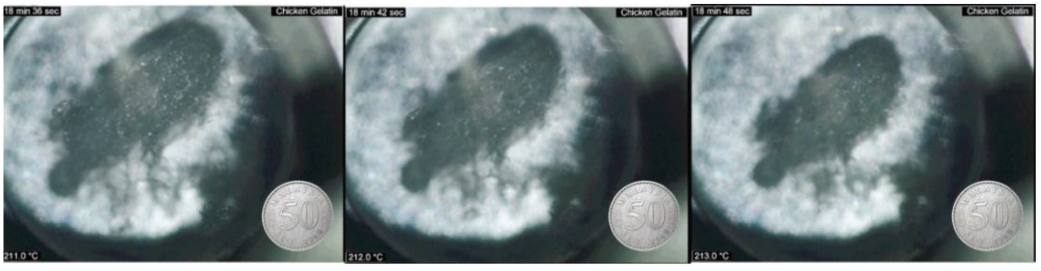


FIGURE 3(a). DSC thermo gram of CST gelatine and BS gelatine closed pan with nitrogen air. a) bovine skin gelatine (onset = 251.29°C, peak = 253.76°C) and b) chicken gelatine (onset = 242.16°C, peak = 242.55°C)

(a) (b) (c)



(d) (e) (f)



(g) (h) (i)

FIGURE 3(b). DSC microscopic view of CST gelatine in an unclosed pan (a)-(i): at different temperatures showing changes (picture in environmental air)

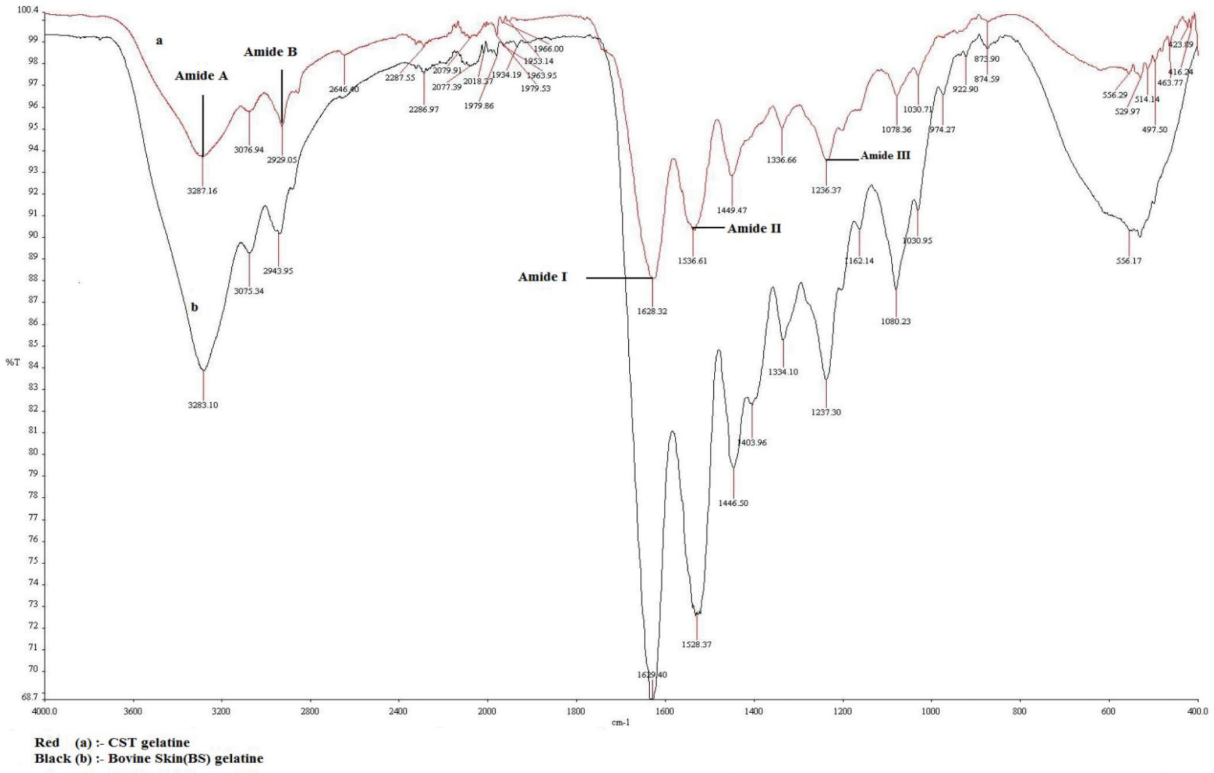


FIGURE 4. Fourier transform infrared (FTIR) spectra comparison between CST gelatine (a) and BS gelatine (b) (standard)

