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Differentiation of bovine and porcine gelatins in processed products via Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and principal component analysis (PCA) techniques

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Abstract: Gelatin is widely used in food and pharmaceutical products. However, the addition of gelatin especially in food products becomes a controversial issue among Muslims due to its animal origin. Thus, the present study was aimed to detect and differentiate the origin of gelatin added in processed foods using a combination method of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Principal Component Analysis (PCA). Porcine gelatin had exhibited 11 prominent polypeptides compared to bovine gelatin with 2 prominent polypeptides. Polypeptides of both gelatin sources at molecular weight ranged from 53 to 220 kDa can be used to differentiate between porcine and bovine gelatins using PCA. The efficiency in extracting gelatin from processed foods by different solutions was also evaluated. Extraction of gelatin in processed foods by cold acetone and deionised water had exhibited a similar polypeptide patterns, suggesting both solutions are suitable. The study indicated that approach of a simple gelatin extraction combined with SDS-PAGE and PCA, may provide robust information for gelatin species differentiation of processed foods.

Keywords: SDS-PAGE, PCA, experimental sample, gelatin extraction, acetone precipitation

Introduction

Commonly, gelatin is used as food additives (stabiliser, emulsifier, thickener, gelling agent), an adhesive in sweets and foaming agent. It mainly derived from mammals, especially from pig skin and cow hide (Fernandez-Diaz *et al*., 2001) due to availability and attainable quality (Schrieber and Gareis, 2007). As the sources of gelatin become an issues for certain consumers, such as religious proscription, occurrence of bovine spongiform encephalopathy (BSE) disease, swine influenza, allergic reaction (Sakaguchi *et al*., 1996) and vegetarians' lifestyle, thus had made it as one of the most controversial food proteins in the food industry. Furthermore, regarding to the labelling (generally the label does not mention the gelatin source) and authenticity problems, it becomes the fear of consumers to consume the gelatin products. Consequently, certain consumers normally avoid the gelatin products unless they are really confident about its sources. For these reasons, the discovery of gelatin species biomarker as well as protein extraction method from the processed products have become as important studies to ensure the consumer rights are protected.

Recently, a number of studies with respect to distinguish the gelatin species have been reported, such as Fourier transform infrared (FTIR) spectroscopy coupled with Principal Component Analysis (PCA) (Hashim *et al*., 2010), high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) (Zhang *et al*., 2009), enzyme-linked immunosorbent assay (ELISA) (Venien and Levieux, 2005), Reversed-phase (RP) HPLC coupled with PCA (Nemati *et al*., 2004) and pH drop method after calcium phosphate precipitation (Hidaka and Liu, 2003). Mostly, the above mentioned methods were based on gelatin raw material and limited study has been done to determine its source in processed products (Doi *et al*., 2009).

Collagen denaturation causes destruction of hydrogen bonds, thus separate the rods and total or partial separation of the chains, causing loss of the triple helix conformation (Papon *et al*., 2007). As a result, the industrial gelatins consist from the mixtures of different compounds of α-chains (~ 100 kDa), β-chains (~240 kDa) and γ-chains (~ 400 kDa) (Papon *et al*., 2007; Waber *et al*., 2010). While the distributions of these chains are determined by the conditionings process either acid or alkali and the intensity of the hydrolysis used (Schrieber and Garies, 2007). As the production of pig skin gelatin is limited to acid process (Grobben *et al*., 2003), we take this advantage to distinguish it with the bovine

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alkali process based on electrophoretic separation in order to indicate the gelatin source in processed products.

Over the last several decades, polyacrylamide gel electrophoresis (PAGE) has been established as the most widespread method for polypeptides separation and molecular weight determination due to its simplicity, efficiency and sensitivity (Mackie *et al.*, 2000; Etienne *et al.*, 2000; Chen *et al*., 2010). Moreover, densitometer together with sophisticated analysis software had eliminated the difficulties associated with the gel data analysis, thus enhance its robustness.

In the present study, the efficiency of solutions used to extract the gelatin from the experimental samples (homemade jellies) and commercial processed foods was assessed based on their electrophoretic patterns with the pure gelatin. The results obtained will further analyse using Principal Component Analysis (PCA) to predict the source of gelatin. PCA will recognise the electrophoretic patterns and group the samples based on their similarity and interprets the relationship between the variables (Marina *et al*., 2010). Therefore, the study was aimed to detect and differentiate the source of gelatin added in processed foods using a combination method of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and PCA.