peptide chains. The amide I peaks are indicated by C=O stretching coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes. CN stretch and in-plane NH deformation modes of the peptide groups indicated the amide II peaks (glycine backbone and proline side-chains). The amide III represented 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. Thus, it can be concluded that the secondary structure of gelatines obtained from the CST was affected by acid pre-treatment and extraction time. The low molecular weight peptides formed during the extraction for long time were more likely able to form covalent cross-links during freeze-drying process (Ahmad & Benjakul 2011).

MOLECULAR WEIGHT ANALYSIS

SDS-Page protein patterns of extracted CST gelatine and BS gelatine are shown in Figure 5. Gelatine is made up of fractions of known amino acids which are joined by amide linkage to form linear polymer varying in molecular weight from 15 to 300 kDa. Gelatine is composed of α-chain, β-chain and higher molecular weight polymer including V̊-components (α-chain trimers) and some lower molecular weight fragments. It was shown that the CST gelatine had α-chain with a molecular weight range of about 120-150 kDa as the major protein and β-chain band range between 250 kDa (Figure 5). There were other bands obtained, containing lowest α-chain band intensity and slightly higher band intensity of β-chain. The molecular weight of standard protein (spectra multicolour high range protein ladder) was used as a marker for the molecular weight of gelatine. This indicated that the sufficiently high temperature used in gelatine extraction was more likely to cause the hydrolysis of α- and β-chains. This sufficient percentage of β-chain was observed for CST gelatine extraction at 65 ± 2°C. Extraction temperature has an important role in protein component of resulting gelatine where by extraction at higher temperatures had shorter chains which is indicated by lower content of α- and β-chains. Gelatine with higher contents of both α-type chains possessed better functional properties including gel strength, emulsifying and foaming properties. It was reported that chicken skin collagen extraction by pepsin digestion, confirmed the presence of both distinct α-chain which were α1 and α2 (Cliche et al. 2003). At the same time, CST gelatine showed scarcely the presence of peptides with molecular weight of less than α- chains. The formation of peptide fragments are associated with lower viscosity, low melting point, low setting point, high setting time as well as decreased Bloom strength of gelatine. This molecular characteristics contributes to their functional properties (Nagarajan et al. 2012).

POLYPEPTIDE PROFILE OF THE AMINO ACIDS

Gelatine's physical and chemical properties are influenced by the 18 distinct amino acid compositions and their

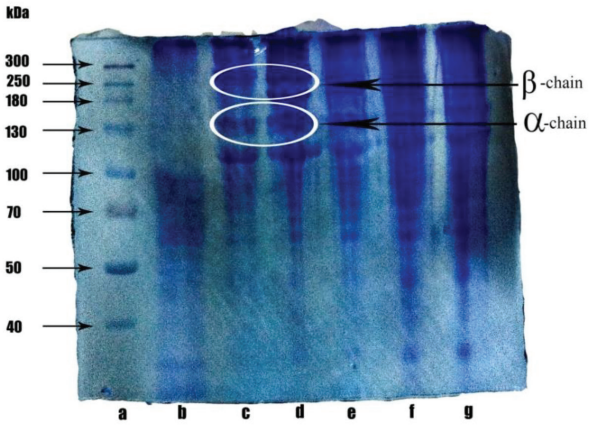


FIGURE 5. SDS-polyacrylamide gel electrophoresis pattern of CST gelatine and BS gelatine. Lane a: spectra multicolour high range protein ladder; lane b: CST gelatine extraction 2; lane c: CST gelatine extraction 4; lane d: CST gelatine extraction 5; lane e: BS gelatine; lane f: BS gelatine and lane g: BS gelatine

molecular distribution. A typical structure of gelatine is represented as: -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- (Kommareddy et al. 2005). Structurally, gelatine molecules contain repeating sequences of glycine-X-Y triplets, where X and Y are frequently proline and hydroxyproline amino acids. The sequence has glycine as the every third amino acid (Goodsell 2000). These sequences are responsible for the triple helical structure of gelatine and its ability to form gels in which the content of imino acid (proline and hydroxyproline) and glycine are important for gel strength of gelatine materials (Ahmad & Benjakul 2011; Kommareddy et al. 2005). The amino acid profile of CST gelatine is comparable to BS gelatine as shown in Figure 6. As mentioned above for CST gelatine type is made up of 21.54% imino acid (Pro+Hyp) and 19.54 % glycine, whereas BS gelatine was found to contain 23.69% imino acid and 16.95 % glycine. Table 2 shows CST gelatine has moderately similar Bloom strength as compared to BS gelatine, but with a higher content of glycine (Cheow et al. 2007; Wangtueai & Noomhorm 2009). The stability of the triple helix structure in denatured gelatines has been reported to be proportional to the total content of imino acids. Hydroxyproline also plays an important part in the stabilization of triple helix strand due to its hydrogen bonding ability through its hydroxyl group (Nagarajan et al. 2012).