Materials and Methods

Samples

Porcine skin gelatin (Type A) and bovine skin gelatin (Type B) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercial food colouring (rose pink), flavouring (rose flavour) (Star Brand, FFM Berhad) and sugar were purchased from a local retail store. Commercial hard shell gelatin capsules (colour and colourless) were purchased from local pharmacy in Malaysia. Two types of gummies were purchased from Rome, Italy and Melaka, Malaysia respectively. Marshmallow was purchased from retail store in Selangor, Malaysia.

Chemicals

Sodium dodecly sulphate (SDS), glycine, tris-base, 2-mercaptoethanol, N,N,N',N'-tetramethylethylenediamine (TEMED), glycerol were purchased from Merck (Darmstadt, Germany). Ammonium persulfate, 1.5 M Tris-HCI buffer pH 8.8 and 0.5 M Tris-HCI buffer pH 6.8 were purchased from Bio-Rad (Hercules, CA, USA). Ethylenediaminetetraacetic acid disodium salt (EDTA) was purchased from VWR International Ltd. (Poole, England). Bromophenol blue was purchased from GE Healthcare (Uppsala, Sweden). Urea and acrylamide/bis-acrylamide 30% were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Determination of protein concentration

Protein concentration determination was performed following a method of Bradford (1976). Determination was done in triplicate from at least 4 independent experiments for each sample.

Preparation of samples

Control samples

Control samples of pure porcine (PSS) and bovine (BSS) gelatins were prepared by dissolving gelatin powder in 1 ml of 8 M Urea-SDS sample buffer containing 8 M urea, 2% SDS, 10 mM ETDA, 0.5 M Tris-HCl, 1.114 g/ml 2-mercaptoethanol, 10% glycerol, 0.05% bromophenol blue. The mixture was then vortexed until completely dissolved. Subsequently, the mixture was centrifuged at 3000 rpm for 3 min using a centrifugation (Eppendorf Centrifuge 5424, Hamburg, Germany) and 10 µl of the supernatant that contained approximately 8 µg of protein content was collected and loaded onto the well for electrophoresis procedures.

Experimental sample (Jelly)

Gelatin powder was mixed with 50 g sugar in a beaker. The mixture was soaked in 250 ml deionised water for 10 min to allow gelatin swelling. The mixture was then continues stirred until it completely dissolved in a hot water bath. Pink food colouring and rose flavouring (250 µl) agents were subsequently added and the solution was mixed thoroughly. The jelly was then placed at 4°C to solidify.

Processed food

The confectionery (gummies and marshmallow) were cut into small pieces by using clean and sharp scissors and transferred into conical flask containing deionised water. While, for hard shell capsule, the cap and body from each capsule was separated and the content was discarded. The cap and body of capsule were then cleaned using a piece of lens tissue prior to dissolve them in the deionised water. The mixture was stirred on the hot plate until it completely dissolved. This experiment was performed at approximately 37°C.

Extraction of gelatin

Two solutions of cold acetone (acetone precipitation) and deionised water were used to extract the protein (gelatin) from the studied samples. Their efficiency was examined by comparing the obtained polypeptide bands patterns with the commercial pure

gelatins. Acetone precipitation was done according to Fic *et al.* (2010), with slight modifications. 800 µL of cold acetone (-20°C) were added into 200 µL of melted jelly or diluted processed foods. The mixture was vortexed and incubated for overnight at -20°C and was then centrifuged at 14,000 rpm using a centrifuge (Eppendorf Centrifuge 5424, Hamburg, Germany) for 10 min. Subsequently, the supernatant was discarded and the pellet was air-dried from the acetone residue in the fume hood. The protein pellet was then dissolved in 8 M Urea-SDS sample buffer. While, gelatin extraction using deionised water was done by mixing 200 µL of melted jelly or diluted processed foods with 8 M Urea-SDS sample buffer. The mixture was then vortexed until its mix well. The volume of sample buffer was different in each sample for each of the gelatin extraction method in order to get clear band patterns.