Alanine has an influence in viscoelastic properties of gelatine, whereby its content were 8.863% for CST gelatine compared with 7.434% for BS gelatine. This coincides with the slight difference in viscosity and Newtonian flow of

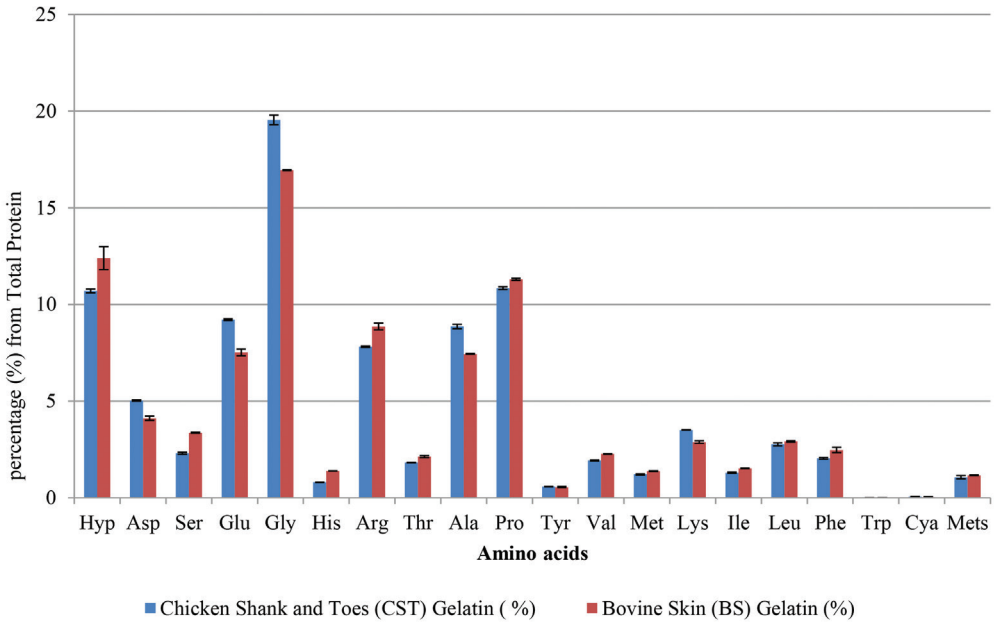


FIGURE 6. Amino acid composition of chicken shank and toes (CST) gelatine and bovine skin (BS) gelatine (total CST gelatine 91.38 ± 1.007% , total BS gelatine 90.65 ± 1.564% of total protein content of each type gelatine)

TABLE 3(a). Fourier-transform infrared spectra peak locations and assignment (Ahmad & Benjakul 2011)

Region	Peak range	Peak Wave number (cm^{-1})				Vibration mode
		CST gelatine	BS gelatine	Chicken feet gelatine	Ox leather gelatine	
Amide A	3600-3300	3287.16	3283.10	3399.56	3391.84-3467.09	N-H stretch coupled with hydrogen bond
Amide B	2945-2926	2929.05	2943.95	2923.72	2921.49	CH_2 asymmetrical stretch
Amide I	1658-1628 (1700-1600)	1628.32	1629.40	1652.01	1651.32	CH_2 asymmetrical stretch, C=O stretch
Amide II	1560-1335	1536.61	1528.37	1539.87	1556.53	N-H bend coupled with C-N stretch
Amide III	1242-1234 (1245-670)	1236.37	1237.30	1241.29	-	N-H bend coupled with C-N stretch (complex system mainly associated with CH_2 residual groups from glycine and proline)

TABLE 3(b). Uniquely identified peptide from CST gelatine by MALDI-TOF. Top Score: CO1A2_CHICK, Mass: 129618, Score: 77 (68 + 9), Expect: 0.012, Queries matched: 10, Collagen alpha-2(I) chain (Fragments) OS=Gallus gallus GN=COL1A2 PE=1 SV=2

Observed	Mr [expt]	Mr [calc]	Delta	Start	End	Miss	Ions	Peptide
1489.9390	1488.9317	1488.6853	0.2464	84	- 98	0	---	K.AADFGPGPMGLMGPR.G + Oxidation (M)
1577.9412	1576.9339	1576.7593	0.1746	380	- 397	0	---	R.GSNGEPGSAGPPGPAGLR.G
1577.9412	1576.9339	1576.7593	0.1746	380	- 397	0	---	R.GSNGEPGSAGPPGPAGLR.G
1604.9825	1603.9752	1603.8106	0.1646	977	- 994	0	68	R.GDPGPVGPVGPAGAFGPR.G
1604.9825	1603.9752	1603.8106	0.1646	977	- 994	0	--- ---	R.GDPGPVGPVGPAGAFGPR.G
1677.0354	1676.0281	1675.8277	0.2004	180	- 197	0	---	R.GHNGLDGLTGQPGAPGTK.G
1718.0295	1717.0222	1716.8179	0.2044	905	- 924	0	---	R.GPSGPVGSPGPNGAPGEAGR.D
1735.0321	1734.0248	1733.8267	0.1982	431	- 448	0	9	K.GPNGDAGRPGEPGLMGPR.X
1735.0321	1734.0248	1733.8267	0.1982	431	- 448	0	---	K.GPNGDAGRPGEPGLMGPR.X
3756.1162	3755.1089	3754.8423	0.2666	1022	- 1062	0	---	K.GHNGLQGLPGLAGQHGDQGPPGNNGPAGPRGPPGPSGPPGK.D

CST gelatine compared with BS gelatine as shown in Figure 1(a) and 1(b). Glutamine, asparagines (aspartic acid) and arginine were found at high levels in both CST and bovine gelatine. On the other hand, other amino acids such as phenylalanine, lysine, isoleucine, leucine, valine, tyrosine, serine, threonine, histidine and methionine were present in low amounts in both gelatines. Negligible amounts of cysteine were found in both gelatines which may be due to contamination from other proteins or small quantity of stroma proteins, such as elastin which is highly insoluble and stable in salt. Tryptophan was found in negligible amounts in both gelatines.

SPECTROMETRY ANALYSIS OR PROTEIN BIOMARKERS DETERMINATION BY MALDI-TOF

After treatment with trypsin the proteins were co-crystallized with UV-absorbing compounds and vaporized by a pulsed UV-laser beam. These ionized proteins undergo variable modification such as carbamidomethyl (C) and oxidation (M) are then accelerated in an electric field and the mass to charge ratio of different protein ion species can be deduced from their velocity. The peptide mass tolerance was ± 200 ppm, fragment mass tolerance was ± 0.2 Da and maximum missed cleavages were 1. A quick comparison between the results obtained from MALDI-TOF analysis and in the inbuilt database showed that the mass of the gelatine obtained was from collagen α-2(I) chain fragments of *Gallus gallus*. The score obtained was significantly high of 77 thus confirming the product obtained was CST gelatine. All specific peptides are listed in Table 3(b). Peptides GDPGPVGPVGPAGAFGPR (68 ions) and GPNGDAGRPGEPGLMGPR (9 ions) could only be found in collagen α-2 chain in the *Gallus gallus*. Enzymatic digestion and peptide identification with high resolution MALDI-TOF are common strategies used for protein identification in proteomics (Buckley et al. 2012). It also confirmed the presence of α-I compared as indicated by SDS-Page.

CONCLUSION

CST gelatine has characteristics and properties which are similar to BS gelatine. However, CST gelatine shows higher amount of total protein. The extracted CST gelatine was vitreous and has better gelling properties as compared with other commercially available gelatines. This is beneficial for the poultry industry whereby unconsumed by-products such as CST can be processed into valuable commercial gelatine. These products can be used in food industry as food coating materials as well as in pharmaceutical applications and cosmetics. The optimized extraction developed resulted in the reduction of extraction stages and moderately higher gelatine yield quality. These procedures will give value-added benefit to related industries (especially in poultry industry) by saving energy through reduction of process stages, and maximizing utilisation of industrial by-product thus reducing environmental pollution. Through this research it can be concluded that acceptable yield were obtained during the extraction at different temperatures with reduction in time of production. This research shows that the gelatine obtained from CST also complies with standard pharmacopoeia requirements for base products and hence could be used in pharmaceutical formulations. This study shows that transforming waste from poultry industry into everyday food and life-saving products is a possibility.

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