Electrophoresis

SDS-PAGE was performed as described by Laemmli (1970). Gelatin polypeptides were separated on a slab gels consisted of 4% stacking gel and 6% resolving gel. Ten µL of completely dissolved samples (extracted gelatin) in 8 M Urea-SDS sample buffer was collected and loaded onto the well. SDS-PAGE was carried out in a Mini Protean II tetra cell (Bio-Rad Laboratories, CA, USA), at a constant voltage of 80 V for 2 h. The molecular weight of the polypeptide bands were estimated using high molecular weight standard marker for SDS electrophoresis (GE Healthcare, Buckinghamshire, UK) consisted of myosin (220 kDa), α2- macroglobulin (170 kDa), β- galactosidase (116 kDa), transferring (76 kDa) and glutamic dehydrogenase (53 kDa). Polypeptide bands were visualised by using silver stain solution in order to detect the low abundance polypeptides. Silver staining (Bio-Rad Laboratories, Hercules, CA) was performed according to the manufacturer's instructions. Subsequently, the stained gel was scanned using a densitometer (GS-800 Calibrated Densitometer Bio-rad, Hercules, CA) and each polypeptide band was analysed using Quantity One Software (Bio-rad, Hercules, CA).

Statistical analysis

The results from the qualitative comparison (SDS-PAGE) will further analysed using Principal Component Analysis (PCA), by using Unscrambler 9.7 (Camo, USA) software. Molecular weight regions were set as the variables, whereas the qualitative presence or absence of polypeptide band molecular weight values in each variable of each sample was used as input data. 14 high molecular weight regions

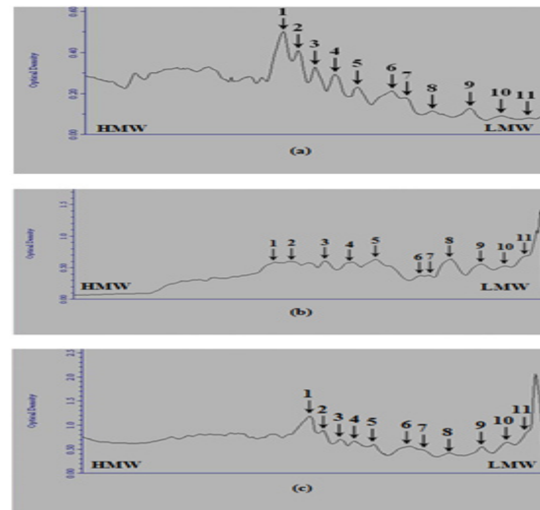

Figure 1. Electrophoretic profiles of gelatin polypeptides. (a) PSS gelatin as a control; (b) experimental sample: gelatin extraction using deionised water; and (c) experimental sample: gelatin extraction by acetone precipitation; peak 1-11: prominent bands; HMW: high molecular weight; and LMW: low molecular weight.

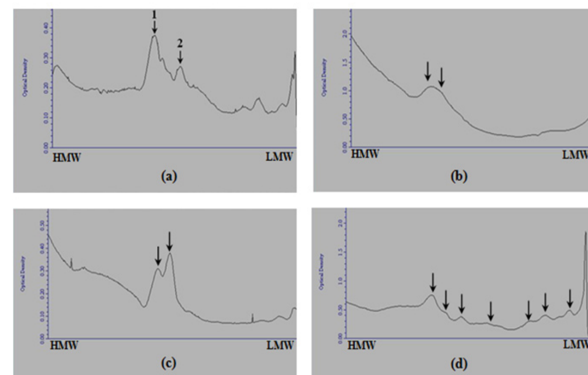

Figure 4. Electrophoretic profiles of pure gelatin and gelatin commercial processed foods.
(a) Pure bovine gelatin (peak 1-2 were prominent bands); (b) gummy B (LG); (c) colourless hard shell capsule (CC); (d) colour hard shell capsule (RC); HMW: high molecular weight; LMW: low molecular weight; each arrow represent a prominent band in (b), (c) and (d). Proteins from processed foods were extracted by acetone precipitation.

ranged between 53 to 220 kDa (180, 170, 160, 150, 140, 130, 120, 110, 100, 90, 80, 70, 60 and 50 kDa) were set as the variables.

Results and Discussion

Electrophoretic patterns of gelatin polypeptides

The differentiation between porcine skin gelatin (PSS) and bovine skin gelatin (BSS) gelatins at molecular weight region ranged from 53 to 220 kDa was studied under our developed SDS-PAGE conditions, which by using 6 % resolving gel, 8 M Urea-SDS sample buffer, 8 µg of protein amount together with sensitive silver staining. The PSS gelatin was exhibited wider molecular weight distribution compared to BSS gelatin, which PSS gelatin contained

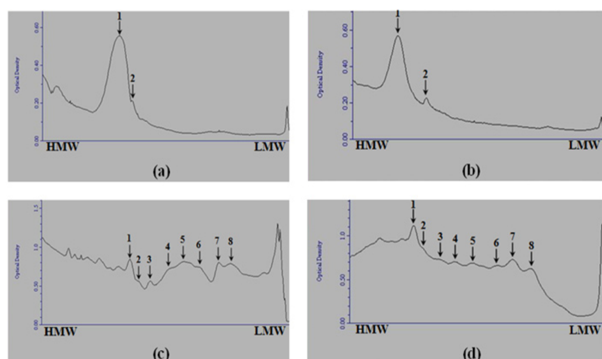

Figure 2. Electrophoretic profiles of commercial processed foods. (a) and (b): marshmallow (HM); (c) and (d): gummy A (HG); (a) and (c): acetone precipitation; (b) and (d): gelatin extraction using deionised water; HMW: high molecular weight; and LMW: low molecular weight.

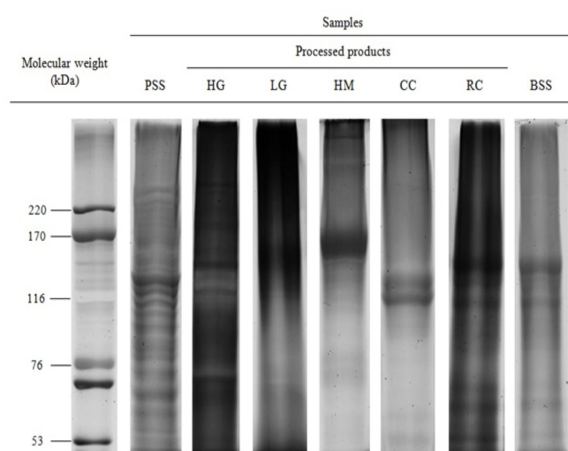

Figure 3. Electrophoretic polypeptide patterns of pure gelatin and gelatin commercial processed foods.
PSS: pure porcine gelatin; HG: gummy A; LG: gummy B; HM: marshmallow; CC: colourless hard shell capsule; RC: colour hard shell capsule; BSS: pure bovine gelatin.

11 prominent bands (approximately 125, 120, 114, 106, 96, 87, 83, 76, 70, 64 and 58 kDa) (Figure 1a) rather than 2 prominent bands (approximately 135 and 110 kDa) in BSS gelatin (Figure 4a). The presence of polypeptide bands with molecular weight less than α-chain in PSS gelatin were in good agreement with Cole and Roberts (1996). While the molecular weight distribution of BSS gelatin was consistent with the result of Muyonga *et al*. (2004), who found the major molecular weigh fractions of type B gelatin from bovine bone is in the α-chain region. In addition, the size exclusion chromatography–multi angle laser light scattering (SEC-MALLS) analysis by Eysturskaro *et al*. (2009) showed that the bovine type B gelatin exhibited well-defined peak compared to porcine type A gelatin (Figure 1a and 4a). The distinct electrophoretic polypeptide patterns between PSS and BSS gelatin could be used as bioindicator to indicate the source of gelatin in the studied processed products.

Effect of protein extraction on electrophoretic pattern of gelatin polypeptides

The efficiency of two solutions of cold acetone (acetone precipitation) and deionised water to extract the protein were assessed by comparing the obtained polypeptide band patterns with the commercial pure gelatin. As a preliminary study, the gelatin from the experimental sample of homemade jelly was extracted using both solutions. Interestingly, peaks that represented the polypeptide bands were consistent with the 11 prominent bands of PSS gelatin (Figure 1). The finding indicated that both solutions were successful in extracting protein gelatin from the experimental of homemade jelly.

Subsequently, the efficiency of both solutions was accessed on using commercial products of marshmallow (HM) and gummy A (HG). No distinct difference was observed (based on the number of polypeptide bands) except for different intensities exhibited by each peak (Figure 2). It could be assumed that the cold acetone (acetone precipitation) and deionised water were suitable for protein extraction from the processed products. This circumstance also indicated that the SDS-PAGE is tolerant with the impurity contents as well as could offer simplicity in sample preparation. However, for further examination we decided to apply acetone precipitation method for precautionary as ingredients in the commercial processed products were more complex.

Comparison of polypeptide band patterns of processed foods

High Bloom gelatin is the main ingredient of hard shell capsule production (Goedknegt and Grobben, 2003). According to this information, two types of hard shell capsule, RC (coloured) and CC (colourless) were examined. As a result, they successfully exhibited similar bands pattern to that of BSS gelatin. Three different confectionery products (2 types of gummies and a marshmallow) were analysed. The bands pattern exhibited by gummy B (LG) and marshmallow (HG) were similar to that of BSS gelatin, while band pattern of gummy A (HG) was similar to that of PSS gelatin. As a pre-estimation (based on bands patterns observation), RC and CC hard shell capsule, LG gummy and HM marshmallow contained gelatin from bovine source, while HG gummy contained gelatin from porcine source (Figure 2, 3 and 4). The qualitative differences were further confirmed by PCA.

Principal Component Analysis

PCA is a multivariate analysis that presents the

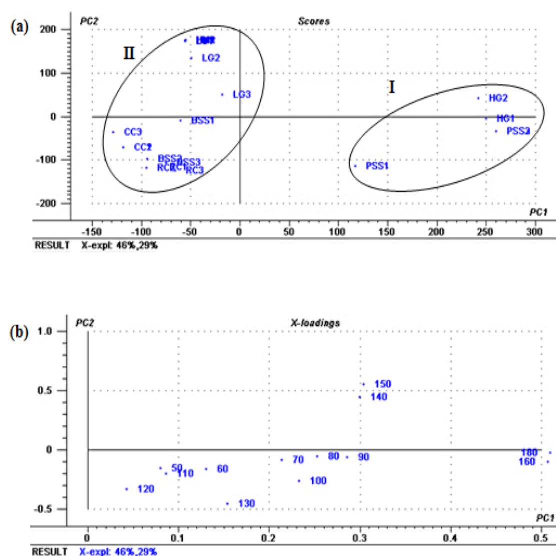

Figure 5. PCA grouping of processed foods. (a) Score plot; (b) loading plot; I: porcine based products and II: bovine based products.

relationships between variables and between samples (Garrido-Relgado *et al.*, 2009). Commonly, the results are presented in terms of score and loading plots. Score plot shows the samples location in each model component while loading plot interprets the relationship of variables. PCA was able to detect the patterns, grouping, similarities and differences of the input data (Kara, 2009). The combination of various analytical methods such as FTIR (Hashim *et al.*, 2010), HPLC (Nemati *et al.*, 2004) and GC (Lee *et al.*, 1998) with PCA are commonly utilised in order to interpret the poor analytical differences.

Based on first two principal components, PC 1 and PC 2 (carry the maximum variability in the data) (Luca *et al.*, 2011), two groups of processed foods were obtained; processed food (HG gummy) that exhibited similar bands patterns to that of PSS at the positive score (group I), and processed foods (LG gummy, HM marshmallow and RC and CC hard shell capsules) that exhibited similar bands pattern to that of BSS at the negative score (group II) (Figure 5a). PC 1 and PC 2 accounted for 46 and 29 % of the variation, respectively; thus, 78 % of the variance was accounted for the first 2 PCs. As can be seen in the loading plot in Figure 5b, the main molecular weight regions that strongly contributed for samples grouping were 130 and 150 kDa based on PC 1 and 180 and 160 kDa based on PC 2. All the mentioned regions above were consistent with regard to the location of the polypeptide bands of PSS gelatin (but not prominent bands) and thus indicated that they had potential to be another region for being biomarker for gelatin species differentiation. The samples grouping by PCA were consistent with qualitative differentiation by SDS-PAGE.

Conclusion

The differentiation of electrophoretic polypeptides between PSS and BSS gelatins at molecular weight region ranged from 53 to 220 kDa was manipulated in order to evaluate the efficiency of solution used in gelatin extraction methods as well as to estimate the gelatin origin of the processed products. Assessment of two solutions of cold acetone (acetone precipitation) and deionised water to extract the gelatin from the experimental samples (homemade jelly) suggested that both of them were suitable, as the extracted gelatin exhibited similar polypeptide bands with the PSS gelatin. However, as the commercial processed foods may contain more complex ingredient compared to experimental samples, acetone precipitation method was applied. SDS-PAGE combined with PCA may provide robust information for gelatin species differentiation of processed food and non food products.

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